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STUDIES ON THE FIBRINOLYSIS IN TUMOR BEARING MICE

I. Fibrinolytic System, Ascites Retention and Intraperitoneal Hemorrhage in Tumor Bearing Mice : Suppressing Effect of Epsilon Aminocaproic Acid

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INTRODUCTION

It has been known that a dramatic hemorrhage may frequently occur in the abdominal cavity in the mice with ascites tumor cells.

This paper presents the pathogenical aspect of such a bleeding, relating with the disturbed fibrinolytic system in the tumor bearing mice and a hemostatic effect of epsilon aminocaproic acid.

Suggestion had been given from the evidence presented by the previous workers. In 1952, Soulier et al.[1] and Ratnoff[2] respectively, described a fatal hemorrhage associated with the accelerated fibrinolysis of blood in patients ; then, a number of papers have been published, indicating that some of the patients having neoplastic growth undergo the severe fibrinolytic hemorrhage. A fundamental study, however, had been made by Cliffton et al.[3] in 1955 ; and the potent fibrinolytic activity was demonstrated by them, using the fibrin plate method, in some of the human cancer tissues obtained by surgical operations. Thus, in order to know whether or not the fibrinolytic system would be directly related to the occurrence of the hemorrhage into ascites of the tumor bearing mice, the present experiments were carried out.

METHODS

Animal experiments

Swiss albino-mice of both sexes, which line (DD) had been bred in this laboratory, were used throughout the experiments. Mice, weighting between 18 gm and 23 gm, were fed a solid food (CLEA ; oriental commercial pellets for breeding purpose) and water ad libitum.

The ascites form of Ehrlich tumor cells was obtained from the Growth Research

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Institute, Kobe Medical College, and was maintained by serial intraperitoneal passage. Tumor transplantation was carried out aseptically with drawing ascitic fluid from a donar mouse bearing a 5th day Ehrlich ascites tumor growth. Diluting the obtained fluid with Ringer–Locke solution after measuring the number of tumor cells, a solution of 1.0 ml containing 1×10^6 or 5×10^6 tumor cells was prepared and was given intraperitoneally to a each mouse of the experimental series.

Mice were killed under ether anesthesia at different intervals of time, the ascites being carefully collected by a pippet opening the abdominal cavity. A small amount of blood sample was withdrawn by opening the heart. Counting of red cells or tumor cells was made after the usual method of the blood cells counting.

Epsilon aminocaproic acid was given to a series of mice dissolving it in the drinking water at 5% concentration.

The antifibrinolytic assay

The antifibrinolytic activity of serum or ascites was examind after the streptokinase activation test by U. Okamoto et al.[4]; a modification of the method described by Colgan et al.[5]. The ascitic fluid obtained was centrifuged by 3000 r. p. m. for 10 min at 0°C and the ascitic plasma obtained was used for the assay. Placing test tubes in the ice cold water bath, 0.10 ml of borate saline buffer (pH 7.4) containing 100 u. of streptokinase (Varidase, Lederle Lab.), 0.10 ml of ascitic plasma or serum, 0.45 ml of borate saline buffer (pH 7.4) and 0.05 ml of borate saline solution containing of 5 units of thrombin (Nippon Blood Bank Co.) were admixed together in the test tube and kept for 5 min in the ice cold condition. Then, adding 0.10 ml of 0.6% solution of bovine fibrinogen (Cohn Fraction I Armour) to each reaction mixture, test tubes were incubated at 25°C. The time required for the complete disappearance of the formed fibrin clot was observed by min.

The reaction mixture containing the saline, instead of ascitic plasma or serum, was taken as the control, and the difference between the lysis time of the aliquots and that of the controls was expressed by minutes and used for an index of the antifibrinolytic or anti-streptokinase activity of each sample.

RESULTS

1. *Decrease of the antifibrinolytic activity of the ascitic plasma after the inoculation.*

Results shown in Fig. 1 indicated that the antifibrinolytic activity of the ascitic plasma was considerably high in the earlier stage of the tumor growth in mice, and that the antifibrinolytic activity was decreased to a great extend in the later stage. This was proved in the two series of mice inoculated with 1×10^6 tumor cells or 5×10^6 tumor cells, even though the decreasing rate of antifibrinolytic activity was more rapid in the series

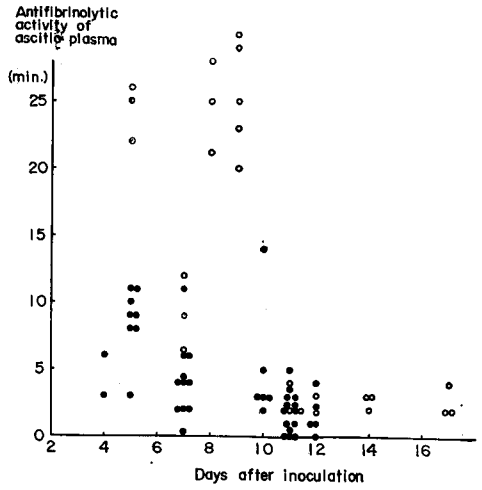

Fig. 1. Decreasing tendency of antifibrinolytic activity in the ascitic plasma following the inoculation of Ehrlich tumor cells to a couple of series of mice. Abscissa indicates the days after inoculation. Ordinate indicates the antifibrinolytic activity of ascitic plasma expressed by min. Decrease of the antifibrinolytic activity is observed in the earlier period when the dose of tumor cells inoculum increases. Dose of tumor cells inoculum: 1×10^6 cells in open circles and 5×10^6 in solid circles.

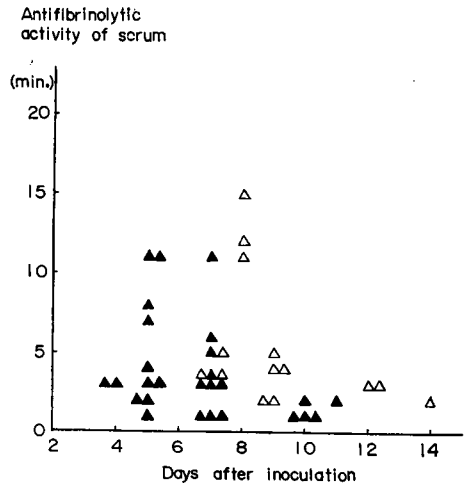

Fig. 2. Decreasing tendency of antifibrinolytic activity in the serum of blood taken by heart puncture following the growth of Ehrlich tumor cells.

Abscissa indicates the days after inoculation; ordinate indicates the antifibrinolytic activity of the serum expressed by min. Decreasing tendency of antifibrinolytic activity is also observed, but not so obviouse as the ascitic plasma. Dose of tumor cells inoculum: 1×10^6 cells in open triangles and 5×10^6 cells in solid triangles.

to which a larger number of tumor cells was inoculated.

As shown in Fig. 2, the examination of serum of the blood samples obtained from these two series of mice also indicated some trends of decreasing the antifibrinolytic activity in the later days after the inoculation, though it was not so conspicuous as that in the ascites.

2. *The amount of ascites retention and the decrease of the antifibrinolytic activity of ascitic plasma*

The amount of ascites retention, examined in the experimental series described above, was plotted against the logarithm of the antifibrinolytic activity of ascitic plasma in Fig. 3A.

Disregarding both the number of tumor cells inoculated and the days after the inoculation, a linear correlation was statistically found between the amount of ascites expressed by ml and the antifibrinolytic activity expressed by the logarithm of the delay (min) in the lytic process.

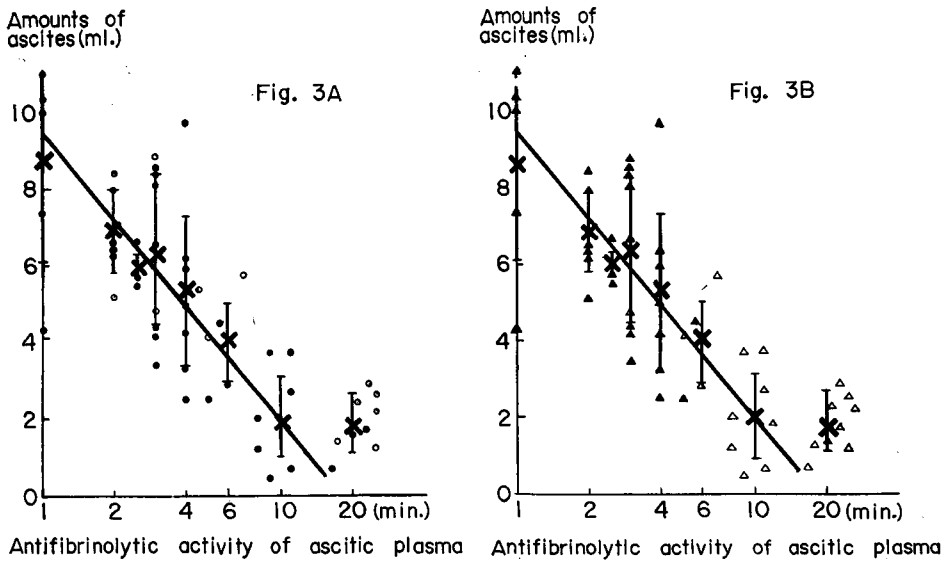

Fig. 3. A linear correlation between the amout of ascites and the antifibrinolytic activity of ascitic plasma. Decrease of the latter (plotted in logarithmic scale) parallel with the increase of the former.

In Fig. 3A and 3B, abscissa indicate the antifibrinolytic activity of ascitic plasma. Ordinates, amount of ascites. Cross indicates mean value and vertical bar, standard deviation. Fig. 3A also indicates that the correlation mentioned above exisits disregarding the dose of cells inoculum which is 1×10^6 cells in open circles and 5×10^6 cells in solid circles.

Fig. 3B. Indicates that the remarkable hemorrhage is frequently observed with the decrease of the antifibrinolytic activity. Solid triangles indicate that the number of erythrocytes in ascites of a mouse is more than 1×10^9. Open triangles, less than 1×10^9.

A more significant correlation was found relating with the number of red cells appeared in the ascites as shown in Fig. 3B. The markedly increased number of red cells in ascites (more than 1×10^9 of red cells when calculated of the whole ascites of a mouse) was observed in those cases, where the decreased antifibrinolytic activity of ascitic plasma and the increased retention of ascites were both observed.

Results shown in Fig. 3B indicated that, when the antifibrinolytic activity of ascites decreased less than 5 min, a severe hemorrhage into the abdominal cavity had occurred in most instances, yet some exceptional cases being remained. However a distinctive correlation was observed with an emphasis on the sum of the antifibrinolytic activity of the mice's sera and of the ascites as described in the following item.

3. *Hemorrhage into ascites and its relation to the antifibrinolytic activity of ascitic plasma and serum of the blood*

Determining the number of red cells appeared in ascites, its quantitative correlation to the antifibrinolytic activity of ascitic plasma or serum was examined in the following experiments. No satisfactory correlation could be found when the number of red cells

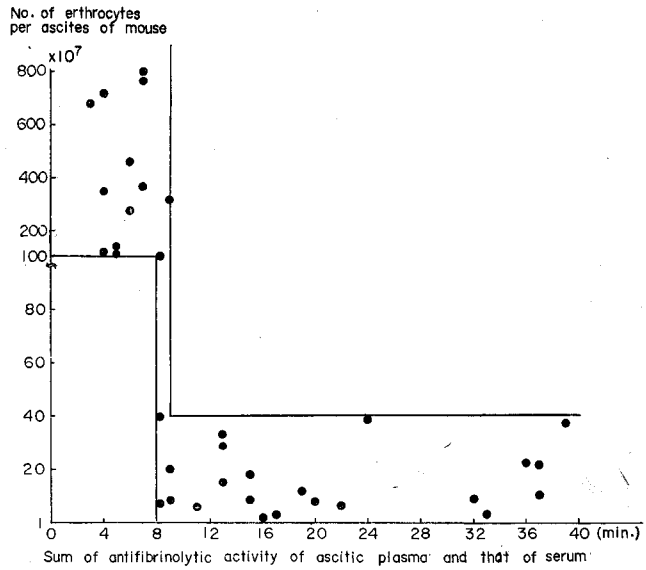

Fig. 4. A distinctive correlation of the ascltes hemorrhage to the sum of the antifibrinolytic activity in ascitic plasma and that of serum. Ordinate indicates the number of erythrocytes per ascites of a mouse. Abscissa, the sum of the antifibrinolytic activity of ascites plasma and that of serum. Each solid circle plotted indicates the result obtained from a mouse, disregarding the dose of cells inoculum. When the sum is less than 8 (min), a drastic hemorrhage is observed without any extraception. Such a hemorrhage however is not observed when the sum is more than 8 (min).

were respectively plotted against the antifibrinolytic activity of ascitic plasma or that o' serum, or against the logarithm of the antifibrinolytic activity of these samples.

As shown in Fig. 4, an interesting correlation, however, was successfully demonstrated by plotting the number of erythrocytes in ascites against the sum of antifibrinolytic activity of ascitic plasma and that of serum. The number of erythrocytes in ascites was remaining under 4×10^8 when the sum of antifibrinolytic activity of ascitic plasma and that of serum remained over 8 min. The number of erythrocyte measured was, however, abruptly increased over 1×10^9 when the sum of antifibrinolytic action of ascitic plasma and that of serum diminished less than 8 min. This noticeable correlation suggested that the intraperitoneal hemorrhage would be controled by the addition of the decreased antifibrinolytic activity in ascitic plasma and that in serum. But, in these series of mice, hardly was any noticeable correlation detectable between the antifibrinolytic activity of ascitic plasma and that of serum.

4. *Suppressing effect of the oral administration of epsilon aminocaproic acid on the ascites and the intraperitoneal hemorrhage*

It was indicated that the decrease of the antifibrinolytic activity of ascitic plasma and/or serum was, more or less, related to the increase of the ascites retention and the intraperitoneal hemorrhage. Therefore, epsilon aminocaproic acid having a specific inhibitory action on the plasmin system was orally administered to a series of tumor bearing mice in order to know the influence of artificial increase of antifibrinolytic activity.

Immediately after the inoculation of 5×10^6 tumor cells to a series of 29 mice, the administration of the agent was started by giving them the drinking water containing 50 mg of epsilon aminocaproic acid per ml. The average amount of up-take of the agent containing drinking water per mouse was ca 6 ml on the 1st day of the experiment, ca 4 ml on the 5th and ca 2 ml on the 10th ; that is, 300 mg~100 mg of epsilon aminocaproic acid to a mouse daily. Another series of 31 mice, which inoculated with the same number of tumor cells without the agent was taken as the control.

At different intervals after the inoculation, mice were sacrificed and the amount of ascites, the number of red cells and antifibrinolytic activity of the ascitic plasma and the serum were examined.

In the series of mice administered with epsilon aminocaproic acid, antifibrinolytic activity of serum was more than 30 (min) when examined on the next day after inoculation. This indicated the distinct increase of antifibrinolytic activity of serum, while that of control remained within certain variation (10~20 min).

The antifibrinolytic activity of the ascitic plasma was, to a great extent, showed an increase throughout the experiment in the series of the agent administered mice. The lysis time examined from the 10th day to 12th day after inoculation was found to be unusually prolonged, and it was more than 24 hr. in all which examined on the 11th day. In the control series, however, the antifibrinolytic activity of the ascitic plasma showed the remarkable decrease after the inoculation as expected.

From 10th to 12th day after inoculation, in 21 mice treated with epsilon aminocaproic acid, and 26 mice without it, the following results were obtained. The amount of ascites in each of the mice belonging to epsilon aminocaproic acid group was less than 5 ml in 71% and 5~10 ml in 29% ; while, in the control group, it was less than 5 ml in 19%, 5~10 ml in 52% and 10~15 ml in 29%. In reviewing these results, mean value and standard deviation in these two groups were $5.4\ \text{ml}\pm1.7$ ml in the epsilon aminocaproic acid group, and $8.2\ \text{ml}\pm2.7$ ml in the control. Thus the statistical examination indicated that the ascitic retention in the epsilon aminocaproic acid group was not significantly less than that in the control group so far as examined in the limitted number of the mice used even though the mean value obtained from the former was less than that of the latter.

As shown in Fig. 5, more significant was the influence of epsilon aminocaproic acid on the number of red cells appeared in ascites. The number of red cells per mm^3 of ascites of respective mouse, sacrified from 10th to 12th day after the inoculation, were that ; the mean value and standard deviation were $(10.0\pm6.1)\times10^4$ in the epsilon

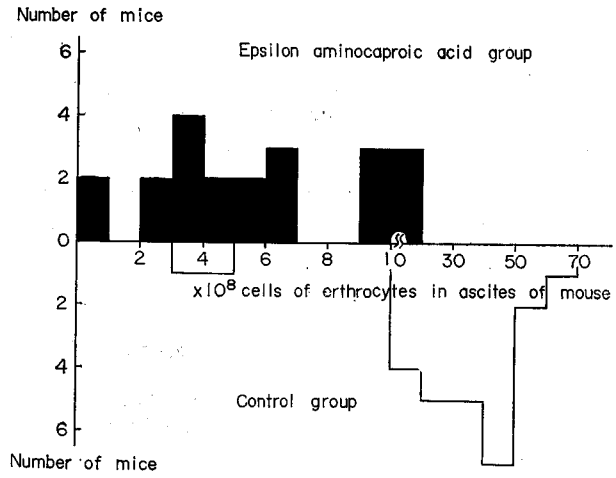

Fig. 5. The suppressing effect of epsilon aminocaproic acid upon the intraperitoneal hemorrhage of the tumor bearing mice. The frequency distribution is shown in this Fig. indicating the discrepancy of the grade of hemorrhage between the control group and the group administered with epsilon aminocaproic acid through drinking water.

aminocaproic acid group and $(45.9\pm21.1)\times10^4$ in the control group, indicating the significantly decreased hemorrhage in the former than the latter. In order to know an index of the total volume of blood appeared in ascites, the total number of red cells in the whole ascites were calculated, and results obtained were shown in Fig. 5. It was obviously demonstrated that the number of red cells in the whole ascites in a mouse of the epsilon aminocaproic acid group were far less than that of the control group, suggesting the anti-hemorrhagic effect of agent on the tumor bearing mice. Furthermore, the authors have observed that the fibrin formation in the abdominal cavity was difinitely firm in the epsilon aminocaproic acid group while in the control group, hardly any was shown.

DISCUSSION

A new aspect of the neoplastic growth, i. e. the involvement of the fibrin metabolism in the tumor tissues, has aroused a focus of attention by several workers. Following the finding of fibrinolytic activity in some human tumors (Cliffton et al. in 1955)[3], a certain relation between the metastasis and the fibrinolytic activity was also studied by the same authors in 1956[6]. Later, Pressman and his co-workers[7] in 1960, using the fluorescen labeled antibody to fibrin, demonstrated the fibrin deposition in some tumor tissues of patients. The another evidence was presented by Bale et al. in 1960[8] that the I^{131} radioactive antibody to rat fibrin was localized in tumor cells of rats after the intravenous administration of the antibody.

Furthermore, a remarkable finding was made by Mütshler and Bale in 1962[9]; that is, the retention of I^{131} rat fibrinogen and I^{131} antibody to rat fibrin was increased in some rat tumors. They also reported that I^{131} fibrin deposition around the tumor tissues was increased when epsilon aminocaproic acid was given orally.

The authors have started the examination on the relation of the fibrinolytic system to the intraperitoneal hemorrhage inoculated with Ehrlich tumor cells into the abdominal cavity of mice. Results obtained by streptokinase activation test were described in order to outline the general trend of the disturbance of the fibrinolytic system in the ascitic plasma and the serum of blood as well. The more detailed study of the disturbance of the fibrinolytic system in the ascites should be necessary. The results of the studies pursued by the same authors will be soon published.

In the tumor bearing animals, the decrease of the antifibrinolytic activity was distinctively observed in the ascitic plasma in the later period of each experimental series (Fig. 1). The decreasing trend of the antifibrinolytic activity, however, was not so obvious in the serum (Fig. 2). Besides, results shown in Fig. 3A and Fig. 3B suggested that the decrease of the antifibrinolytic activity in the ascitic plasma might be one of the factors in producing the ascitic retention as well as the hemorrhage. The another series of mice have shown that the logarithm of antifibrinolytic activity was inversely proportional to the amount of ascites produced (Fig. 3A).

A potent effect of epsilon aminocaproic acid for suppressing hemorrhage and decreasing the antifibrinolytic activity has been clearly demonstrated by examining the group of mice to which the agent was administered after the inoculation and continued throughout the experiment (Fig. 5).

The quantitative data shown in Fig. 4 have shown that severe hemorrhage into ascites has been induced in these mice in which the sum of the antifibrinolytic activity of ascitic plasma and that of serum was less than a certain critical value (i. e. 8 min). These results would imply that the interraction between the antifibrinolytic activity of ascites and that of blood serum may be more significant in producing the bleeding into ascites through the peritoneal wall. This seemed to suggest some enlightment in elucidating the hemorrhage mechanism. It is also expected that the mentioned mechanism of the intraperitoneal hemorrhage would operate even in man having a certain kind of abdominal tumor.

SUMMARY AND CONCLUSION

Inoculating Ehrlich tumor cells in the abdominal cavity of mice, the variation of fibrinolytic system in ascitic plasma or serum of the circulatory blood were studied in relating to the ascitic retention and intraperitoneal hemorrhage. In order to know variable fibrinolytic system, a certain amount of ascitic plasma or serum was added to the fibrino-

lytic system containing streptokinase, then the delay of lysis time produced by adding ascitic plasma or serum was estimated and expressed as an index of the antifibrinolytic activity.

1) In the later days after the inoculation, apparent decrease of the antifibrinolytic activity in ascitic plasma was observed in mice invariably and such a decrease was always accompanied by the ascitic increase and hemorrhage.

2) Statistical examination revealed that a logarithm of antifibrinolytic activity of the ascitic plasma was inversely proportional to the amount of the ascites produced by the tumor cells inoculation.

3) Severe hemorrhage into ascites was necessarily produced in these mice in which the sum of the antifibrinolytic activity of ascitic plasma and that of serum was less than a certain critical value (i. e. 8 min.).

4) The oral administration of epsilon aminocaproic acid to a series of mice had initiated soon after the inoculation and continued through the experiment, and it was known that the ascitic plasma examined retained a powerful antifibrinolytic activity. Furthermore, the authors had demonstrated that hemorrhage into the abdominal cavity could be greatly suppressed by administering epsilon aminocaproic acid to the tumor cells bearing mice.

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