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A Study on Fibrinolysis in Experimental Bladder Tumors*

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Summary. The fibrinolytic activity in the N-butyl-N-butanol (4)-nitrosamine (BBN) induced bladder tumors in rats and normal rat bladders was investigated at cellular level by the histochemical fibrin slide technique, and the ratio of plasminogen activator and urokinase inhibitor contained in tissue was estimated by the fibrin plate method. The plasminogen activator activity in the bladder epithelium gradually decreased as the neoplastic process became established, and the cancer cells lost the fibrinolytic activity of the normal tissue from which they sprang. The ratio in the cancerous tissue was strikingly on the side of inhibitor (1 : 434) as compared with that in the normal tissue (1 : 32). Concomitantly, a highly significant reduction of fibrinolytic activity in blood, assayed by euglobulin lysis time, was observed after the development of the carcinoma. It may be reasonable to assume that the ratio in the cancerous tissue serve for antiplasmin in the fibrinolytic system in blood. Antifibrinolysis induced with tranexamic acid had no significant effect on the incidence and growth of the bladder tumors.

Key words: Experimental bladder tumors, urokinase inhibitor, fibrinolysis, tranexamic acid.

Extravascular fibrin deposition in human carcinoma was found by O'Meara (1. 2) to be related to an increased thromboplastic activity in the tumor tissue, and he suggested that fibrin at the periphery of a growing malignant tumor favoured infiltrating growth and spread. This concept stimulated experimental study of the effect of fibrinolysis on the growth and spread of tumors. Since the work of Kreisler (3), a considerable literature on the effect of anticoagulants, induced fibrinolysis or antifibrinolysis on the growth and spontaneous metastasis formation of experimentally transplanted carcinomas and intravenously injected tumor cells has been published. The results however are not in close agreement and moreover some of them are quite contradictory. On the other hand, little is known about these effects upon the induction and subsequent growth of experimental tumors, or about the alterations in the fibrinolytic activity in tissue and blood in the experimental neoplastic process.

Druckrey et al. (4) found that oral administration of N-butyl-N-butanol (4)-nitrosamine (BBN) selectively induced bladder tumors in rats. Ishikawa et al. (5) reported that rat bladder tumors induced by BBN were transitional cell carcinomas, the most common type in the human bladder.

The epithelial lining of the bladder was found to have fibrinolytic activity in rat, man (6, 7) and guinea pig (8), using the histochemical fibrin slide technique designed by Todd (9). On the other hand, Hisazumi (10) demonstrated that normal mucous membranes and cancerous tissues of the human bladder exhibit selective inhibition of urokinase, and they also inhibit tissue plasminogen activator to some extent.

The aim of the present study was to investigate the influence of induced antifibrinolysis on the incidence, growth and histological picture of the rat bladder tumor induced by oral administration of BBN. The alterations in the fibrinolytic activity and urokinase inhibitory activity in tissue and blood during development of the BBN induced bladder tumor were studied as well.

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Materials and Methods

Animals: Male rats of Wistar strain, weighing approximately 250 g, were used. They were allowed free access to water and a standard pellet ration.

Carcinogenic Substance: 10 g of N-butyl-N-butanol (4)-nitrosamine (BBN; Junseikagaku, Co., Tokyo, Japan) and 0.2 ml of Tween 80 (Wako pure chemical industries, LTD., Tokyo, Japan) were mixed, and the mixture was then dissolved in their drinking water in a concentration of 0.05 per cent.

Fibrinogen: Bovine plasminogen-rich fibrinogen (Armour Pharmaceutical Co. Kankakee, Ill.) was dissolved in borate-saline buffer (pH 7.75) and used for the evaluation of fibrinolytic activity by the following methods.

Antifibrinolytic Agent: Tranexamic acid (Daiichi Seiyaku Co., Tokyo, Japan) was dissolved in a concentration of 0.05 M in the drinking water. It was also dissolved in the same concentration in the drinking water containing 0.05 per cent BBN. This concentration was virtually sufficient to inhibit the urinary fibrinolytic activity when administered to the rats ad libitum.

Urinary Fibrinolytic Activity: The rat urine was collected in metabolic cages, using phenyl mercuric nitrate (1 : 1 000) as a preservative. After filtration through a Buchner funnel with Whatman's No. 4 filter paper urinary fibrinolytic activity was estimated by the fibrin plate method of Astrup and Müllertz (11) with slight modifications and expressed in Ploug units of urokinase per one ml of 24 h urine.

Fibrinolysis Autograph: A slight modification of the histochemical technique of Todd (9) was used to study the topographical distribution of plasminogen activator in tissues as described in a previous paper (7). For the control preparations, plasminogen-free fibrinogen was obtained by affinity chromatography on L-lysine-substituted Sepharose (12).

Fibrinolytic Activity in Blood: A modification of the euglobulin lysis time method described by von Kaulla and Shultz (13) was used. Serum was obtained from the blood drawn from the plexus veineux ophthalmique of rats under ether anesthesia by the use of a capillary glass tube and immediately assayed for fibrinolytic activity. The solution of euglobulin (0.1 ml) from the serum was pipeted into two test tubes, and then one ml of borate-saline buffer and one ml of 0.1 per cent fibrinogen solution were added. These mixtures were then clotted by means of adding 0.01 ml of a solution of thrombin (20 NIH units/ml). The complete dissolution time of the clot was read at 37 C in a water-bath. The average of two determinations was calculated.

Plasminogen Activator Content in Tissue: Plasminogen activator was extracted from the induced tumor and normal bladder according to the technique described by Funahara et al. (14), using 2M KCl as vehicle. Thirty microliters of the extract were put on unheated and heated fibrin plates, and incubated at 37 C for 18 hours. The lyzed areas on the fibrin plates were measured, and plotted against urokinase concentrations on log-log paper, and expressed in Ploug units of urokinase.

Urokinase Inhibitory Activity in Tissue: The extracts from the induced tumor and normal bladder tissue were prepared according to the methods described in a previous paper (10). After blood coagula and fat were removed, the tissues were minced, and homogenized with a 10-fold volume of cold borate-saline buffer (pH 7.75) in a wet grinder for 10 min at 4 C, and then centrifuged for 30 minutes at 23 000 X G. Tissue extracts were obtained as the supernate. With fibrin plates, the inhibitory activity of the extracts against urokinase was calculated by the method of Kawano et al. (15). One inhibitory unit was defined as the activity necessary to inhibit one Ploug unit of urokinase (Green Cross Co. Osaka, Japan).

Urokinase Inhibitory Activity in Blood: The serum obtained was serially diluted with borate-saline buffer, and mixed with the same volume of a standard urokinase solution (30 Ploug units per ml). The fibrinolytic activity of these mixtures was estimated by the method of Brakman and Astrup (16), and the inhibitory activity was calculated by the method of Kawano et al. (15).

Animal Experiment 1: Histochemical studies of fibrinolytic activity in experimental tumors. Six rats were fed ad libitum with the BBN-containing water and a diet of rat pellets. They were kept in a metabolic cage, in a stable air-conditioned room at about 22 C. Two treated and 2 control animals were sacrificed on the 90th, 130th and 160th days after the administration of BBN. The bladders removed were immediately examined for the localization of fibrinolytic activity by means of the fibrinolysis autograph. Three preparations from each sacrificed group were incubated at 37 C for 30, 60, 120 and 180 min respectively, and the localization and degree of fibrinolytic activity were compared with one another. In addition, in order to determine the influence of the induced antifibrinolysis on the epithelial fibrinolytic activity of the bladder, 3 rats were fed ad libitum with the tranexamic acid-containing water for 30 days, and the bladders removed were subjected to the histochemical study.

Animal Experiment 2: Effects of the induced antifibrinolysis on the incidence, growth and histological picture of the experimental tumor. Forty rats were divided into four groups of ten rats each; one group served as the control, and the other three groups received tranexamic acid, BBN, and tranexamic acid and BBN respectively mixed with drinking water as described before.

Necropsy was performed at the end of the 181-day experimental period, except in two animals which succumbed to an unknown cause after 32, and 115 days of tranexamic acid and BBN, and BBN administrations respectively. Blood was collected from the plexus ophthalmique, and the bladder was quickly removed and explored for the development of tumor. Tumor tissues were conventionally fixed, and examined by routine histology. The blood obtained was assayed for fibrinolytic activity and urokinase inhibitory activity. The tumor tissues were homogenized (10 per cent weight per volume homogenate) in borate-saline buffer at 4C. These samples were assayed for urokinase inhibitory activity.

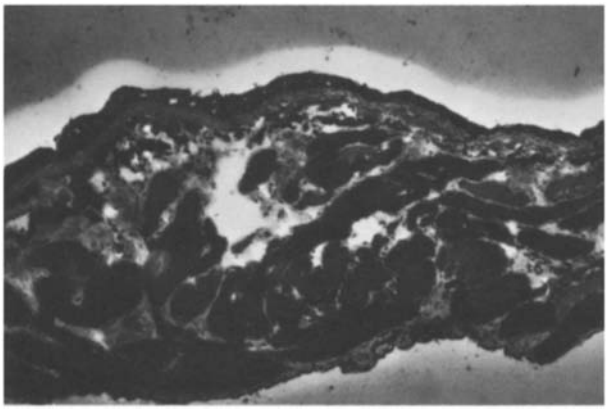

Fig. 1. Fibrinolysis autograph of the normal bladder of rat showing striking fibrinolytic activity related to the epithelium after 15 min of incubation. Original magnification X 50

Results

Histochemical studies of fibrinolytic activity in the bladder during the development of experimental tumors:

After the administration of BBN for 90 days, the bladder epithelium showed hyperplastic proliferation but no histological evidence of malignancy. In this group, the epithelium was fibrinolytically inactive after 15, and 30 min of incubation. After 60 minutes of incubation, however, zones of lysis were observed inconstantly and seen to be unevenly distributed along the mucosal lining. By prolongation of the incubation time up to 120 min the epithelial lining was surrounded by diffuse areas of lysis, and this activity appeared to correspond to that seen in the control bladder after 15 min of incubation.

The administration of BBN for 130 days resulted in the development of benign papilloma, which was quite similar to the common type of bladder tumor in men. Fibrinolytic activity did not appear after incubation for up to 60 min. and only a few small areas of focal lysis related to tumor cells appeared after 120 min of incubation as shown in Fig. 2.

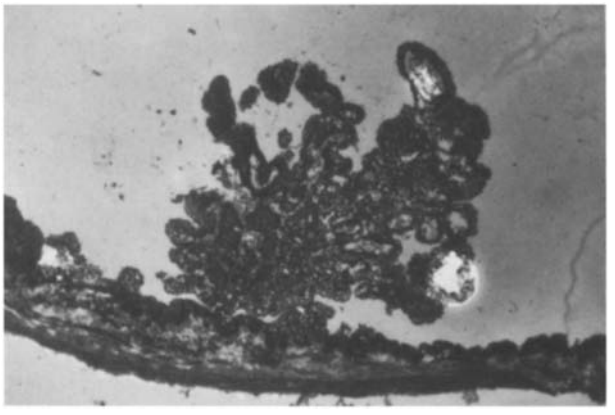

Fig. 2. Autograph showing zones of lysis arising haphazardly at very few sites from BBN induced tumor cell after 120 min of incubation. Original magnification X 20

In the transitional cell carcinoma induced by 160-day administration of BBN, the fibrinolytic activity from the tumor cells was infrequent and showed haphazard distribution even at prolonged incubation of more than 120 min. There was no difference between the activities of tissues sections in the 130-day, and the 160-day administration group.

None of the sections produced zones of lysis on plasminogen-free fibrinogen, indicating the absence of nonspecific protease activity or plasmin, and therefore, the activity was caused by an activator of plasminogen.

These results indicate that the fibrinolytic activity in normal bladder epithelium gradually decreased in the process of tumor development and the absence of fibrinolytic activity was followed by the establishment of cancer.

In the bladders of control animals, diffuse areas with intense lysis of fibrin were observed along the mucosal lining even after 15 min of incubation as shown in Fig. 1. With prolongation of incubation time, lysis areas were widespread and well-demarcated. Among the control bladders, no discernible difference in the epithelial fibrinolytic activity was found.

In the three rats that received antifibrinolytic treatment with tranexamic acid containing drinking water for 30 days, the epithelial activity was quite similar to that in the control bladder in terms of the fibrinolysis autograph.

Effects of the induced antifibrinolysis on the incidence, growth and histological picture of the experimental tumor:

In the group treated with tranexamic acid only, the bladders showed no noticeable histological change. Of the nine rats in the BBN treated group, two showed transitional cell carcinoma, three had epidermoid carcinoma, and the others had the concomitant occurrence of both carcinomas. Of the nine rats in the BBN and tranexamic acid treated group, two had transitional cell carcinoma and epidermoid metaplasia concurrently induced, and others showed epidermoid carcinoma and transitional cell carcinoma. There was no significant difference in the histological grading and staging of the tumor between the two groups; namely, Grades 1, 2, and 3 were seen in five, three, and one rats, and Stages A and B in eight and one rats in the BBN treated group, while Grades 1, 2, and 3 in five, two and two rats and Stage A in all rats in the BBN and tranexamic acid group.

These results indicated that the induced antifibrinolysis showed no significant effect on the incidence and growth of bladder tumors induced by the administration of BBN.

The effect of BBN administration on the fibrinolytic system in rats:

7 of 9 tumors induced provided a sufficient tissue volume for the determination of urokinase inhibitory activity in tumor tissue. Tissue extracts from these 7 tumors and 8 normal bladders which served as control were obtained by centrifuging their homogenates, and the inhibitory activity was determined by the fibrin plate method. As shown in Table 1, the average values for BBN group and control group were 8 540 $\pm$ 980 (in the range of from 7 740 to 10 710) and 79 $\pm$ 10 (in the range of from 60 to 90) urokinase inhibitory units per gm of tissue, respectively, the difference being of statistic significance ($p < 0.005$).

Plasminogen activator in tissue was determined in the 7 BBN induced bladder tumors and the 8 normal bladders. Extracts from these tissues with 2 M KCl were examined. The plasminogen activator activity was 19.7 $\pm$ 2.1 (in the range of from 16 to 23) Ploug units per gm of tissue in the BBN induced tumors and 2.5 $\pm$ 0.7 (in the range of from 2.to 4) in the normal bladders. There was a highly significant increase of plasminogen activator in the BBN tumor group ($p < 0.005$). As evidenced by the ratio of activator and inhibitor, the tumor tissue was found to be strikingly shifted to the side of inhibitor as compared with the normal bladder.

The urokinase inhibitory activities in the blood samples from the 9 rats in the BBN group and 10 rats as control were estimated. As shown in Fig. 3, the average values for the BBN group and control group were 364 $\pm$ 259 (in the range of from 100 to 936) and 38 $\pm$ 20 (in the range of from 12 to 88) urokinase inhibitory units per ml, respec-

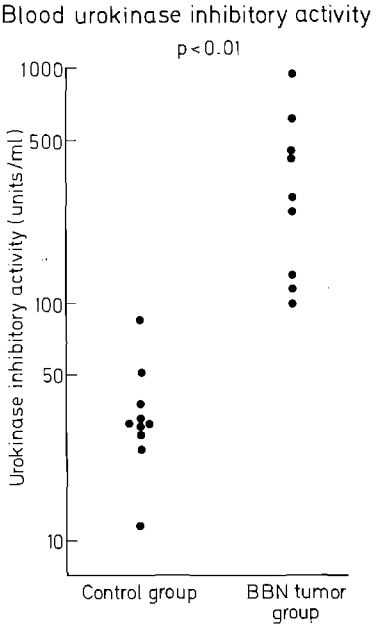

Fig. 3. Urokinase inhibitory activity in blood from rats of control and BBN tumor groups

Table 1. Plasminogen activator and urokinase inhibitor contained in 1 gm of tissue

Tissue	No. of cases	Plaminogen activator (units)	Urokinase inhibitor (units)	Ratio of activator and inhibitor
Normal	8	2.5 $\pm$ 0.7	79 $\pm$ 10	1 : 32
BBN induced bladder tumor	7	19.7 $\pm$ 2.1	8540 $\pm$ 980	1 : 434

tively, the former being significantly higher than the latter ($p < 0.01$).

As shown in Fig. 4, the fibrinolytic activities in the blood samples from the control and BBN groups were 7 ± 2 (in the range of from 4 to 11) and 98 ± 14 hours (in the range of from 80 to 120) of euglobulin lysis time, respectively; there was a highly significant prolongation of the lysis time.

Urinary fibrinolytic activity was estimated on the 18th, 40th, 62th, 104th, 126th, and 146th days after the administration of BBN. The activity varied a great deal, and the urines of some rats were fibinolytically inactive. However, no significant relationship was found between the administration of BBN and urinary fibrinolytic activity.

These experiments indicate that BBN administration results in a reduction of general fibrinolytic activity, and shifts the activity in the direction of antagonism of the fibrinolytic system in rats through the development of bladder tumors containing a large amount of fibrinolysis inhibitors.

Discussion

According to the theories of O'Meara (1, 2), that deposition of fibrin in tumor tissue favors tumor growth, experimental studies on the effect of the induced antifibrinolysis with tranexamic acid or epsilonaminocaproic acid on the growth rate of tumors have been presented by several workers. However, these experimental results have not been in close agreement, and some data are contradictory. If must be taken into account that the discrepancy might be caused by differences between the thromboplastic and fibrinolytic properties of tumor cells and animal organs used. In the present study, the use of tranexamic acid afforded no significant effect on the incidence, growth and histological findings of the BBN induced bladder tumors. This is probably due to the fact that the antifibrinolytic treatment employed resulted in complete depression of urinary fibrinolytic activity, but no inhibitory effect upon the epithelial activity.

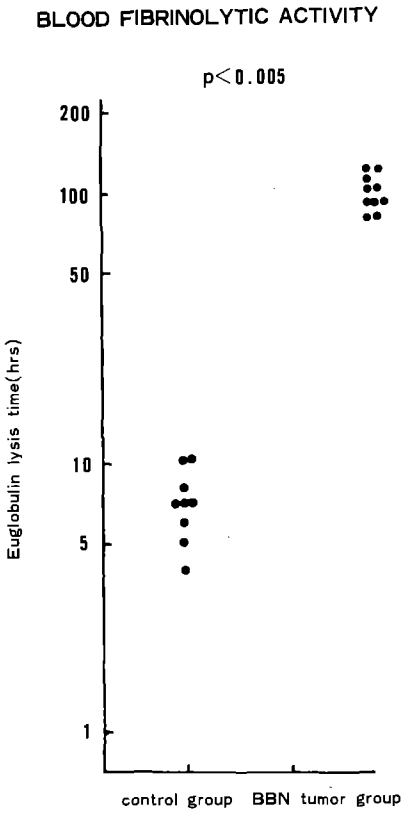

Fig. 4. Fibrinolytic activity in blood from rats of control and BBN tumor groups

Epithelial cells of the rat bladder have been found to be fibrinolytically active as a result of plasminogen activator. The authors also observed diffuse areas of intense lysis related to the mucous membrane. This epithelial activity was gradually reduced during the induction of bladder cancer by the administration of BBN, as estimated by the incubation time necessary for the production of lysis, and the cancers lost the fibrinolytic activity of the normal tissue from which they sprang. The cancerous tissues possessed small amounts of plasminogen activator but very large quantities of urokinase inhibitor, antagonistic to plasminogen activator, therefore the abolition of fibrinolytic activity at cellular level is probably due to the markedly inhibitory ratio of inhibitor to activator in the induced cancer cells. This abolition may be thought of as one of the biological properties of cancer cells, and the ratio of activator and inhibitor in the induced cancer cells would serve for antiplasmin in the fibrinolytic system of blood. In effect, there was a relation between the low fibrinolysis in the cancerous tissue and the prolongation of euglobulin lysis time of the blood. These experimental results may assist the investigation of coagulation disorders noted in patients with cancer.

Recently, Worowski and Farbiszewski (17) reported that serum antiplasmin and antitrypsin levels were found to be increased in malignant diseases of humans and experimental animals, and this phenomenon seemed to be due to an enhanced release of the protease inhibitors from the neoplastic cells into the circulating blood. In addition, they assumed that the enhanced release of the proteolytic enzyme inhibitors into the blood resulted from the increased permeability of the neoplastic cell membranes; the rise of permeability was due to the necrosis concurrent with the neoplastic process, inflammatory foci and disturbances in the circulation. Concerning the release of urokinase inhibitor into the blood, the same mechanisms may be assumed.

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