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Fibrinolytic Activity of in Vitro Cultivated Human Bladder Cell Lines

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Summary. Three human bladder carcinoma cell lines, T 24, RT 4, and MANO, a human bladder non-malignant epithelial cell line, HCV-29, and a human lung fibroblast line, 460 Hl, were investigated for their ability to induce fibrinolytic, urokinase and plasmin inhibitory activities in cell culture, using serum-free medium, for up to 36 h. Generally, the non-malignant cell line and the fibroblast line had a greater ability to produce urokinase inhibitor than d'd the malignant cell lines. The amount produced varied greatly between cells and over the study period. A low concentration of plasminogen activator, immunologically identical with urokinase, and its accumulation in culture supernate were found with RT 4 after 12 h and 24 h cultivations, whereas no plasminogen activator was detected in all other cell lines for periods up to 36 h. No plasmin, non-specific protease or plasmin inhibitory activities were detected in any of the supernates from the cell lines.

Key words: Plasminogen activator - Urokinase inhibitor - Human bladder cell lines - Fibrinolysis.

In a previous study (8) Hisazumi et al. investigated the localization of fibrinolytic activity in normal and cancerous tissues of the bladder using the histochemical fibrin slide techique designed by Todd (24). They found that intact normal bladder epithelium as well as non-injured carcinoma tissues are fibrinolytically inactive. However, an activator of plasminogen was released on destruction of the epithelial cells or carcinoma tissue. In addition, an extract from the carcinoma tissue had a potent inhibitory effect on urokinase. Bernik and Kwaan (2, 3) detected an inhibitor of urokinase in primary tissue cultures of the human bladder and lung. They showed that this inhibitor acted specifically against urokinase. It accumulated in the supernatant and was derived from cells in the culture. They were unable to identify which cell produced the inhibitor. In 1973, Peterson et al. (20) detected fibrinolytic activity in single cells freshly isolated from human malignant tumours. Enhanced fibrinolytic activity was also found in many cell lines derived from human tumours (14). It is believed at present that many malignant cells produce a plasminogen activator and that the identification of fibrinolytic activity may be a useful additional criterion for malignancy in cell culture.

In the present study, 3 human bladder cancer cell lines, a human bladder non-malignant epithelial cell line, and a human lung fibroblast line were investigated with respect to their ability to induce fibrinolytic activity and inhibitory activity in cell culture.

MATERIAL AND METHODS

Cell and Culture Conditions

Five cell lines grown in monolayer culture were used in this study. The cells were routinely grown in Parkers 199 medium with 10 per cent irradiated calf serum with antibiotics (100 i. u. penicillin and 100 µg strepto-

mycin per ml). T 24 and RT 4 were established cell lines from bladder carcinomas and were obtained and originally described by Bulbenfk et al. (4) and Rigby and Franks (21), respectively. These have been used as target cells in many immunological studies (17, 18, 19). MANO was obtained from a grade III bladder carcinoma and supplied by Dr. M. Troye, Wenner-Gren Institute, Stockholm University. In our laboratory, MANO has now reached the 36 passage. HCV-29 was obtained by Dr. J. Fogh from human bladder epithelium, is an established line and has been used as a control in lymphocyte cytotoxicity studies of bladder carcinoma patients (19). 460 Hl was a line of human lung fibroblasts in mid phase II (6). T 24, RT 4 and HCV-29 were originally kindly supplied by Dr. C. O'Toole, Wenner-Gren Institute, Stockholm University. Two days following subcultivation, the cells were rinsed four times in Hams F 10 medium without serum and antibiotics and finally placed in this medium containing 0.2 per cent gelatin (to prevent absorption of the activator to the plastic surfaces) and antibiotics (see above). The medium was then sampled at 12 hour intervals for a period of 36 hours. At each sampling, the culture was examined by phase-contrast microscopy.

The sampled medium was immediately filtered through a 0.22 µ Millipore filter (Millipore Corp. Bedford, Mass., USA) and stored at -20° C until assayed. Following the final sampling the cell viability and numbers in each bottle were estimated using a cytograf 6300 A (Biophysics systems, Inc., Baldwin, Place Road, Mahopac, N. Y., USA). Control medium was prepared by incubation in bottles without cells.

Topographical Observation of Fibrinolytic Activity

For this study, we chose two of the cell lines RT 4 and 460 Hl, as the former was a plasminogen activator producer and the latter an urokinase inhibitor producer (see Results). Two days prior to the experiment the cells were subcultivated into Petri dishes containing glass pieces 25 x 40 x 2 mm. These were rinsed in serum free Hanks B. S. S. four times to ensure the complete removal of serum inhibitors and then placed in a new Petri dish. Two such glass pieces were examined parallel, one being examined with living cells and the other following fixation in 50 per cent methanol in normal saline for 30 s followed by rinsing in saline.

A modified histochemical fibrin slide technique according to Bernik and Kwaan (2) was applied for the detection of plasminogen activation using both fixed and living cells as described above. The fixed cells were examined as follows. Ten ml of human thrombin (Topostasin, Roche, Basel, Switzerland), 25 NIH units per ml, were pipetted onto the preparation and 100 µl of human plasminogen-rich fibrinogen, 90 per cent clottable, (Kabi Co., Stockholm, Sweden) 0.7 per cent, pH 7.6, ionic strength 0.15, were added and rapidly mixed with thrombin to form a fibrin film approximately 0.06 mm thick. The preparations were incubated in a moist chamber at 37° C for 120 min. For control preparations, plasminogen-free fibrinogen obtained by the affinity chromatography on L-lysine-substituted Sepharose (Daiichi pure chemical Co. LTD., Tokyo, Japan) was employed.

The living cultures were examined as follows. A drop of human thrombin was placed on the microscope slide on which the cells were growing and allowed to spread over the surface. Then, 100 µl of human fibrinogen was added and mixed with the thrombin by gently tilting the dish. Lysis of the fibrin by cells in culture was monitored by continuous phase-contrast microscopy at 37° C for 2 h. This procedure was also performed using plasminogen-free fibrinogen to differentiate between plasminogen activator and non-specific proteases.

The Assay of Fibronolytic Activity in Supernatant Culture Media

Serum-free supernates from cultures were assayed by the fibrin plate method (1) using bovine plasminogen-rich and plasminogen-free fibrinogen as substrates. Bovine plasminogen-rich fibrinogen 97.3 per cent clottable, (IMCO Co. LTD., Stockholm, Sweden) was dissolved in saline barbital buffer, pH 7.75, total ionic strength 0.15, at a concentration of 0.2 per cent. Plasminogen-free fibrinogen was obtained by the affinity chromatography as mentioned above, and was used for the control preparations. Bovine thrombin (Thrombin, Sigma chemical Co., St. Louis, MO., USA) was dissolved in 0.9 per cent saline solution at a concentration of 25 NIH units per ml. Fibrin plates were prepared as described previously (7). A drop (30 µl) of the culture supernates was applied on both the plasminogen-free and plasminogen-rich fibrin plates and the plates were incubated at 37° C for 18 h. The results were plotted against urokinase standards of different strengths (Urokinase reference standard, Calbiochem, San Diego, Calif., USA) and were expressed as Ploug units[1].

[1] One Ploug unit, in terms of fibrinolytic activity, is approximately equivalent to 1.4 CTA units, 3 Abbott units or 17 Sterling-Wintrop units.

Inhibitory Activity of the Culture Supernates

Having verified the there was no plasmin activity which might interfere with assays of inhibitor, culture supernates were studied for plasmin inhibition. For this determination, both the fibrin plate technique and caseinolytic method (12) were employed.

In the fibrin plate technique, culture supernates and control supernates that had not been exposed to cells were mixed with the same volume of human plasmin (kindly supplied by Kabi Co., Stockholm, Sweden) standards, 0.25-1.0 casein units per ml, respectively. A drop (30 µl) of these mixtures and plasmin standards was placed on plasminogen-free fibrin plates in triplicate, and the plates were incubated at 37° C for 18 h. The plasmin inhibitory activity in supernates was assessed by a decrease in lytic areas as compared to controls and was expressed in units per ml of plasmin inhibited by supernates.

The determination of caseinolytic activity was carried out according to the procedure described by Bernik and Kwaan (3). 0.5 ml of the supernates was mixed with an equal volume of serial dilutions of plasmin standards (0.5 - 2.0 casein units per ml) and incubated at 37° C for 30 min. After incubation, 1.5 ml of Tris (0.06 M) - NaCl (0.09 M) buffer, pH 7.5, and 2.5 ml of alpha casein (Worthington Biochem. Co., Freeholm, N.J., USA) 1.4 per cent, in Tris buffer, were added to each of the mixtures to give a final volume of 5.0 ml. The tubes were then incubated at 37° C. A 2.0 ml aliquot was sampled from each tube after 2- and 32-min incubations, and by adding 3.0 ml of 0.5 M perchloric acid the reaction of the mixtures was stopped. These mixtures were allowed to stand at room temperature for 30 min and then filtrated through Munktell filter paper, No. OOH (Grycksbo Pappersbruk, Sweden), corresponding to Whatman No. 42 filter paper. Caseinolytic activity was determined in the filtrates at 275 mµ. Plasmin inhibitory activity in the supernates was evidenced by a decrease in casein proteolysis as compared to that of the simultaneously assayed plasmin standards in Tris buffer and control medium without cells.

As mentioned above, plasminogen activator inhibition was determined in supernates that showed no plasmin inhibition in either caseinolytic or fibrinolytic assays. These supernates were examined against urokinase and tissue activator from pig heart (kindly supplied by Prof. Per Wallén, Umeå University, Sweden) having a specific activity of more than 10,000 Ploug units per mg protein. Both culture supernates and control supernates were mixed with equal volumes of urokinase standards, 2.4 - 9.6 Ploug units per ml, or of tissue activator standards, 7.5 - 30.0 Ploug units per ml. These mixtures were incubated at 37° C for 30 min and then a drop (30 µl) of these mixtures was applied to plasminogen-rich fibrin plates in triplicate. These plates were incubated at 37° C for 18 h. Control standards in buffer were assayed simultaneously. The inhibitory activity in supernates was determined by a decrease in lytic areas as compared with controls and expressed in units per ml of urokinase or tissue activator inhibited by the supernates.

Immunological Study of Plasminogen Activator in Supernatant Culture Media

Antiserum (supplied through the courtesy of Dr. Y. Wakayama, Kyoto University, Kyoto, Japan) prepared by the subcutaneous injection of purified human urokinase into rabbits, was used to identify immunologically the activator activity in supernates of cultures.

Supernates showing plasminogen activator activity were assayed with urokinase (4.8 - 38.5 Ploug units per ml) standards on 2 series of fibrin plates incorporating antiserum and control serum respectively in final concentrations of 1 : 1000. The degree of specific neutralization of activator in culture media was compared to that of the urokinase standards and the results were expressed as percentage neutralization of activator activity.

RESULTS

Cell Cultures in Serum Free Medium

Following the placement of the cultures in the serum free medium, in the case of the malignant cell lines cell division decreased but did not cease This was not the case for HCV-29 and 460 Hl which ceased to divide in the serum free medium. In all cases except for 460 Hl, a few dead cells could be found floating in the serum free medium at all times up to 36 hours.

The Fibrinolytic Activity of the Culture Medium

As is shown in Table 1, in all the supernates no plasmin nor non-specific proteolytic activity was detected by the plasminogen-free fibrin plate method. Plasminogen activator activity was found at 24 and 36 h in the supernates from RT 4. The production of this activator appeared to increase with time in the serum free condition showing a maximum of 12.61×10^{-5} Ploug units per cell per 12 h after estimation at 36 h.

Table 1. Fibrinolytic activity measured at different times in the supernatant medium when exposed to cells for 36 h

Plasmin or non-specific protease activity			
Sampling time (h)	12	24	36
All cell lines	0	0	0

Plasminogen activator activity (Ploug units/cell/12 h)			
Sampling time (h)	12	24	36
RT 4	0	8.46×10^{-5}	12.61×10^{-5}
All other cell lines	0	0	0

Table 2. Inhibitory activity measured at different times in the supernatant medium when exposed to cells for 36 h

Plasmin inhibitory activity			
Sampling time (h)	12	24	36
All cell lines	0	0	0

Urokinase inhibitory activity (Ploug units)

	Sampling time (h)	/ml	(cells/ml)	total produced	$\times 10^{-7}$/cell/12 h
T24	12	0.3	(480,150)	3.6	74.9
	24	0.6	(720,225)	+2.4	33.3
	36	0	(1,440,450)	-2.4	-16.6
RT4	12	0.5	(56,033)	6.0	1070.8
	24	0.5	(84,050)	+0	0
	36	3.6	(168,100)	±12.4	737
MANO	12	0.5	(217,283)	6.0	276.1
	24	0.6	(325,925)	+0.8	24.5
	36	0.6	(651,850)	±0	0
HCV -29	12	27.7	(10,466)	332.4	300000
	24	19.3	(15,700)	-67.2	-42802
	36	37.3	(31,400)	+72.0	22929
460 Hl	12	3.2	(69,637)	38.4	5514.3
	24	2.7	(104,456)	-4.0	-382
	36	8.5	(203,912)	+23.2	1110

Plasmin Inhibition

Using both caseinolytic and fibrinolytic assays, all supernates were examined for antiplasmin activity. As shown in Table 2, they exerted no inhibitory effect on plasmin.

Plasminogen Activator Inhibition

Activator inhibitory activity was determined in all supernates as they had no inhibitory effect on plasmin (see Table 2). The supernate from all cell lines showed some urokinase inhibitory activity, those from 460 Hl and HCV-29 revealing potent activity. As can be seen from Table 2, the general tendency in all supernates was a decreased activity with time when the total activity is related to the cell number present. In some cases, however, transient decreases were found in supernates at 24 h as compared with the 12 h value. The same supernates at 36 h again showed increased activity even if the amount was then always less than that observed at 12 h (RT 4, HCV-29, and 460 Hl). Total loss of urokinase inhibitory activity was also observed under a 12 h period in the same supernate sampled at 24 and 36 h (T 24).

Inhibition of tissue activator was simultaneously examined using the supernates. No inhibitory effect on tissue activator was demonstrated which indicates that inhibitory activity appears to be directed selectively against urokinase.

As shown in Figures 1 and 2, in immunological assays with the anti-urokinase serum, there was 100 per cent neutralization of activator activity in supernates of RT 4 as compared to that of the urokinase standards. Therefore, it is quite possible that the plasminogen activator is antigenically identical to urokinase.

Local Fibrinolytic Activity of Cells in Culture

Fixed cells from RT 4 and HCV-29 lines showed no fibrinolytic activity after 2 h incubation with plasminogen-rich fibrin. When the living cells were exposed to plasminogen-rich fibrin during culture in Petri dishes, the fibrin was not lysed by the cells in culture even after 2 h of incubation.

DISCUSSION

Some authors have suggested serum-containing medium for the investigation of fibrinolysis in cells cultured from various tissues since they consider serum essential for satisfactory

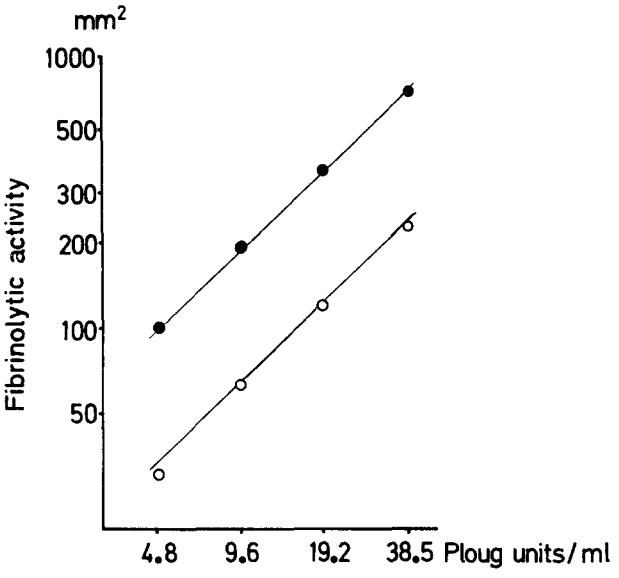

Fig. 1. Inhibitory effect of urokinase antisera on human urokinase activity. Fibrin plate assay •—• fibrin plates incorporating control serum (1 : 1000) ○—○ fibrin plates incorporating antiurokinase serum (1 : 1000). Percentage neutralization of urokinase: 73%

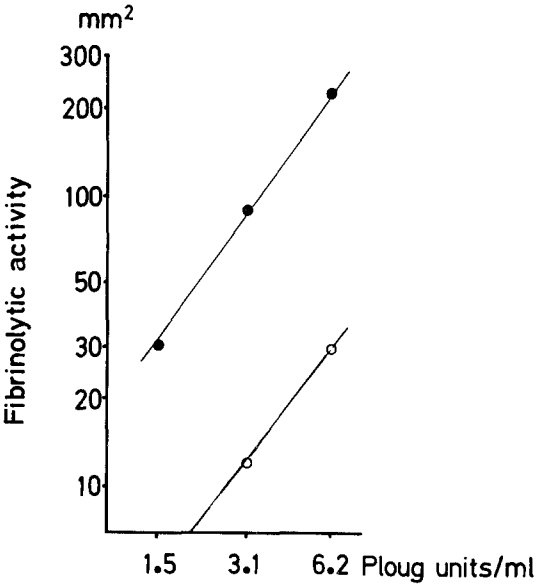

Fig. 2. Inhibitory effect of urokinase antisera on plasminogen activator in RT 4 supernate. Fibrin plate assay. •—• fibrin plates incorporating control serum (1 : 1000) fibrin plates incorporating antiurokinase serum (1 : 1000). Percentage neutralization of plasminogen activator: 75%

growth conditions and cell function. However, for the analysis of components of the fibrinolytic system, especially inhibitors of fibrinolysis, the presence of abundant plasmin inhibitor in serum produces considerable problems. For that reason, we have adopted the serum-free condition in common with most researchers (2, 3). Whether cell function in these experiments reflects the cell function in the presence of serum cannot be judged with certainty since the influence of serum factors on cell growth and function is not known. During the course of the experiments reported here the cells showed no significant decrease in numbers or viability, the malignant lines showing some increase in cell numbers.

So far very little is known about the fibrinolytic and anti-fibrinolytic properties of bladder carcinoma cells in culture or otherwise. Recently, Laug et al. (14) using two cell lines derived from human bladder carcinoma, (HT-1197 and HT-1376), showed that both cell lines exhibited high levels of fibrinolytic activity after 12-hour and 24-hour cultivations. They stated that malignant cells are more potent in inducing fibrinolysis than their normal counterparts with the exception of normal cells derived from the lung and kidney. There is convincing evidence that many malignant cells produce plasminogen activators (5, 13, 14, 16, 25, 26). According to Bernik and Kwaan (2), cells from fetal and adult tissues produced activator antigenically identical with urokinase, which they called urokinase-type activator, as well as an activator which differed from urokinase in immuno-assay, called tissue-type activator. The latter activity was released following the injury or death of cells, whereas urokinase-type activator was produced in cultures, containing live, metabolizing cells.

The data presented in Table 1 show that cell lines derived from bladder carcinoma do not invariably possess the ability to produce or secrete plasminogen activator. These data together with that obtained in the topographical observation of the fibrinolytic activity of cells in cultures suggest that even if they are activator producers, this production may be very weak. Jones et al. (13) have reported a similar result.

No demonstrable antiplasmin activity was found, but all the cells produced urokinase inhibitors. The amount produced varied greatly between the cell lines and even in the amounts detected in the same medium exposed to the cells for different lengths of time. The greatest production was found in the HCV-29 and 460 H1 cultures which is in agreement with Bernik and Kwaan's findings (3). From Table 2, it is evident that in some samples the inhibitor production had decreased markedly in the

second assay, but it could increase again folfowing a further 12 hour observation. This indicates that it is not only a matter of declining production with time, but a more complex reaction that requires further study.

Our present immunological studies show that a cancer cell line, e.g. RT 4, can produce an activator in culture antigenically identical to urokinase. The fact that cells growing without serum and in the artificial conditions which in vitro cultivation implies, still produce plasminogen activators and/or urokinase inhibitors suggests that these are basic functions of the cell and are retained even after malignant transformation.

In contradistinction to the low production of urokinase inhibitor by bladder carcinoma cells in the in vitro conditions described here, a high level of urokinase inhibitor occurs in the blood of patients with advanced carcinoma of the bladder (11). Soong and Miller (23) reported significantly enhanced plasma levels of urokinase inhibitor in a group of 100 patients with disseminated tumours of various kinds. In an experimental study of N-butyl-N-butanol (4)-Nitrosamine induced bladder tumours in rats, Hisazumi et al. (10) found a correlation between the local production of plasminogen activator or urokinase inhibitor in the tumour, and the plasma level of activator or inhibitor respectively, indicating that the enzyme production in the tumour tissue has an influence on the fibrinolytic potential of plasma. As mentioned in the introduction, Hisazumi, in a previous study (9), found fragments and extracts from human bladder carcinoma to contain urokinase inhibitor in a significantly higher concentration than in normal bladder tissue. He also reported a greater anti-tumour effect of intracavitary thio-tepa treatment in superficial bladder tumours if urokinase was added to the instillation fluid. This combined therapy with increased fibrinolytic activity may result in a better access of simultaneously administered anticancer agents into cancerous tissues and cells and by promoting the production of plasmin (a possible lysosomal labiliser), might result in intracellular lysosomal release (15, 22). To obtain the optimum results from such a therapy it is advisable to provide an excess of urokinase. As yet we have no basis on which to assess the amount of urokinase required.

The observation of high urokinase inhibitor concentrations in bladder carcinoma tissues is not in agreement with our present finding of very low inhibitor production in tumour cells in serum free medium in vitro. It must be considered, however, these tissue culture experiments involve only a few cell lines under artificial conditions, whereas the tumour, like normal tissues, contains various tissue compartments such as connective tissue and blood vessels which are potential producers of factors which may influence the local fibrinolytic environment. For the investigation of a single cell compartment, tissue culture can be superior under certain conditions but the analysis of tissue fragments may give a more complete picture of the biochemical properties and interactions of tumours.

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