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Establishment of a Human Renal Pelvic Cancer Cell Line Producing Tissue Thromboplastin and Plasminogen Activator

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Summary. A new epithelial cell line derived from undifferentiated carcinoma of human renal pelvis, designated KP 1, was established in vitro. The cell line has been passaged 190 times in vitro for 5 years and 9 months. The predominant cell in KP 1 was a tear-drop-shaped cell. Doubling time of the cell line was 35 h. The malignant epithelial character of this line was verified by carcinogenictiy in the subcuticular layer of nude mice and by karyotypic analysis which revealed the cells to be completely aneuploid with a model chromosome number in the hypertriploid range. KP 1 cells were shown to produce both tissue thromboplastin and plasminogen activator which was immunologically identical to urokinase, the plasminogen activator in urine.

Key words: Cultured human cancer cells, Renal pelvic cancer, Thromboplastic activity, Fibrinolytic activity, Plasminogen activator.

Introduction

Cancer cell lines cultured in vitro are useful tools for studying oncology. However, long-term cultures of human urinary tract tumours have been difficult to establish. Only limited success has been achieved with human renal pelvic cancer [6].

Fibrin deposition has been considered to be one of the important factors influencing growth and spread of tumour, although there is still controversy as to its significance [8, 10, 15, 16, 18, 20, 22]. O'Meara [15, 16] observed extravascular deposition of fibrin in human malignant tumours and suggested that fibrin at the advancing border of growing tumour favoured infiltrating growth and spread. Peterson [18], on the other hand, indicated that fibrin deposition in tumour tissue might be a defence mechanism against the growing tumour. Fibrin deposited around tumour cells has also been considered to act as a glue facilitating the adhesion of blood-borne metastases to capillary walls [22]. Thus, much attention has been given to the thromboplastic and fibrinolytic properties of tumour cells which might influence such fibrin metabolism [7, 9, 10, 18, 20, 21].

This paper describes the establishment and some biological characteristics – including thromboplastic and fibrinolytic activities – of a new human renal pelvic cancer cell line (KP 1).

Materials and Methods

Tumour and Cell Culture

Tumour tissue of the renal pelvis of a 55-year-old female was obtained at surgery of July 29th, 1975. As the tumour was composed of a whitish part and a yellowish part macroscopically, the two parts were subjected to cell culture separately. Microscopic examination of the two parts revealed different histological appearances, the whitish part undifferentiated carcinoma and the yellowish part papillary transitional cell carcinoma with squamous differentiation. Each tissue was washed, minced and dispersed by 0.1% collagenase (Type IV, Worthington Biochemical Corp.) in Joklik modified minimum essential medium (GIBCO) at 37 °C. The cells (1×10^6) suspended in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Japan) supplemented by 10% foetal calf serum (GIBCO) and antibiotics (penicillin G, 100 unit/ml; streptomycin, 100 μg/ml) were plated into a 60 mm culture dish and incubated at 37 °C in 5% CO_2 in air with medium changes every 3 or 4 days. Monolayers were subcultured by treatment with calcium- and magnesium-free phosphate buffered saline containing trypsin (0.1%) and ethylenediaminetetraacetic acid (0.2%) at 37 °C for a few minutes and re-seeded. Phase contrast microscopy of the living cells attached to culture dishes was carried out with a Nikon inverted microscope.

Growth Kinetics

Growth curves were established by seeding 4×10^4 cells into tissue culture tubes, followed by counting adherent cells at appropriate intervals in three replicate tubes.

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Table 1. Correlation of wet weight of cancer cells to cell number and protein content

Cell line	Wet weight (mg)	Cell number ($\times 10^5$)	Protein content	
			Saline extract (mg)	2 M KSCN extract (mg)
KP 1	100	72. 8 ± 6.3	3.21 ± 0.34	2.06 ± 0.10
KU 1	100	74.3 ± 5.4	2.65 ± 0.11	1.79 ± 0.30

Values are means ± standard deviation of 5 samples

Heterotransplantation

Congenitally athymic nude mice, BALB/c origin, about two months old, supplied from CLEA JAPAN INC., were used to examine the carcinogenicity of the cultured cells. These mice, bred and maintained in sterile conditions in a vinyl isolator, were given subcutaneous injections of the 5×10^6 cultured cells suspended in Hanks' balanced salt solution (Nissui Pharmaceutical Co., Japan). The tumours which developed were histologically examined.

Chromosome Analysis

Monolayer cultures were treated with 0.05 μg Colcemid (GIBCO)/ml for 6 h and exposed to a hypotonic solution (0.075 M potassium chloride solution) for 10 min at room temperature. The cells were fixed with freshly prepared methanol/acetic acid (3:1), dropped on clean slides and air-dried. G banding was accomplished by treating the cells with a 0.25% trypsin solution for 3 min. The slides were rinsed with phosphate buffered saline, allowed to stand at room temperature for 24 h, and then stained with buffered Giemsa.

Thromboplastic and Fibrinolytic Activities

Thromboplastic and fibrinolytic activities of the cultured cells were estimated both in cancer cell lysate and serum-free supernatant fraction as compared with those of a malignant epithelial cell line, KU 1, which was established from transitional cell carcinoma of human urinary bladder in the Department of Urology, Faculty of Medicine, Keio University, Japan [23, 24].

Anti-urokinase immunoglobulin G, was prepared as described previously [13]. Antiserum against human urokinase (UK), the plasminogen activator in urine, was produced by injecting goats with highly purified human UK (M.W. 51,000; Specific activity, 90,100 IU/mg)[1]. The immunoglobulin G (IgG) fraction was isolated from the sera by gel filtration through a Sephacryl S-200 superfine column after precipitation with saturated ammonium sulphate. Normal IgG fraction was also prepared from untreated goat sera in the same manner.

Plasminogen-rich fibrin agar plates (FAPs) were prepared using commercially obtained plasminogen-rich human fibrinogen (The Green Cross Corp., Japan) by the method reported previously [14]. For the fibrinolysis neutralisation experiment described below, FAPs containing 0.2 mg of the anti-UK IgG or normal IgG at a final concentration of 1:50 to the fibrinogen were prepared.

Purified plasminogen-free fibrinogen was prepared from human plasma by the method of Barnhart and Forman [3] after chromatography on lysine-Sepharose by the method of Deutsh and Mertz [5]. The purified plasminogen-free fibrinogen was 98% clottable by an excess of bovine thrombin. Plasminogen-free FAPs were also prepared in the same manner.

The method used to prepare the lysate of confluent cell cultures for the assay of thromboplastic and fibrinolytic activities and the fibrinolysis neutralisation experiment was similar to that reported by Kinjo et al. [9]; thromboplastic and fibrinolytic activities were extracted with 0.9 ml of physiological saline and lml of 2 M potassium thiocyanate solution respectively from 100 mg wet weight of cancer cells by freeze and thawing, and sonication. The number of these cancer cells and the protein content of each lysate are shown in Table 1.

To prepare the serum-free supernatant fraction 5×10^5 cells were plated in 60 mm petri dish and allowed to attach and grow for 24 h. The medium was then removed. The cells were washed three times with warm serum-free culture medium and incubated again for 24 h at 37 °C in 3 ml of the serum-free culture medium. The serum-free supernatant was collected and clarified by centrifugation at 900 x g for 10 min and used for the assay of thromboplastic and fibrinolytic activities and the fibrinolysis neutralisation experiment.

Thromboplastic activity of the cell lysate and serum-free supernatant fraction prepared as above was recorded as plasma recalcification time (PRT) using standard human plasma as reported by Kinjo et al. [9]. In order to determine whether coagulation mainly depended upon the extrinsic or intrinsic system, the samples were subjected to an assay system using Factor VII or Factor IX deficient human plasma in addition to standard human plasma.

Assay and neutralisation experiments of fibrinolytic activity of the cell lysate and the serum-free supernatant fraction prepared as above were performed using standard FAP, FAP containing the anti-UK IgG and FAP containing normal IgG as reported previously [13, 14]; dilution series of the samples (0.01 ml) were applied to these three types of FAPs. After incubation at 37 °C for 18 h, the diameter of the lysed area examined on standard FAP was compared with that of standard UK (Mochida Pharmaceutical Co., Japan). The fibrinolytic activity of the cell lysate was expressed in International units of UK per one milligram protein and that of the serum-free supernatant fraction in International units of UK per one millilitre. Specific neutralisation of the fibrinolytic activity by the anti-UK IgG was estimated by decrease or absence of lysed area on FAP containing the anti-UK IgG as compared to that examined on standard FAP or FAP containing the normal IgG.

The samples were applied to plasminogen-free FAP in order to differentiate plasminogen activator from non-specific proteases.

Results

Growth in Vitro

The cell line KP 1 developed from the whitish part of the tumour, whereas growth of the cells from the yellowish

[1] Kindly donated by Mochida Pharmaceutical Co., Japan

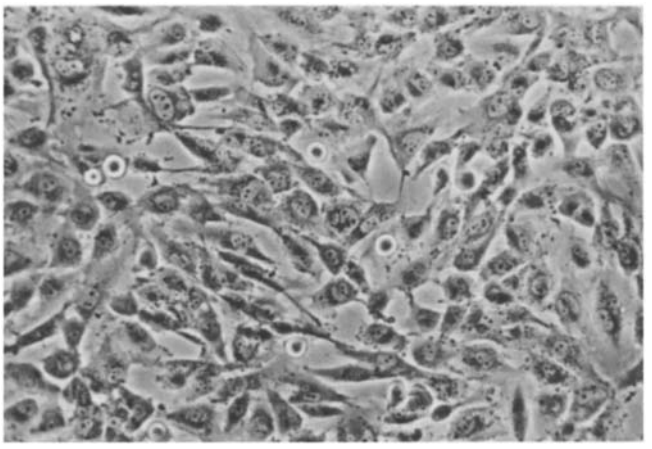

Fig. 1. Phase-contrast micrograph of KP 1 cells at the 49th passage. x 27^5

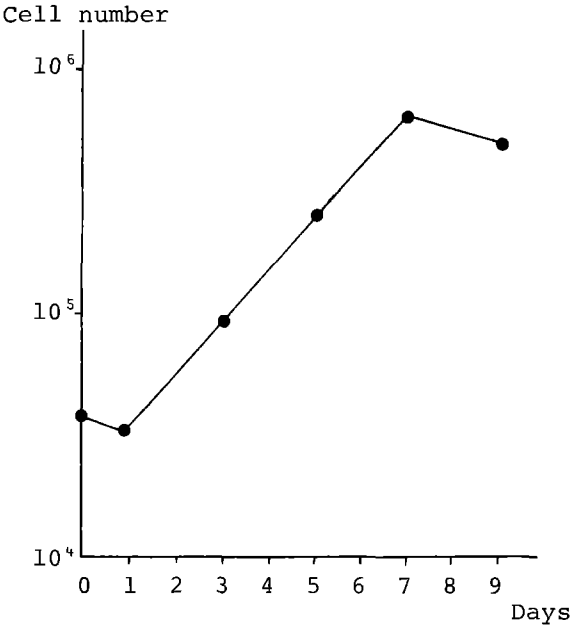

Fig. 2. Growth curve of KP 1 cells at the 120th passage. An average of three counts for each time period was used to define each point

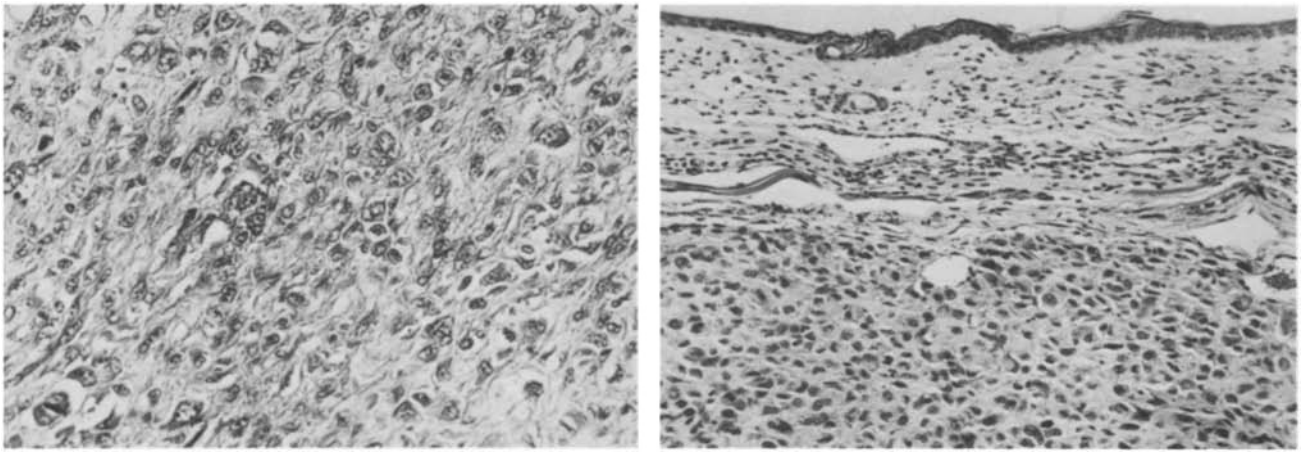

Fig. 3. Histology of the whitish part of the original tumor characterized by pleomorphic cells (left x 200) and nude mouse tumor induced by KP 1 cells at the 135th passage (right x 130)

part was not obtained. Good attachment and growth of cells from the whitish part of the tumour were observed in primary culture within 4 days and the secondary culture was made after 3 weeks. Subculture was initially performed at a very conservative rate in order to maintain a high cell density in the dish. After the 10th passage, however, the cells were subcultured at a 1:8 ratio. The morphology of KP 1 cells by phase-contrast microscopy is shown in Fig. 1. In several early passages contaminating fibroblasts were observed. However, they disappeared gradually and only teardrop-shaped cells continued to grow. KP 1 cells had large oval or round nuclei with a few predominant nucleoli. KP 1 cells lacked contact inhibition, and multilayering was seen if the cells were not subcultured when they reached a monolayer. The growth curve of KP 1 cells at the 120th passage is shown in Fig. 2. The population doubling time calculated from the best-fit line for logarithmic growth was approximately 35 h. KP 1 has now been subcultured 190 times during the past 5 years and 9 months (up to April 1981). The morphology and in vitro growth properties of cell line KP 1 have changed little during this period.

Growth in Nude Mice

Nude mice injected subcutaneously with KP 1 cells at the 7th and the 135th passages all developed subcutaneous tumours a few centimetres in diameter within 90 days. The tumours were well circumscribed and surrounded by thin fibrous connective tissue. The histological appearances of the tumours produced by KP 1 cells at the 7th and the 135th passages were quite similar to each other and to that of the whitish part of the original tumour (Fig. 3).

Table 2. Chromosome frequency distribution in KP 1 cells

Passage number	Chromosome number																			No. of cells examined
	63	64	65	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	88	
53	–	–	–	1	–	–	1	–	2	2	1	3	7	4	4	2	21	2	2	1	53
110	1	1	1	1	1	3	4	15	20	22	33	35	12	4	5	1	–	1	–	–	160

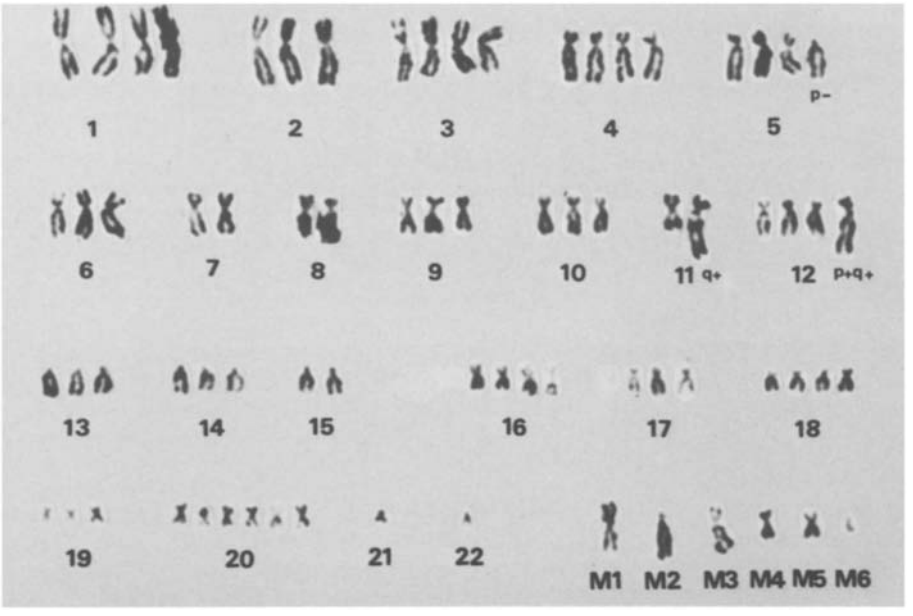

Fig. 4. Trypsin-Giemsa banding karyotype of KP 1 cells at the 110th passage with 74 chromosomes

Table 3. Thromboplastic activity of cell lysate and serum-free supernatant fraction

Cell line	Sample	Plasma recalcification time (sec)		
		Standard human plasma	Factor VII deficient human plasma	Factor IX deficient human plasma
KP 1	Cell lysate	24.5 ± 0.9	249.9 ± 12.6	27.4 ± 1.6
	Serum-free supernatant fraction	161.9 ± 5.5	Not examined	Not examined
KU 1	Cell lysate	28.9 ± 0.5	154.8 ± 3.9	30.1 ± 0.4
	Serum-free supernatant fraction	132.6 ± 7.1	Not examined	Not examined

Values are means ± standard deviation of 5 samples

Chromosome analysis

Table 2 shows the chromosome frequency distribution at the 53rd and the 110th passages. The chromosome number was 100% aneuploid. At the 53rd passage there was a wide frequency distribution of chromosome numbers extending from 67 to 88, the modal number being 80 (40%). Also, at the 110th passage, there was a wide frequency distribution of chromosome numbers extending from 63 to 81. However, the modal number has shifted from 80 to 75 (22%) with another peak at 74 (21%).

Detailed karyotypic analysis by G banding was performed at the 110th passage. As shown in a typical karyogram (Fig. 4), several characteristic marker chromosomes (M1 ~ M6) and chromosomes with multiple rearrangements resulting in unusual G banding pattern (5p–, 11p+, 12p+q+) were detected. Of these marker chromosomes, M1 and M2 were most commonly observed. Sex chromosome was not clearly identified.

Table 4. Fibrinolytic activity of cell lysate and serum-free supernatant fraction

Cell line	Samples	Fibrinolytic activity
KP 1	Cell lysate	0.24 ± 0.03
	Serum-free supernatant fraction	Not detectable
KU 1	Cell lysate	0.24 ± 0.06
	Serum-free supernatant fraction	0.01 ± 0.00

Values are means ± standard deviation of 5 samples. The fibrinolytic activity of the cell lysate is expressed in International units of UK per one milligram protein and that of the serum-free supernatant fraction in International units of UK per one millilitre

Table 5. Neutralisation assay of the fibrinolytic activity of KP 1 cell lysate by anti-UK IgG

Dilution of the lysate	Diameter of lysed area (mm)		
	Standard FAP[a]	FAP[a] containing normal IgG	FAP[a] containing anti-UK IgG
1	10.0	9.6	0
1/2	8.0	8.7	0
1/4	6.9	7.4	0
1/8	6.3	6.2	0
UK 10 IU/ml	16.6	16.7	0

[a] Fibrin agar plate

Thromboplastic Activity

Plasma recalcification time (PRT) was recorded on five samples at the 120th passage. Average values and standard deviation of PRT are shown in Table 3. The cell lysate of KP 1 showed considerable thromboplastic activity. In comparison with the thromboplastic activity examined with standard human plasma, the cell lysate showed similar activity when using Factor IX deficient human plasma, but lower activity when using Factor VII deficient human plasma. Considerable thromboplastic activity, which was also Factor IX-independent and Factor VII-dependent, was recognized in the cell lysate of KU 1 as well. The thromboplastic activity was also recognised in the serum-free supernatant fractions from these two cell lines, although it was low as compared with that of the cell lysates.

Fibrinolytic Activity

Five samples at the 126th passage were assayed. Average values and standard deviation of the fibrinolytic acitivity are shown in Table 4. The cell lysate of KP 1 showed mild fibrinolytic activity. Lysed areas were absent when the assay was performed with plasminogen-free FAP, indicating that the activity was attributable to plasminogen activator. The cell lysate of KU 1 also showed mild plasminogen-dependent fibrinolytic activity. Fibrinolytic activity could not be detected in the serum-free supernatant fraction from KP 1 cells. The serum-free supernatant fraction from KU 1 cells, however, showed low plasminogen dependent fibrinolytic activity.

Fibrinolysis Neutralisation Experiment

Five samples of the cell lysate and serum-free supernatant fractions were tested. As shown in Table 5, the lysed area was absent when the dilution series of the cell lysate of KP 1 and UK (10 IU/ml) were applied to the FAP containing the anti-UK IgG. The diameter of lysed area by the cell lysate of KP 1 or UK on FAP containing the normal IgG was almost the same as that examined on the standard FAP. This indicated that the IgG prepared in this study was not contaminated by various types of fibrinolysis inhibitors contained in the serum. Therefore, the complete inhibition of the fibrinolytic activity of the cell lysate of KP 1 on the FAP containing the anti-UK IgG was considered to be due to the specific reaction between the plasminogen activator in the cell lysate of KP 1 and the anti-UK IgG. Such specific complete inhibition of the fibrinolytic activity by the anti-UK IgG was recognised in the cell lysate and serum-free supernatant from KU 1 as well. The lysed area by the seum-free supernatant fraction from KP 1 was not detected at all on the three types of FAPs.

Discussion

We have reported here some of the characteristics of a human renal pelvic cancer cell line KP 1 which was established and has been maintained in our laboratory for 5 years and 9 months. It was proved that this cell line was malignant epithelial in origin by the following facts: 1) the cells produced solid tumour in nude mice at the site of subcutaneous injection, the histological appearance of which was similar to that of the original tumour; 2) the chromosomes of the cultured cells were abnormal in number and structure; 3) the cells had been in continuous culture for 5 years and 9 months without any evidence of a decreased growth rate and lacked contact inhibition in the early passages. Since HeLa cells have never been brought into or propagated in our laboratory, and extensive karyotypic analyses have shown that there are no HeLa marker chromosomes in KP 1 and that its overall karyotype is distinct from that of HeLa, the possibility of HeLa contacmination is not likely.

Recently we reported that we had directly transplanted the whitish part and yellowish part of the original tumour used in this study separately into nude mice and had estab-

lished serially transplantable strains (NKPP 1 and NKPP 2) [14]. The histological appearance of the tumour grown in nude mice by the subcutaneous injection of KP 1 cells in early and late passages was similar to that of the serially transplantable tumour NKPP 1 as well as to that of the whitish part of the original tumour.

It has been demonstrated that human or animal tumour cells have variable degrees of thromboplastic and fibrinolytic activities [7, 9, 10, 13, 14, 18, 20, 21]. This study also revealed that KP 1 cells had both activities, although no definite fibrinolytic activity could be detected in the serum-free supernantant fraction. From the results of coagulation studies using standard Factor VII deficient and Factor IX deficient human plasma, the thromboplastic activity of the cell lysates of KP 1 and KU 1 seemed to be due to tissue thromboplastin of the tumour cells themselves.

It is well known that human brain has extremely high thromboplastic activity [2]. The thromboplastic activity of KP 1 cell lysate was only slightly lower than that of human brain which was assayed previously in the same manner [9]. We reported previously that KP 1 belonged to the group showing moderate thromboplastic and moderate fibrinolytic activities [9]. However, in the present study, on the basis of the previous criteria [9], KP 1, as well as KU 1 can be classified into the group showing high thromboplastic and moderate fibrinolytic activities. The thromboplastic activity of this cell line seems to have changed during the past two years.

Many clinical studies have demonstrated that patients with cancer have a hypercoaguable state [4, 12] and an abnormally high incidence of vascular thrombosis [11, 17, 19]. Davis et al. [4] and Miller et al. [12] postulated the presence of low grade, chronic disseminated intravascular coagulation initiated by slow thromboplastin release from the tumour. This study also suggests that tissue thromboplastin released from viable cancer cells or destroyed cancerous tissue in vivo may be one of the important factor inducing such events.

The results of fibrinolysis neutralisation experiments with the anti-UK IgG indicated that the plasminogen activator of KP 1 as well as KU 1 cells was immunologically identical to UK. Åstedt and Holmberg [1] reported that the plasminogen activator released from six lines of human ovarian cancer cells was immunologically identical to UK. Hisazumi et al. [7] also demonstrated that the plasminogen activator released from human urinary bladder cancer cell line, RT 4, was immunologically identical to UK. On the other hand, Tucker et al. [21] showed immunological differences between the plasminogen activator of both cell lysates and supernatant fractions from eight lines of cultured human brain tumour cells and UK. Recently we also showed that the plasminogen activator released from four lines of gastric cancer, two lines of lung cancer and one line of renal cancer was immunologically identical to UK but that from one line of lung cancer was not. The nature of the plasminogen activator of human cancer cells is little known. Further investigations must be made to discover whether or not plasminogen activator of all urothelial cancer cells is invariably identical to UK.

Thromboplastic and fibrinolytic activities of tumour cells may influence the deposition and removal of fibrin in tumour tissue. Therefore, in order to investigate the significance of coagulation-fibrinolysis system in the growth and spread of tumour using experimental tumours, it may be necessary to analyse the characteristics of the tumour cells themselves beforehand. This KP 1 line showing carcinogenicity in nude mice and characterised by high thromboplastic and moderate fibrinolytic activities will provide useful in vitro and in vivo model systems for studying the significance of coagulation-fibrinolysis system in human cancer. Furthermore, this line may offer possible opportunities for the evaluation of chemotherapeutic agents.

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