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Original Articles

Immunological Analysis of Plasminogen Activators from Cultured Human Cancer Cells

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Summary. Immunological similarities or differences between urokinase and plasminogen activators from 9 lines of cultured human caner cells with varying degrees of fibrinolytic activity were examined with antibodies against human urokinase.

The antibodies completely inhibited the fibrinolytic activity of 4 lines of gastric cancer, 2 lines of lung cancer, 1 line of urinary bladder cancer and 1 line of renal cancer, indicating that the plasminogen activators from these cell lines were immunologically identical to urokinase. In 5 out of these cell lines, immunological identity was also confirmed by double diffusion analysis.

The plasminogen activator from 1 line of lung cancer was found to be immunologically dissimilar to urokinase by a neutralization experiment and double diffusion analysis.

These findings indicate that there are at least two immunologically distinguishable forms of plasminogen activators from human cancer cells.

Key words: Plasminogen activator – Fibrinolysis – Cultured human cancer cells.

Introduction

The relationship between increased plasminogen activator synthesis and malignant transformation by chemical carcinogens or oncogenic viruses has been demonstrated in mammalian and avian cells in culture (Unkeless et al., 1973; Ossowski et al., 1973; Christman et al., 1975). Furthermore, plasminogen activator has been demonstrated to be present higher at levels in malignant tumors than in corresponding normal tissues (Laug et al., 1975; Nagy et al., 1977). Fibrinolytic activity of tumor cells is considered to be one of the important factors for growth and spread of tumor (Peterson et al., 1973, 1975; Tanaka et al., 1977).

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Recently, Åstedt and Holmberg (1976) demonstrated that the plasminogen activator from cultured ovarian cancer cells was immunologically identical to urokinase (UK). Åstedt (1979) also found no immunological crossreaction between circulating plasminogen activator and UK, and mentioned that this was important for the development of immunological assay techniques for the detection of UK-like plasminogen activator released from tumors. However, in applying and evaluating such assays in vivo, one must know whether the plasminogen activator of tumor cells originating from various tissues is always immunologically identical to UK or not.

In the present study, we examined the immunological similarity or dissimilarity between plasminogen activators from 9 lines of cultured human cancer cells and UK.

Materials and Methods

Cancer Cells. The cancer cell lines used in this study were composed of 4 lines of gastric cancer (MKN 1, MKN 28, KATO III, OKAJIMA), 3 lines of lung cancer (QG 56, QG 90, PC 9), 1 line of urinary bladder cancer (KU 1) and 1 line of renal cancer (KPK 1). All the cell lines except for OKAJIMA and KPK 1 are described as established cell lines in the literature (Yajima, 1970; Yasumoto et al., 1976; Hojo, 1977; Sekiguchi et al., 1978; Kinjo et al., 1979). OKAJIMA and KPK 1 have demonstrated their tumorgenicity in nude mice, in the histological identity between the original tumors and transplanted tumors in nude mice, and in progressive growth in in vitro culture systems (unpublished data). Histological cell types of the original tumors at operation and the passage number at the time of the neutralization experiment described below are shown in Table 1. KPK 1 was grown in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Japan) supplemented by 10% fetal calf serum (GIBCO). The others were grown in RPMI 1640 (GIBCO) supplemented by 20% fetal calf serum (FCS).

Preparation of Anti-UK Immunoglobulin G. Antiserum against human UK was produced by injecting goats with highly purified human UK (M.W. 51,000; Specific activity, 90, 100 IU/mg) which was kindly granted from Mochida Pharmaceutical Co., Tokyo, Japan. Subcutaneous injection of 1 mg of the UK preparation with complete Freund's adjuvant was given every 2 weeks. Two weeks after the 4th injection, the sera were collected. Serial dilution, up to 256 times, of the antisera produced one precipitin line by double diffusion analysis against the original UK-antigen.

Table 1. Histological types of original cancers and in vitro passage number of cultured human cancer cell lines

Cell line	Passage number	Original organ	Histological types
MKN 1	132	Stomach	Adenosquamous carcinoma
MKN 28	73	Stomach	Well differentiated adenocarcinoma
KATO III	286	Stomach	Signet ring cell carcinoma
OKAJIMA	143	Stomach	Poorly differentiated adenocarcinoma
QG 56	>100	Lung	Squamous cell carcinoma
QG 90	74	Lung	Small cell anaplastic carcinoma
PC 9	198	Lung	Squamous cell carcinoma
KU 1	>100	Urinary bladder	Transitional cell carcinoma
KPK 1	68	Kidney	Renal adenocarcinoma

Immunoglobulin G (IgG) was isolated from the sera by gel filtration through a Sephacryl S-200 superfine column after preparation with saturated ammonium sulfate.

Normal goat IgG was also prepared in the same manner.

Preparation of Fibrin Agar Plates. Plasminogen-rich fibrin agar plates were prepared by the method of Nilsson et al. (1972). For the neutralization experiment described below, 0.2 mg of the anti-UK IgG or normal IgG was incorporated in the fibrin agar plates at a final concentration of 1:50 to the fibrinogen.

Purified plasminogen-free fibrinogen was prepared from human plasma by the method of Barnhart and Forman (1955) after chromatography on lysine-Sepharose by the method of Deutsch and Mertz (1970). The purified plasminogen-free fibrinogen was 92% clottable by bovine thrombin. Plasminogen-free fibrin agar plates were also prepared in the same manner.

Assay of Fibrinolytic Activity and Immunological Study. Two hundred thousand cells were plated into 60 mm petri dish in 3 ml of complete growth medium and allowed to grow for 72 h. The medium was then removed, the cells were washed three times with warm serum-free culture medium and incubated at 37° C in 3 ml of serum-free culture medium for 72 h. The medium was then collected and centrifuged at $200 \times g$ for 10 min in order to remove the non-adherent cells, and the aliquots of the supernates were used for the assay or neutralization experiment of fibrinolytic activity. The remaining medium was concentrated and dialysed against phosphate buffered saline at PH 7.4 and the protein concentration was determined by the method of Lowry et al. (1951). The adherent cells were dispersed by trypsinization, and cell number and cell viability including non-adherent cells were examined.

Dilution series of the cell supernate (0.01 ml) were applied to the fibrin agar plates incorporating the anti-UK IgG. They were also applied to the standard fibrin agar plates and fibrin agar plates incorporating normal IgG as controls. In addition they were applied to the plasminogen-free fibrin agar plates in order to differentiate plasminogen activator from non-specific proteases. After incubation at 37° C for 18 h, specific neutralization by the anti-UK IgG was estimated from a decrease or absence of lysed area as compared to controls. The diameter of the lysed area examined on standard fibrin agar plates was compared with that of standard UK (Mochida Pharmaceutical Co., Japan) and the fibrinolytic activity was expressed in International units of UK per one milliliter.

For cell lines which showed high fibrinolytic activity (more than 1 IU/ml), the supernates prepared as above were collected and concentrated by ultrafiltration up to the following activity: MKN 1, 5,000 IU/ml; MKN 28, 5,000 IU/ml; QG 56, 11,000 IU/ml; QG 90, 8,000 IU/ml; PC 9, 15,000 IU/ml; KPK 1, 26,000 IU/ml. The immunological identity between the plasminogen activators of these supernates and UK was then examined by double diffusion analysis.

Results

Cell number, cell viability, and protein concentration and fibrinolytic activity of the supernate of each cell line are shown in Table 2. The values are mean $\pm$ standard deviation of 5 samples taken from the different culture dishes. In all cell lines, the majority of the cancer cells were viable and the cell number increased 5 to 10 times when compared with the initially plated cell number. Protein concentration in each supernate was considerably different from one line to another. The fibrinolytic activity of each supernate was also different from one line to another. Two lines of gastric cancer (MKN 1, MKN 28), 3 lines of lung cancer (QG 56, QG 90, PC 9) and 1 line of renal cancer (KPK 1) showed high fibrinolytic activity. KPK 1 showed extremely high fibrinolytic activity, although the protein concentration was low. However, 2 lines of gastric cancer (KATO III, OKAJIMA) and 1 line of urinary bladder cancer (KU 1) revealed low fibrinolytic activity. The lysed area examined on plasmino-

Table 2. Cell number, cell viability, and protein concentration and fibrinolytic activity of the supernates of cultured human cancer cell lines

Cell Line	Cell number ($\times 10^4$)	Cell viability (%)	Protein concentration (μg/ml)	Fibrinolytic activity (International unit/ml)
MKN 1	124.67±9.07	98.6±0.5	26.3±5.4	1.67±0.12
MKN 28	185.00±9.90	96.6±2.4	10.7±3.0	1.25±0.13
KATO III	176.34±1.00	86.6±2.3	12.3±0.6	0.09±0.01
OKAJIMA	103.17±8.81	87.7±2.9	10.5±0.8	0.17±0.03
QG 56	126.33±2.57	92.2±0.9	20.6±1.6	7.60±1.15
QG 90	196.33±8.08	99.0±0.5	35.7±0.5	4.43±0.25
PC 9	192.08±7.07	88.8±2.3	47.7±4.5	4.55±0.21
KU 1	178.8±5.74	97.7±0.5	10.0±1.1	0.26±0.09
KPK 1	153.25±1.56	100	5.7±0.1	48.67±5.03

Table 3. Neutralization assays of fibrinolytic activity of cultured human cancer cells by anti-UK IgG

Cell line	Dilution of supernate	Diameter of lysed area (mm)		
		Standard fibrin agar plate	Fibrin agar plate containing normal IgG	Fibrin agar plate containing anti-UK IgG
QG 56	1	12.8	13.1	0
	1/2	11.4	11.5	0
	1/4	9.1	9.3	0
	1/8	6.5	7.0	0
	UK (10 IU/ml)	13.9	14.0	0
QG 90	1	10.6	11.1	11.1
	1/2	9.6	9.9	9.9
	1/4	8.2	8.1	7.8
	1/8	6.9	7.1	6.8
	UK (10 IU/ml)	13.6	13.5	0

gen-free fibrin agar plates was consistently absent in all cell lines, indicating that the fibrinolytic activity was due to plasminogen activator.

The results of the fibrinolysis neutralization experiment using the supernates of the representative 2 cell lines (QG 56, QG 90) are shown in Table 3. The anti-UK IgG completely inhibited the fibrinolysis of QG 56 as well as UK. The lysed diameter examined on the fibrin agar plates containing normal IgG was almost the same as that examined on the standard fibrin agar plates at various dilution series. This indicates that the IgG prepared as above is not contaminated by various types of fibrinolysis inhibitors. Therefore, this complete inhibition was considered to be due to the specific reaction between the plasminogen activator from QG 56 and the anti-UK IgG. The fibrinolysis by the supernate of QG 90 was not inhibited by the anti-UK IgG at all.

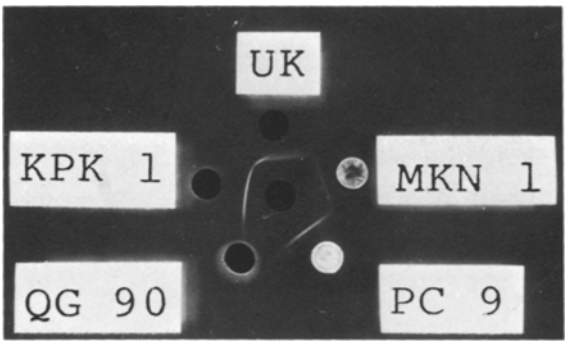

Fig. 1. Double diffusion pattern of plasminogen activators from representative four lines of cultured human cancer cells. The center well contains 8 times diluted anti-UK goat serum. The outer wells contain UK (5,000 IU/ml) and the concentrated supernates of each cell line. Diffusion was performed in 1% Special Agar Noble (DIFCO) buffered at PH 7.4 with phosphate buffered saline. The well diameter is 3 mm and volume is 10 µl

Fibrinolysis by the supernates of the other 7 cell lines was also completely inhibited by the anti-UK IgG.

In double diffusion tests with the anti-UK serum, concentrated supernates of MKN 1, MKN 28, QG 56, PC 9 and KPK 1 gave a single immune precipitin line. However, the supernate of QG 90 did not give a detectable precipitin line (Fig. 1).

Discussion

Although, in this study, FCS was omitted in the culture media because of its content of fibrinolysis inhibitors (Shulman, 1952; Mullertz, 1957), the cancer cells survived and continued to divide and grow in the media.

Varied degrees of plasminogen dependent fibrinolytic activity was detected in the supernatant fluid of cultures. Rifkin et al. (1974) found that cultures of normal embryonic lung cells produced very high levels of plasminogen independent fibrinolysis and mentioned that this phenomenon was due to the action of other proteases. Nagy et al. (1977) also found a similar phenomenon in the lysates of cervical cancers and thought that this could be attributed to residual blood plasminogen contaminating tumor tissue. However, in both the previous (Kinjo et al., 1979) and the present studies there were no cancer cell lines producing plasminogen independent proteases.

Neutralization assays with anti-UK IgG revealed that the plasminogen activator in the supernates of the 8 cell lines was immunologically identical to UK, as Åstedt and Holmberg (1976) reported in 8 lines of cultured ovarian cancer cells. As to the cell lines with high fibrinolytic activity, such identity was also confirmed by double diffusion analysis. However, the neutralization assay and double diffusion analysis demonstrated that the plasminogen activator in the supernate of 1 line of lung cancer (QG 90) was immunologically different from UK, suggesting that it also differs from the plasminogen activators from the other cell lines examined here.

The present results indicate that there are at least two immunologically distinguishable forms of plasminogen activators from human cancer cells, although we do not yet know how many different forms of plasminogen activators

are present in human cancer cells. Tucker et al. (1978) demonstrated the immunological difference between the plasminogen activator from 8 lines of cultured human brain tumor cells and UK.

It is not yet known whether both types of plasminogen activators possess a different biological significance in the growth and spread of tumors. However, this must be taken into consideration in applying and evaluating the immunological assay of plasminogen activators of cancer patients.

In this study, the plasminogen activator from 2 lines of lung cancer was immunologically identical to UK, but that from 1 other line of lung cancer was not. This may suggest that the types of plasminogen activator produced are not necessarily characteristic to the tissue from which the cancer cell lines were derived.

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