HIGH EXTRACELLULAR FIBRINOLYTIC ACTIVITY OF TUMORS AND CONTROL NORMAL TISSUES[1]

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Summary - In search of whether high extracellular proteolytic activity is a general tumor phenotype, we examined the levels of extracellular fibrinolytic activity due to plasminogen activation in the primary cultures of 10 lines of immunogenic and non-immunogenic transplanted sarcomas of mice. The levels of activity were compared with those of their normal control tissues, muscle and skin. Both the normal control muscle and skin, as well as all tumors had high and moderate levels of extracellular fibrinolytic activity. The data therefore do not support the hypothesis that high extracellular proteolytic (fibrinolytic) activity is either a general tumor phenotype, or a property which may offer significant advantage over normal cells for positive selection in tumor growth and invasiveness.

Cells liberate proteolytic activators which are capable of converting the plasma proenzyme, plasminogen, to the active protease, plasmin, which has been measured by its ability to lyse labeled fibrin (1-3). Transformed cells in culture have been reported to exhibit high levels of this extracellular proteolytic activity, compared to low levels from nontransformed cells (3,4). However, the level of extracellular fibrinolytic activity was shown not to correlate with the transformed or nontransformed state of extensively passaged cells (5). Recently, it has been stressed that primary cultured cells from malignant tissues generally have higher extracellular fibrinolytic activity than have primary cultured cells from normal tissues (6).

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The present investigation was therefore undertaken to determine whether or not high extracellular fibrinolytic activity is a general tumor phenotype. We examined the levels of extracellular proteolytic activity in the primary cultured cells of ten lines of transplanted sarcomas, and compared them to the levels in primary cultures of their normal control tissues.

Materials and Methods - Transplanted Tumors and Mice - The tumors used in this study were of 10 lines of transplanted sarcomas that were produced and passaged in vivo in the laboratory of Dr. Richmond T. Prehn of this Institute Five of the sarcoma lines were originally induced by subcutaneous pellets of methylcholanthrene (MCA) in male C3H mice. In the studies of Dr. Prehn and his associates, 3 of the sarcomas (MCA 2043, 2046, 2050) are immunogenic, one (MCA 2027) is of low immunogenicity, and one (MCA 2045) is non-immunogenic. The remaining 5 lines were of non-immunogenic sarcomas which were originally derived by spontaneous transformation of C3HxC57BL mouse embryo cultures. The sarcomas had been stored in the frozen state, and had been serially passaged subcutaneously 3 to 19 times in thymectomized and 550 R X-irradiated male C3HxC57BL mice (Table 1). One to 3 weeks prior to each passage, the mice were thymectomized; and one day before implantation, they were X-irradiated.

Primary Cell Cultures. Cells of minced tumors, muscle, and skin were dissociated with 20 ml of collagenase (1 mg/ml) in Hank's buffered saline at pH 7.4. Cells were seeded at different densities in plastic 25 cm^2 flasks (Falcon) in growth medium containing Eagle's minimal essential medium, 10% fetal bovine serum, penicillin and streptomycin. Medium was replaced every 1 to 3 days until the cultures were 1/2 to 3/4 confluent, when they were assayed for extracellular fibrinolytic activity.

Assay of Fibrinolysis. One day after the replacement of complete medium, the cells were placed in serum-free medium and were assayed for fibrinolytic activity by the method of Unkeless et al. (3), as previously stated in part (5). After incubation of the cells for 18 hr in 5% CO_2 at 37°, the cell-free supernatants ("harvest media") were isolated. The densities of the tumor cells

ranged from 0.6 x 10^4 to 5.4 x 10^4 cells/cm^2, and those of normal cells from 1.0 x 10^4 to 5.0 x 10^4 cells/cm^2.

In the subsequent sterile enzymatic digestions of films of bovine ^{125}I-fibrin, 2 ml of harvest media, diluted with serum-free medium as indicated below, was incubated with 61 µg of purified human plasminogen (7) in ^{125}I-fibrin coated 35 mm plastic dishes for 18 hr in 5% CO_2 atmosphere. The ^{125}I in the clear digest fluids was then counted. The concentration of plasminogen activator in harvest media was compared on the basis of 10^5 cells per 5 cm^2 surface per ml of medium (100% harvest medium), and was measured by activity at 3 or more dilutions of harvest media. More than 25% solubilization of ^{125}I-fibrin with 9% harvest medium was rated to be the result of "high fibrinolytic activity," and the same with 91% or 100% harvest medium that of "moderate activity." (In a previous study (5), the activity ranged down to "undetected.") Corrections (<9% with tumors; < 4% with normal tissues) were made for the radioactivity released by serum-free medium that had not been in contact with cells, and by 100% harvest medium alone without added plasminogen. The dependence of the fibrinolysis on the presence of added plasminogen indicated that the measured fibrinolysis reflected the level of plasminogen activator produced by the cells. Further, the low background levels of proteolysis demonstrated the low order of other types of extracellular proteolytic activity.

Results - Nine of the 10 of the transplanted sarcomas in primary cell cultures had high levels of extracellular fibrinolytic activity. One tumor, MCA 2027, exhibited moderate activity. Table 1 contains the transplant generation number, number of individual tumors in individual mice, and the lowest concentration of cell-exposed harvest medium (after dilution with fresh serum-free medium) that gave the % fibrinolysis shown. Considerable extracellular fibrinolytic activity was found with all tumors, irrespective of the origin of the tumors, their histological composition, transplant generation, and their degree or lack of immunogenicity. Thus, our findings at first seemed to be consistent with the hypothesis that high fibrinolytic activity is a tumor phenotype, and that the

Table 1 Extracellular fibrinolytic activity in primary cultures of sarcomas transplanted in thymectomized and X-irradiated mice

Tumor	Transplant Gen.	No. Tumors	Fibrinolytic Activity: Lowest Conc. Active Harvest Medium % (v/v)	Fibrinolytic Activity: Fibrin Solubilized %	Fibrinolytic Activity: Rating
MCA					
2027	12,13	4	91	35-41	moderate
2043	7	2	9-27	35-64	high
2045	15	2	9	60-63	high
2046	8	2	9	37-50	high
2050	19	3	9	13-36	high
Spontaneous TC					
69	6	3	9	22-30	high
72	5	2	9	61-67	high
75	5	3	9	28-71	high
76	5	2	9	69-70	high
78	3	2	9	27-31	high

Tumors were induced subcutaneously by methylcholanthrene (MCA) or by spontaneous transformation of embryo cultures (TC), and were transplanted serially into compatible mice for the number of passages shown. Subconfluent primary cultures of tumor cells were maintained in serum-free medium for 18 hr, after which extracellular medium was removed to yield cell-free "harvest medium." Densities were then 0.6×10^4 to 5.4×10^4 cells/cm^2. Two ml of harvest medium, diluted (v/v) as indicated, and 61 µg of purified human plasminogen were added to ^{125}I-fibrin coated 35 mm dishes, incubated for 18 hr at 37° in 5% CO_2 atmosphere, and the ^{125}I in the supernatant digest fluids was counted. The concentration of plasminogen activator in harvest medium was compared on the basis of activator liberated by 10^5 cells per 5 cm^2 per ml of medium (100% harvest medium). The table indicates the lowest concentration of % (v/v) of harvest medium that yielded significant % solubilization of ^{125}I-fibrin, and the rating of level of fibrinolytic activity. Details are provided in the text.

serial passaging of tumors *in vivo* involves a positive selection of malignant cells with high levels of extracellular proteolytic activity due to plasminogen activation.

However, we then examined the primary cultures of normal control tissues. Muscle and skin of the adult mice of the same hybrid strain were taken as the normal control tissues. This choice was based on the following: (i) The tumors

were diagnosed by Dr. R. Philip Custer of this Institute as poorly differentiated fibrosarcomas, others as poorly differentiated rhabdomyosarcomas, and still others as primitive mesenchymal tumors. (ii) The transplanted MCA tumors originated as a result of subcutaneously implanted pellets of methylcholanthrene. (iii) Comparisons of the subcutaneously transplanted tumors with adjacent muscle and skin would possibly relate to advantages conferred by high extracellular proteolytic activity on tumor growth and invasiveness.

As shown in Table 2, the primary cultured cells of adult muscle and skin of normal and control mice also exhibited moderate and high levels of extracellular fibrinolytic activity. Normal muscle was from mice which had no tumor, and which had not been thymectomized or X-irradiated. Control muscle and skin

Table 2 Extracellular fibrinolytic activity in primary cultures of normal and control muscle and skin

Organ	Mouse C3HxC57BL	Tumor	No. Exper.	Fibrinolytic Activity: Lowest Conc. Active Harvest Medium % (v/v)	Fibrinolytic Activity: Fibrin Solubilized %	Rating
muscle	normal	none	3	100	53-91	moderate
muscle	normal	MCA 2027	3	100	69-77	moderate
muscle	Tx, 550 R	MCA 2027	3	100	73-76	moderate
skin	Tx, 550 R	MCA 2027	1	91	38	moderate
skin	Tx, 550 R	MCA 2043	1	91	68	moderate
skin	Tx, 550 R	MCA 2045	3	9-55	20-68	high
skin	Tx, 550 R	TC 72	1	9	50	high

Primary cultures of muscle and skin were assayed for extracellular fibrinolytic activity as described under Table 1 and in the text. The cell densities of the cultures ranged from 1.0×10^4 to 5.0×10^4 cells/cm^2.

Tx, 550 R: thymectomized and X-irradiated, as in text.

were from thymectomized and irradiated mice that bore tumors. Thus, the muscle and skin cells of normal and control mice had levels of extracellular fibrinolytic activity that were of similar high order like those of the transplanted tumors.

Discussion - The primary cultures of both tumors and also their control normal tissues exhibit high and moderate levels of extracellular fibrinolytic activity. The data therefore do not support the hypothesis that high extracellular fibrinolytic activity is either a tumor phenotype, or is a property which confers significant advantage to tumor cells over normal cells for positive selection in growth or invasiveness. Neither do the data support the concept that alteration of cell surfaces by soluble extracellular protease is at the basis of the immunogenicity of some tumors.

Reich and associates have stressed that the primary cultures of malignant tissues generally have enhanced soluble extracellular fibrinolytic activity, as compared to the primary cultures of normal tissues (6). The present findings, namely, that primary cultures of control normal adult tissues exhibit levels of extracellular fibrinolytic activity of a similar order of magnitude like those of primary cultures of tumors, is not in accord with that generalization. Actually, it has been known for many years that considerable amounts of plasminogen activator activity is extractable from many organs of human and other species (1,2,8). In addition, Todd presented histochemical evidence to indicate that fibrinolytic activity in tissues is concentrated around blood vessels, particularly veins and venules (9). Further, Bernik and Kwaan in 1969 showed that primary cultures of normal human kidney, lung, and several other tissues produce considerable amounts of extracellular plasminogen activator activity in serum-free medium (2). None of these studies, including our own, relate to any hypothetical function of cell surface-bound protease. It therefore seems reasonable to question the importance of high extracellular fibrinolytic activity as a general tumor phenotype, in the face of the rather extensive distribution of the considerable extracellular fibrinolytic activity of many normal

tissues. This statement is based on the demonstrated lack of correlations between the level of extracellular fibrinolytic activity, and both (a) the malignant or normal state of primary passaged cells (this report), and (b) the transformed or nontransformed state of extensively passaged cells (5).

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