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PLASMINOGEN ACTIVATOR AND MIF-LIKE ACTIVITIES IN KIRSTEN VIRUS TRANSFORMED MOUSE NIH CULTURE FLUIDS*

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Summary:

Kirsten virus transformed mouse NIH cells produce both a macrophage migration inhibition activity for guinea pig and mouse peritoneal exudate cells and a plasminogen activator. The migration inhibition factor activity exhibited thermal stability up to 80°C while the plasminogen activator was inactivated after 15 minutes at 70°C. Separation of these activities was achieved by absorption of the migration inhibition activity on agarose-fucosamine or high speed centrifugation.

Introduction

It had been shown earlier that culture fluids from virus transformed cells inhibit the migration of guinea pig peritoneal exudate cells (GPPE) *in vitro* and that these fluids contain plasminogen activator (4,5). This inhibition was similar to that produced by lymphocyte-produced macrophage migration inhibition factor (MIF) (6). More recently it has been observed that MIF-like activity for GPPE could be generated following incubation of human urokinase or partially purified preparations of plasminogen activator from Simian virus 40 transformed mouse 3T3 cells (SV3T3) with medium containing guinea pig serum (7). These observations have here been extended to Kirsten virus transformed mouse NIH cells (KNIH). Both MIF-like

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activity as measured against GPPE and plasminogen activator activity have been obtained in serum-free culture fluids of these cells and shown to be separate entities by affinity chromatography and differences in thermostability.

Materials and Methods

Cells and Cell Culture: The NIH Swiss mouse embryo cells, Kirsten virus transformed NIH cells (KNIH) (8), and SV3T3 cells were kindly supplied by Dr. Judah Folkman, Children's Hospital Medical Center, Boston, Mass. Cells were grown in Dulbecco's modification of Eagle's minimal essential medium supplemented with 5-10% fetal calf serum either in 75 cm^2 plastic T-flasks (Falcon Plastics, Los Angeles) or in suspension culture in spinners. Confluent monolayer cultures were washed with serum free medium or phosphate buffered saline (PBS) and replaced with same for incubation overnight. Suspension grown cells were similarly washed, resuspended at about 6 x 10^5 cells/ml, and allowed to incubate with agitation in the cold for 4 hours. After centrifugation, culture fluid supernatants from both monolayer and suspension cultures were harvested as starting material for these studies. In some cases these fluids were dialyzed and lyophilized and stored frozen before use.

MIF Assay: Crude or partially purified culture fluids were incubated overnight (16-18 hours) at 37°C in plastic tubes in Eagle's MEM (x4) containing 15% freshly thawed guinea pig serum (GPS). This medium was then assayed for MIF-like activity in chambers containing capillaries of pelleted GPPE cells (7,9). After incubation of chambers at 37°C in 5% CO_2 atmosphere for 18-20 hours, the area of migration was traced and measured by planimetry. Cell viability was assessed by trypan blue exclusion and migration inhibition tests were considered valid only if at least 95% of the GPPE cells remained viable. Replicate assays were run and only inhibition of macrophage migration greater than 20% was judged to be significant.

Plasminogen Activator (PA) Assay: PA activity was determined by a micro-adaptation (10) of the fibrin dish assay of Unkeless et al. (10, 11). Plasminogen-free human fibrinogen was the generous gift of Dr. Yu-lee Hao, American Red Cross, Washington, D.C. Plasminogen was prepared by the method of Deutsch and Mertz (12). Urokinase (WinKinase, 35,000 CTA units/mg protein, Sterling-Winthrop Laboratories, New York) was made available through the generosity of Dr. William P. Blackmore and Dr. Joseph C. Fratantoni. Samples were assayed with and without added plasminogen to distinguish between PA and fibrinolytic activity. The PA activity was expressed as the initial rate of release of ^{125}I-fibrin in terms of CPM x min^{-1} x ml^{-1}. At intervals aliquot of 10-25 µl of reaction solution were removed and counted. A unit of PA activity was defined as the amount of enzyme which led to the solubilization of a microgram of fibrin per hour. Given the specific activity of the fibrin, which was determined by solubilizing random control wells with NaOH and calculating cpm/µ gm fibrin, units of activity were calculated from the initial rates of cpm release by the equation:

$$\text{units (in reaction)} = \frac{V_{\text{initial}} \text{ (cpm x } min^{-1} \text{ x } ml^{-1}) \text{ x } 60 \text{ x Reaction Volume (ml)}}{\text{Specific Activity of Fibrin (cpm/ µgm)}}$$

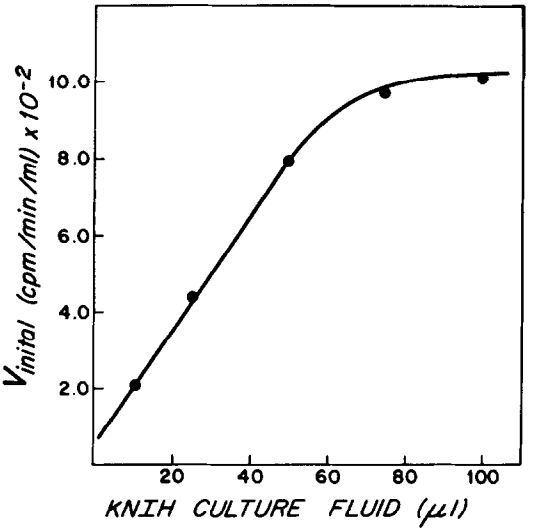

Figure 1. Effect of KNIH cell culture fluid concentration on the initial rate of hydrolysis of fibrin (details in text).

Results and Discussion

Table 1 shows the effect of varying concentrations of KNIH culture fluid protein on the inhibition of GPPE cell migration. Preincubation with guinea pig serum enhanced the migration inhibition observed. Culture fluids from untransformed NIH cells at the same concentration of protein exhibited no MIF-like activity. This is similar to the observations reported earlier that fluids from SV40 transformed 3T3 cultures but not from untransformed 3T3 cultures inhibit migration of GPPE cells. Migration of mouse peritoneal exudate cells was also significantly inhibited (53%) by the KNIH culture fluids (1 mg protein/ 1 ml). Figure 1 demonstrates that these cells also produced PA activity which exhibits linear concentration dependence of the rate of ^{125}I-fibrin hydrolysis at dilute concentrations of culture fluid protein. Consequently all attempts to quantitate the PA activity were restricted to rate data obtained over the linear portion of the concentration rate curve. The KNIH PA

TABLE I

COMPARISON OF MIF-ACTIVITY IN FLUIDS FROM KNIH AND NIH CULTURES; RESULTS OF A TYPICAL EXPERIMENT

Addition	Protein Conc. (mg/ml)	No Preincubation		Preincubated with GPS 12-14 hrs	
		Migration Area	% Inhibition	Migration Area	% Inhibition
No addition		2735	0	2984	0
KNIH Culture Fluid	0.1	2676	2	2604	13
	0.2	2453	10	2909	3
	0.3	2498	9	2284	23
	0.4	2545	7	-	-
	0.5	2646	3	1650	45
	0.6	1701	38	1722	42
	0.8	1686	38	1564	48
	1.0	2592	5	1432	52
No addition				2403	0
NIH Culture Fluid	0.125			2339	3
	0.25			2413	0
	0.50			2155	11
	1.00			2011	16

TABLE 2

THERMAL STABILITY OF MIF AND PA ACTIVITIES OF KNIH AND SV3T3 CELL CULTURE FLUIDS

Treatment	PA Activity		MIF Activity
	V initial $(CPM \times min^{-1} \times ml^{-1})$	% Activity Remaining	% Migration Inhibition
KNIH Culture Fluid			
untreated	861	100	49
70°C, 15 min.	0	0	46
SV3T3 Culture Fluid			
untreated	691	100	28
70°C, 15 min.	34	5	27

activity, similar to that produced by SV3T3 cells, was inactivated by diisopropylfluorophosphate.

Since it was shown earlier that plasminogen activators upon incubation with guinea pig serum generate MIF-like activity (7) an attempt was made to demonstrate the identity or non-identity of the KNIH-MIF-like activity and PA activity. Incubation of crude fluids from KNIH cultures at 70°C for 15 minutes completely inactivated the PA activity but had no effect on the GPPE cell migration inhibition activity (Table 2). The MIF-like activity exhibited considerable thermal stability even up to 80°C for 30 minutes. Culture fluids from SV3T3 cells lost PA activity under conditions similar to the KNIH fluids. Partially purified MIF activity obtained after affinity chromatography (see below) showed similar thermal stability (Table 3). These results strongly suggested that the PA activity and MIF-like activity resided in different molecules.

In an attempt to physically separate the MIF and PA activities, 500 ml of KNIH cell culture fluid which had been dialyzed against

TABLE 3

THE ADSORPTION OF MIF-LIKE ACTIVITY BY AGAROSE- AMINO-CAPROYL-FUCOSAMINE (AF) FROM KNIH CULTURE FLUIDS

Fraction	PA Activity		MIF Activity
	Total Units	% of Total	%Migration Inhibition
KNIH Culture Fluid			
Untreated Starting Material	9600	100	44
Supernatant from AF	9803	102	41
Supernatant, 70°C, 15 min.	0	0	43
Eluate from AF	75	0.8	66
Eluate, 70°C, 15 min.	0	0	51

water were exposed to 20 ml of agarose-E-amino caproyl-fucosamine at 4°C for 18 hr with slow mixing. The resin was collected by centrifugation, washed four times with saline and eluted with 20 ml of 1% L-fucose in the cold overnight. Table 3 shows the PA and MIF-like activities for starting material, supernatant from agarose-fucosamine, and the eluate. Units of PA activity were calculated for all fractions and less than 1% was obtained in the eluate with essentially all the activity remaining in the supernatant. In contrast significant MIF activity was adsorbed and eluted from the resin. The MIF activity remaining in the supernatant was probably due to overloading the capacity of the agarose-fucosamine. The eluted MIF activity exhibited thermal stability comparable to the crude starting material. These results demonstrate that the two activities can be physically separated and thus demonstrate that in addition to any MIF-like activity which might be generated from PA activity, as proposed earlier, there is also present a fairly thermal-stable MIF-like entity.

Finally, these two activities were separated by ultracentrifugation at 35,000 xg for 18 hours. The PA activity of the culture fluids became insolubilized and settled out of solution under these

conditions. Ultracentrifugation yielded a pellet containing the PA activity while the supernatant solution contained the MIF activity. PA was solubilized by treatment with 0.1% Triton X100 and 3M KCl. These observations suggest that the PA activity may be associated with a membrane fraction similar to that reported by Quigley (13) for PA in sarcoma virus-transformed chick embryo fibroblasts and hamster SV40 cells.

Solubilized KNIH PA activity was shown in preliminary experiments to be adsorbed by lysine-sepharose beads, whereas the MIF activity did not bind to this resin. In conclusion, KNIH culture fluids contain separable PA and MIF-like activities which exhibit different thermal stabilities.

References

1. Hammond, M.E., Roblin, R.O., Dvorak, A.M., Selvaggio, S.S., Black, P.H., and Dvorak, H.F. (1974). Science 185: 955-957.
2. Poste, G. (1975). Cancer Res. 35: 2558-2566.
3. Bigazzi, P.E., Yoshida, T., Ward, P.A. and Cohen, S. (1975). Am. J. Path. 80: 69-78.
4. Ossowski, L., Unkeless, J.C., Tobia, A., Quigley, J.P., Rivkin, D.B., and Reich, E. (1973). J. Exp. Med. 137: 112-116.
5. Chou, I.N., Black, P.H., and Roblin, R.O. (1974). Nature 250: 738-741.
6. Dvorak, A.M., Hammond, M.E., Dvorak, H.F., and Karnovsky, M.J. (1972). Lab. Invest. 27: 561-574.
7. Roblin, R.O., Hammond, M.E, Bensky, N.D.,Dvorak, A.M., Dvorak, H.F., and Black, P.H. (1977). Proc. Natl. Acad. Sci USA 74: 1570-1574.
8. Aaronson, S.A. and Weaver, C.A. (1971). J. Gen. Virol. 13: 245-252.
9. David, J.R. and David, R. (1971). In In Vitro Methods in Cell-Mediated Immunity, Bloom, B.R. and Glade, P.R. (eds.), pp. 249-261, Academic Press, New York.
10. Dvorak, H.F., Orenstein, N.S., Rypysc, J., Colvin, R.B., and Dvorak, A.M. (1978). J. Immunol. 120: 766-773.
11. Unkeless, J.C., Tobia, A., Ossowski, L., Quigley, J.P., Rivkin, D.B., and Reich, E. (1973). J. Exp. Med. 137: 85-126.
12. Deutsch, D.G. and Mertz, E.T. (1970). Science 170: 1095-1096.
13. Quigley, J.P. (1976). J. Cell Biol. 71: 472-486.