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CONCOMITANT SECRETION BY TRANSFORMED SVWI38-VA13-2RA CELLS OF PLASMINOGEN ACTIVATOR(S) AND SUBSTANCE(S) WHICH PREVENT THEIR DETECTION

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SUMMARY

SVWI38-VA13-2RA cells have been shown to secrete both plasminogen activator(s) and inhibitory substance(s) which prevent detection of plasminogen activator activity in the widely used ^{125}I-labeled fibrin dish assay. The SVWI38-VA13-2RA plasminogen activator(s) can be detected by assay of individual gel slices following SDS gel electrophoresis of SVWI38-VA13-2RA cell conditioned medium. The inhibitory substance(s) have been detected by the ability of SVWI38-VA13-2RA conditioned medium to inhibit the activity of mouse lung carcinoma (CMT64) cell plasminogen activator(s). Thus, lack of plasminogen activator activity with the ^{125}I-labeled fibrin dish assay alone no longer suffices as proof that cells are not secreting plasminogen activator(s). Concomitant secretion of plasminogen activators and inhibitors must be assessed in attempts to correlate viral transformation, tumorigenicity and plasminogen activator levels.

Investigators in several laboratories have attempted to determine whether a correlation exists between high-level production of the proteolytic enzyme plasminogen activator and various transformed cell characteristics (1,2), or with tumorigenicity *in vivo* (3). In these experiments, plasminogen activator activity has been assayed as plasminogen-dependent release of radioactive fragments from ^{125}I-fibrin dishes (3). On the basis of results with this assay, some investigators have concluded that the level of plasminogen activator activity does not correlate with the transformed or untransformed state of the cells, in part because SVWI38-VA13-2RA cells did not produce any detectable plasminogen activator activity *in vitro* (1). We report here that SVWI38-VA13-2RA cells do in fact secrete plasminogen activator(s), but that this plasminogen activator activity cannot be detected in the ^{125}I-labeled

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fibrin dish assay because SVWI38-VA13-2RA cells concomitantly secrete an inhibitor.

MATERIALS AND METHODS

Cells and media. CMT 64 cells, originally isolated from a spontaneous lung carcinoma arising in a C57BL/Icrf-a^t mouse (5), were obtained from Dr. Leonard M. Franks. These cells were grown in Waymouths MB 752/1 medium (Gibco) containing 5% fetal calf serum (FCS). SVWI38-VA13-2RA cells derived from human WI38 cells following transformation with Simian virus 40 (6) were obtained from the Cell Culture Center, Massachusetts Institute of Technology, Cambridge, Massachusetts. The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. All media also contained 100 U/ml penicillin and 100µg/ml streptomycin. The SVWI38-VA13-2RA cells used in our experiments contained intranuclear SV40 T-antigen demonstrable by the indirect immunofluorescence technique and formed colonies in soft agar using the conditions described by McPherson and Montaguier (7).

Preparation of serum-free conditioned medium and assay of plasminogen activator activity. Nearly confluent 60-mm dishes of SVWI38-VA13-2RA or CMT 64 cell cultures were washed twice in their respective growth media without serum and incubated for 16-18 hr in 2 ml serum-free medium. The following morning the conditioned media were removed, placed in plastic centrifuge tubes, centrifuged at 1000 x g for 10 min at room temperature and assayed immediately for plasminogen activator as described by Vassalli et al. (8). Plasminogen-free bovine fibrinogen (Miles Laboratories, Lot 17), iodinated by the technique of Helmkamp et al. (9), was used to prepare the ^{125}I-fibrin containing wells. Fibrinolysis assays contained 1-50 µl serum-free conditioned medium (CM), 5 µg human plasminogen [purified by affinity chromatography (10) from human serum] and 0.1 M Tris-HCl buffer pH 8.1 in a final volume of 250 µl. Fibrinolysis assays were incubated for 4 hr at 37°. Conditioned medium samples were concentrated and were electrophoresed on sodium dodecyl sulfate (SDS) 10-15% polyacrylamide gradient slab gels as described by Strickland and Beers (11). Plasminogen activator activity in 1-mm slices of SDS-polyacrylamide gels was assayed by incubating the gel slice together with 10 µg human plasminogen and 1 ml 0.1 M Tris-HCl buffer, pH 8.1 in wells containing 2.2 - 2.7 x 10^5 cpm ^{125}I-fibrin for 10-12 hr at 37°. Gel slices from a parallel lane from the same SDS-polyacrylamide gel, containing a replicate sample of conditioned medium, were incubated identically except that the plasminogen was omitted.

RESULTS

Under the standard assay conditions used in these experiments, the plasminogen dependent release of bound ^{125}I-cpm from the ^{125}I-fibrin-containing wells is linear with the amount of CMT 64 cell-conditioned medium added, up to at least 60% digestion of the available substrate (Figure 1). Dilution of the CMT 64 conditioned medium in plastic tubes containing 0.1 M Tris buffer, pH 8.1 results in a more than proportional diminution of plasminogen activator activity, possibly because the plasminogen activator activity has a greater tendency to stick to the plastic tube at low protein concentrations. The reproducibility

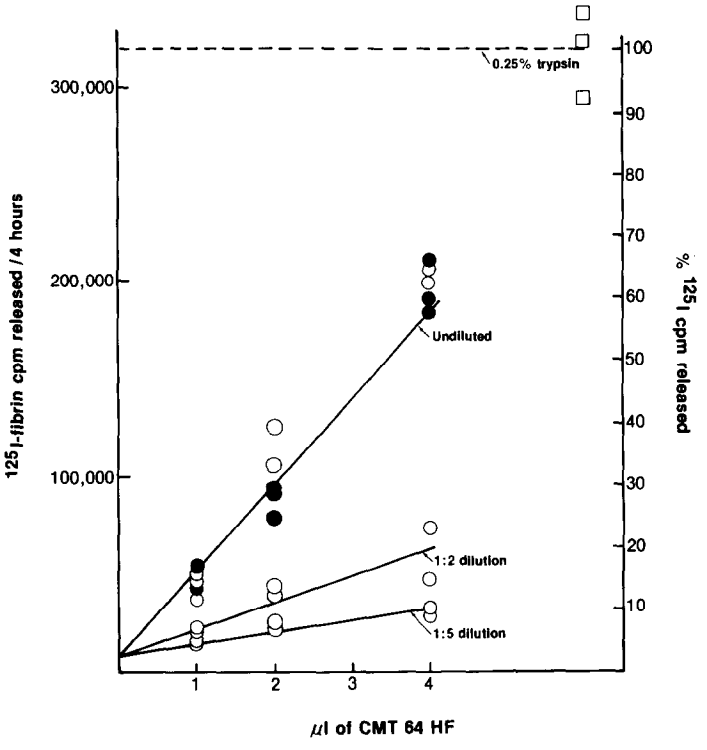

Figure 1. Fibrinolysis Assay Dose Response Curve

o—o samples assayed immediately after harvesting and centrifugation of conditioned medium as described in Materials and Methods. ●—● same preparation of conditioned medium assayed after overnight storage at 4°.

of the assay, also illustrated in Figure 1, makes differences in plasminogen activator activity of more than 2-fold significant.

When up to 50 μl of conditioned medium from SVWI38-VA13-2RA cells was assayed under standard assay conditions, we observed no detectable plasminogen activator activity (Table 1, line B), in agreement with results previously reported by Mott et al. (1). In contrast, 10 μl of conditioned medium from CMT 64 cells gave a readily detectable plasminogen activator activity when assayed on the same ^{125}I-fibrin plate (Table 1, line A). Mixing 50 μl of SVWI38 cell-conditioned medium and 10 μl of CMT 64 cell-conditioned medium in the assay well immediately prior to incubation inhibited the plasminogen

Table 1. Inhibition of Plasminogen Activator Activity by SVWI38-VA13-2RA Conditioned Medium

	^{125}I-fibrin cpm released*	Average % Inhibition †
A. CMT 64 conditioned medium 10 µl	45,177 ± 1710	-
B. SVWI38-VA13-2RA conditioned medium 50 µl	0	-
C. CMT64 CM 10µl + SVWI38 CM 50 µl	9,084 ± 569	80
D. CMT64 CM 10µl + Mock CM 50 µl	34,664 ± 5091	23

*Average of triplicate assays ± standard deviation; calculated after subtraction of ^{125}I-fibrin cpm released by plasminogen alone (5856 cpm). Replicate well incubated with trypsin released 168,540 cpm.

†$\frac{\text{Average cpm released by CMT 64 CM alone - Average cpm released by CMT 64 CM plus inhibitor}}{\text{Average cpm released by CMT 64 CM alone}} \times 100$

activator activity of the CMT 64 cell-conditioned medium by 80% (Table 1, line C). To determine whether this inhibition was a consequence of materials specifically produced by the SVWI38 cells, we prepared a "mock"-conditioned medium by incubating serum-free medium for 16-18 hr in a petri dish without cells. When 50 µl of this "mock"-conditioned medium was added to 10 µl of CMT 64 cell-conditioned medium, a small and variable degree of inhibition was obtained which averaged 23% (Table 1, line D). The degree of inhibition produced by SVWI38 cell-conditioned medium is considerably greater than that produced by the "mock"-conditioned medium, demonstrating that SVWI38 cell-conditioned medium contains substance(s) which inhibit the ^{125}I-fibrin assay.

Plasminogen activators from a variety of cell types retain their activity following SDS gel electrophoresis, and can thus be detected by assay of gel slices (12). When a sample of the SVWI38-VA13-2RA cell-conditioned medium assayed in Table 1 was concentrated and electrophoresed on an SDS polyacrylamide gel, and the gel slices were assayed, several peaks of fibrinolytic

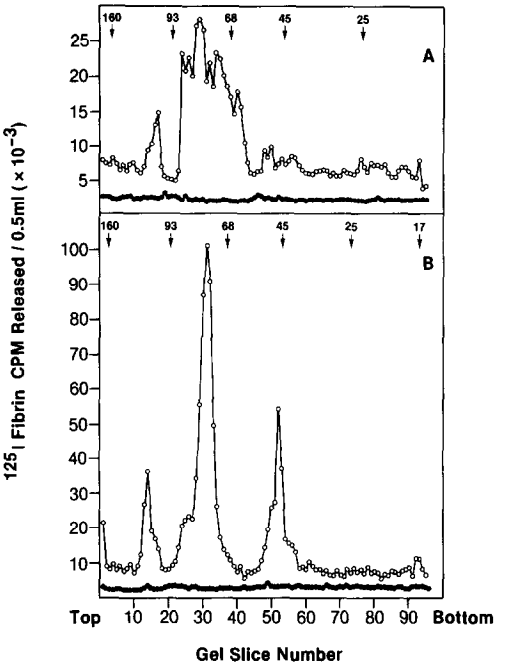

Figure 2. SDS Polyacrylamide Gel Separation of Plasminogen Activators

Samples of serum-free conditioned medium from SVWI38-VA13-2RA cells (A) and CMT 64 cells (B) were concentrated, lyophylized and electrophoresed on SDS 10-15% polyacrylamide gradient slab gels as described in Materials and Methods. Gel lanes containing replicate samples of conditioned medium were sliced into 1 mm segments and assayed for fibrinolytic activity in the presence (o) or absence (•) of human plasminogen.

activity were observed (Figure 2A). The fibrinolytic activity observed was due to plasminogen activators and not to other proteases because incubating slices from a replicate gel lane in the absence of plasminogen produced no peaks of fibrinolytic activity (Figure 2A). The number of plasminogen activator peaks and the predominance of high-molecular-weight species in the gel of SVWI38-VA13-2RA conditioned medium are unusual in light of previous molecular weight characterizations of human cell plasminogen activators (13,14). However, this pattern of plasminogen activator activity peaks has also been observed following electrophoresis of two other separately prepared samples of SVWI38-VA13-2RA conditioned medium. In addition, the unusual pattern of plasminogen activator peaks for SVWI38-VA13-2RA cells is not simply an arti-

fact of our techniques, since electrophoresis of CMT 64 cell-conditioned medium (Figure 2B) produced sharp peaks of 48,000 and 75,000 dalton plasminogen activator activity, as observed previously with other cell types (11,12).

Although we have consistently obtained inhibitory conditioned media from SVWI38-VA13-2RA cells under the cell culture conditions described here, other culture conditions led to different results. For example, growing the SVWI38-VA13-2RA cells and preparing the conditioned medium in an enriched medium (α-MEM, Gibco) produced conditioned media with easily detectable plasminogen activator activity (data not shown). We suggest that SVWI38-VA13-2RA cell-conditioned medium can be active or inhibitory depending upon the ratio of plasminogen activitor(s) to inhibitory substance(s), and that this ratio can be altered by changes in cell culture conditions.

DISCUSSION

Our results show that SVWI38-VA13-2RA cells in culture secrete several high-molecular-weight species of plasminogen activator, whose activity is masked in the conventional ^{125}I-fibrin plate assay by inhibitory substance(s) concomitantly secreted by the cells. Since the ^{125}I-fibrin plate assay is a coupled assay, requiring both formation of plasmin and its subsequent action on ^{125}I-fibrin, we do not yet know whether SVWI38-VA13-RA cells produce substances which inhibit only plasminogen activator, only plasmin, or both enzymes. Since SVWI38-VA13-2RA cell plasminogen activators can be detected following SDS gel electrophoresis, the SDS gel electrophoresis technique may either dissociate an activator-inhibitor complex, irreversibly inactivate the inhibitor, or simply separate the activator from the inhibitor.

Our results have several implications for attempts to correlate virus-induced cell transformation or tumorigenicity *in vivo* with levels of expression of plasminogen activator activity *in vitro* (1,2). First, it is clear that lack of demonstrable fibrinolytic activity using only the ^{125}I-fibrin plate assay can no longer be taken as unambiguous proof that the cells do not produce plasminogen activator(s). Second, an interpretation drawn from previous work

(1), that there was no correlation between the level of fibrinolytic activity and the virus-transformed state of cells, needs to be reexamined, since it depended in part upon finding no plasminogen activator activity secreted by SVWI38-VA13-2RA cells using the ^{125}I-fibrin plate assay. Luskatoff and Edgington (15) recently made a similar suggestion, based upon their finding of an intracellular inhibitor of the ^{125}I-fibrin assay in cultured endothelial cells. Finally, while purifying plasminogen activators from several types of conditioned media, we have at times recovered more total plasminogen activator activity than we estimated was initially present by ^{125}I-fibrin dish assay of the crude conditioned medium (Roblin, R. O., Vetterlein, D. A., Young, P. L. and Bell, T. E., unpublished data). A possible explanation is that partial purification of the plasminogen activator has removed inhibitor(s). Thus, concomitant secretion of plasminogen activators and substances which inhibit the ^{125}I-fibrin plate assay may not be confined to SVWI38-VA13-2RA cells and cultured endothelial cells (15), but may rather be a feature of many different cell types. The fibrinolytic activity of different cell types, as measured with the ^{125}I-fibrin assay, will thus reflect the balance or ratio of activators and inhibitors. Concomitant secretion of plasminogen activators and inhibitors should be looked for by the technique described here when virus-transformed or tumorigenic cells with undetectable plasminogen activator levels are encountered, if accurate correlations between plasminogen activator expression and malignant cell behavior are to be made.

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