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EVIDENCE FOR MEMBRANE ASSOCIATION OF PLASMINOGEN ACTIVATOR ACTIVITY IN MOUSE MACROPHAGES

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SUMMARY

Production and release of high levels of plasminogen activator, a serine protease often referred to as a secretory product, has been considered as a biochemical index for mouse macrophage "activation". Although the mechanism for plasminogen activator release is not known, several characteristics of the release process suggest that the enzyme may be shed from the cell surface of activated macrophages rather than secreted. In this paper, we show that plasminogen activator activity in thioglycollate elicited macrophages is predominantly associated with a subcellular fraction consisting mainly of membranes and granules which are pelletable at 100,000 x g. Furthermore, plasminogen activator activity can be solubilized only by detergents and not by treatments which are known to release granule-bound contents as well as loosely associated peripheral membrane proteins. Thus, these results suggest that macrophage plasminogen activator is firmly found to cellular membranes.

INTRODUCTION

Cultured peritoneal macrophages harvested from mice injected with thioglycollate produce and release high levels of several neutral proteases such as elastase, collagenase and plasminogen activators which are either absent or barely detectable in resident macrophages from untreated control animals (1-3, for reviews see 4,5). Plasminogen activator (PA), a trypsin-like serine protease, catalyzes the conversion of the serum zymogen, plasminogen, into the active enzyme, plasmin, which can be assayed by its fibrinolytic activity (1). Enhanced production and release of PA has been considered as a biochemical index for macrophage "activation" by various means (6-8). In general, macrophage activation results in a much greater increase in the amount of released than cell-associated PA (1).

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Throughout the literature, PA has been referred to as a "secretory" product of activated macrophages (1, 4-9) despite the fact that very little is known concerning the mechanism for its release from cells. Furthermore, a closer examination of the release of PA from elicited macrophages (as defined by Karnovsky and Lazdins (7)) reveals several characteristics which are not commonly observed in other conventional secretory systems: induction of new PA synthesis and its subsequent release is closely associated with macrophage "activation" by various means (1,10-13); the prolonged time course for PA release (1,10) is rather unusual in terms of the "stimulus-secretion coupling" mechanism for exocytosis (14); young and replicating macrophages actively synthesize and release PA (12,15) indicating a relationship between cell proliferation and PA release as has been shown in other cell types (16); most of the newly synthesized PA is destined for extracellular release as evidenced by a rapid turnover of the intracellular PA pool in elicited macrophages (12,13). Such a coupling between synthesis and release of PA has been shown in other stimulated cells releasing PA (17,18).

Taken together, these results strongly suggest that in elicited macrophages, PA may be released by a mechanism(s) other than conventional secretion (exocytosis); i.e., the enzyme may be shed from the cell surface of elicited macrophages (19). In order for a molecule to be shed and not secreted, it must be located on or associated with the cell membrane since, in general, tightly bound membrane proteins become membrane-associated since their synthesis. In this communication we present evidence indicating that in elicited macrophages, PA is associated with a membrane rich subcellular fraction pelletable at 100,000 x g, and that the PA activity can be solubilized only by detergents and not by treatments which would normally release the granule-bound content as well as the loosely associated peripheral membrane proteins.

MATERIALS AND METHODS

Cell culture. Elicited peritoneal macrophages were harvested from C57BL/6 mice 4-5 days post injection with thioglycollate medium (1). The yield was usually 20-30 x 10^6 cells/animal of which approximately 90% were mononuclear cells. The macrophages were plated at 30-35 x 10^6 cells per 150 cm^2 flask contain-

ining 50 ml of Dulbecco's modified Eagle's medium (Dulbecco's medium) + 5% heat-inactivated (56C, 30 min) fetal calf serum + antibiotics. After incubation at 37C in a CO_2 incubator for 24 hrs, the flasks were shaken vigorously to remove the nonadherent cells. The cultures were then washed once, refed with fresh complete medium and incubated for an additional 24 hours before use for fractionation experiments.

Subcellular fractionation. After removing the medium, the cells were washed once with cold 0.05 M Tris-HCl, pH 7.4 and scraped in the same buffer with a rubber policeman. Harvested cells were combined and washed twice by low speed centrifugation in 0.05 M Tris-HCl, pH 7.4. The final cell pellet was suspended in a small volume of 0.05 M Tris-HCl/0.005 M $MgCl_2$, pH 7.4, and allowed to equilibrate for 10 min. The cell suspension was then agitated with a vortex mixer to break the cells. This procedure was carefully monitored by phase microscopy to ensure >90% lysis of the cell without breakage of the nuclei. The vortex-disrupted cells were centrifuged (600 x g, 10 min) and the resulting supernatant was withdrawn. The pellet was washed by resuspending in a small volume of 0.05 M Tris-HCl/0.005 M $MgCl_2$, pH 7.4, agitated with a vortex mixer, and centrifuged. The final pellet, referred to as the nuclear pellet, was resuspended in a small volume of 0.05 M Tris-HCl, pH 7.4, and used for assays. The resulting supernatants from the latter two centrifugations (referred to as post-nuclear supernate) were combined, adjusted to 1 mM EDTA and subjected to centrifugation at 100,000 x g (Spinco type 40 rotor) for 1 hr. The resulting 100,000 x g pellet and supernate were referred to as the membrane plus granule rich and soluble cytoplasmic fractions, respectively. Each subcellular fraction was then assayed for its protein content and PA activity.

Solubilization of PA activity. Aliquots (1 ml) of the post-nuclear supernate were centrifuged at 100,000 x g for 1 hr. The resulting pellets were resuspended, by passing through a 25-gauge needle several times, in 1 ml of 0.05 M Tris-HCl buffer, pH 7.4 (control); 0.5 M NaCl; 3 M KCl; 0.005 M Tris-HCl, pH 7.4; 1 mM EDTA; and 1% Triton X-100 in 0.01 M Tris-HCl, pH 8.1, respectively. The suspensions were allowed to stand for 1 hr at 4C and were then centrifuged at 100,000 x g for 1 hr. The clear supernatants were withdrawn and each of the pellets was resuspended in 1 ml of 0.05 M Tris-HCl, pH 7.4. All the supernatant and pellet samples were dialyzed exhaustively against 0.05 M Tris-HCl, pH 7.4, at 4C and then analyzed for PA activity and protein content. PA activity was determined by the ^{125}I-fibrin plate assay as previously described (15,17), and protein content was determined by the Lowry et al procedure (20). One unit of PA is defined as described (17) and the specific activity of PA was calculated as units of PA per 100 μg protein per 4 hrs.

RESULTS AND DISCUSSION

Concentration of PA activity in the 100,000 x g pellet. After elicited macrophages were fractionated by differential centrifugation into the nuclear, membrane plus granule rich, and soluble (cytoplasmic) fractions, the percent distribution of the total PA activity and protein content in each fraction was determined. As shown in Table 1, the nuclear pellet fraction contained approximately 17% of the total protein but only 7 % of the total PA activity. No attempt was made to further purify the nuclear fraction since it contained <10% of the total PA activity in all experiments. Thus, the majority of

TABLE 1. Selective enrichment of macrophage PA activity in 100,000 x g pellet

Fraction	% Distribution of Total[1]		PA Specific activity
	PA	Protein	
Nuclear pellet	6.9	16.7	5.6 (0.6)[2]
Membrane and granule rich (100,000 x g pellet)	89.9	49.4	66.9 (7.4)
Soluble cytoplasmic (100,000 x g supernate)	3.2	33.9	3.3 (0.4)

[1] The values represent the % distribution in each fraction of the total recovered protein and PA activity. The actual recoveries, based on the original vortexed cells, ranged from 96-101% and 110%-140% for protein and PA activity, respectively.

[2] The values in parenthesis represent the ratio of the specific activity of PA in the isolated fraction to that in the original vortexed cells and served as an indicator of enzyme enrichment. The specific activity of PA of the vortexed cells = 9.1 units/100 μg protein/4 hrs.

PA activity (93%) and protein (83%) remained in the postnuclear supernatant fraction which was then subjected to 100,000 x g centrifugation for 1 hr. As seen in Table 1, the resulting pellet and soluble (cytoplasmic) fractions contained approximately 50% and 34%, respectively, of the total protein present in the original cell homogenate. However, when PA activity was assayed, a significantly different distribution profile was revealed, i.e., the bulk of total PA activity (90%) was found to remain in the 100,000 x g pellet and very little (3%) in the soluble cytosol. Furthermore, the specific activity of PA of the 100,000 x g pellet was also increased markedly (7.4 fold). In fact, the ratio of specific activity of PA of the pellet to that of the cytosol is approximately 20:1. Therefore, these results indicate a close association of PA with a subcellular fraction consisting mainly of membranes and granules which are pelletable at 100,000 x g.

Detergent solubilization of PA activity from the 100,000 x g pellet. In order to determine the nature of the association of PA with cellular membranes or granules, the 100,000 x g pellets were subjected to various treatments including extraction with hypotonic and hypertonic solutions, EDTA or detergents, followed by centrifugation at 100,000 x g for 1 hr. The distribution of PA

TABLE 2. Effect of various treatments on solubilization of PA activity from the 100,000 x g pellet

Treatment	Fraction	% distribution of[1]	
		PA	Protein
0.05 M Tris-HCl, pH 7.4 (Control)	Supernate	1.6	4.6
	Pellet	98.4	95.4
0.5 M NaCl	Supernate	1.9	10.3
	Pellet	98.1	89.7
3 M KCl	Supernate	6.0	11.7
	Pellet	94.0	88.3
0.005 M Tris-HCl, pH 7.4	Supernate	3.1	7.4
	Pellet	96.9	92.6
0.001 M EDTA	Supernate	1.6	7.1
	Pellet	98.4	92.9
1% Triton X-100 in 0.1 M Tris-HCl, pH 8.1	Supernate	69.4	64.8
	Pellet	30.6	35.2

[1] The values represent the % distribution in each fraction of the recovered protein or PA activity. The actual recoveries, based on the starting 100,000 x g pellet prior to treatments, ranged from 90-136% and 60-100% for protein and PA activity, respectively.

activity and protein content in the resulting pellets and supernates was then determined. As shown in Table 2, the bulk of PA activity (94-98%) remained sedimentable at 100,000 x g after extraction with a hypotonic solution (0.005 M Tris-HCl, pH 7.4), a chelating agent (0.001 M EDTA) or high salts (0.5 M NaCl or 3 M KCl). Thus, those treatments which are known to release a substantial amount of granule-bound enzymes (such as β-D-glucosaminidase) or loosely bound surface macromolecules (21,22) failed to solubilize any significant amount of PA activity. In contrast, incubation of the pellet with 1% Triton X-100 in 0.1 M Tris-HCl, pH 8.1, resulted in solubilization of approximately 70% of PA activity and 65% of protein. Similar results were also obtained when 0.1% SDS was used to extract PA activity (data not shown).

Taken together, these results suggest that PA is neither contained (or trapped) within secretory granules nor a peripheral membrane protein (22), i.e., one that is loosely bound to cellular membranes. Thus, because of its firm association with cellular membranes requiring detergent treatment for its solubilization, macrophage PA appears to fulfill the operational definition of an integral membrane protein (22). However, whether PA is bound to the plasma membrane and/or other cellular membranes in these experiments remains to be determined.

The PA from other cell types has also been shown to be in a membrane-bound form (18,23-25). Further purification by sucrose gradient centrifugation has revealed that PA activity in chicken embryo fibroblasts is predominantly associated with a plasma membrane enriched fraction as evidenced by the presence of marker enzymes (21). In mouse 3T3 cells, PA release is correlated with cell growth. Thus, growing and stimulated 3T3 or viral-transformed SV3T3 cells release higher levels of PA than do the confluent 3T3 cells (16,17, see 19 for review). In this connection, it is of interest that the type of cellular membrane to which PA activity is bound has also been shown to be correlated with cell growth state. Thus, PA activity is predominantly associated with a plasma membrane enriched fraction in growing and stimulated 3T3 as well as transformed SV3T3 cells, whereas in confluent 3T3 cells, the majority of PA activity is found to be associated with a "heavy" membrane fraction, presumably the RER (18, see 19 for review). Although PA has not yet been rigorously determined as a truly integral membrane protein, all available evidence indicates a firm association of PA with the plasma membrane (see 19 for review). Thus, the release of PA is likely to occur by a shedding mechanism rather than by secretion (exocytosis).

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