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PLASMINOGEN ACTIVATOR ACTIVITY OF CULTURED MURINE MACROPHAGES AND EFFECTS OF ISOPROPYLMETHYLPHOSPHONOFLUORIDATE (SARIN)

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Abstract—Casein-elicited mouse peritoneal macrophages cultured in the presence of phorbol 12-myristate 13-acetate (PMA) express u-PA. Induction is maximal after 4 hr of stimulation and u-PA activity is mainly recovered with the membrane fraction of the cellular lysate. This enzymatic activity is inhibited by isopropylmethylphosphonofluoridate after stimulation by PMA. The presence of the organophosphorus compound before stimulation does not affect the activity.

The results of the present study on the kinetics of u-PA activity in cultured macrophages, its subcellular localization and the effect of an organophosphorus ester are consistent with the concept that the development of pericellular proteolysis proceeds through a series of stages, namely, (a) synthesis of pro-u-PA, (b) binding to membrane receptors, (c) activation to a double-chain u-PA, and (d) conversion of plasminogen into plasmin. The assay procedure developed here provides a sensitive tool to investigate the mechanism of interference of chemicals with several steps of induction of this enzymatic activity in macrophages.

In the course of many biological processes like organogenesis, tumor metastasis, and inflammatory reactions, some cells migrate to different sites of the body through various histological structures. This migration may require the degradation of components of the extracellular matrix (viz. basement membranes, collagen, elastin networks and proteoglycans) [1].

The exact mechanism of this invasion is not fully understood. Two interdependent processes are apparently required for cellular invasion, i.e. cellular attachment and pericellular proteolysis. Cellular attachment is regulated by specific surface glycoproteins such as laminin and fibronectin [2]. Pericellular proteolysis is principally under the control of the plasmin system and specially the urokinase-type plasminogen activator (u-PA)* [3]. U-PA is a serine proteinase; its molecular weight is about 55,000. The exclusive natural substrate of this enzyme is the ubiquitous molecule of plasminogen, the zymogen of the broad spectrum proteinase plasmin which in turn performs the unspecific degradation of the extracellular matrix.

U-PA is present in biological systems mainly in two different forms, i.e. a single-chain protein with little [4] or no catalytic activity (pro-u-PA), and a double-chain protein corresponding to the active form of the enzyme. Single-chain pro-u-PA is considered as a zymogen that is converted by plasmin into the active double-chain u-PA.

As a serine proteinase, u-PA is phosphorylated and inhibited by organophosphorus esters (OPE) such as diisopropylfluorophosphate (DFP); but the pro-u-PA form is not sensitive to inhibition by OPE (for a review see Ref. 5).

Macrophages play an important role in inflammatory reactions. This implies extensive migratory abilities which probably involve the u-PA activity expressed by these cells. U-PA activity is related to the maturation state of the macrophages and can be modulated by various agents [5]. Current concepts on u-PA activity of macrophages suggest that localization of this activity to the cell surface is governed by the presence of u-PA receptors on the plasma membrane. This specific localization allows limited proteolytic activity around the macrophage [3].

In order to study the influence of xenobiotics on macrophage u-PA activity, we have developed an assay procedure to measure u-PA expressed in culture by casein-elicited mouse peritoneal macrophages after stimulation by phorbol 12-myristate 13-acetate (PMA) and have studied the effects of an OPE, isopropylmethylphosphonofluoridate, on this activity.

MATERIALS AND METHODS

Reagents. Isopropylmethylphosphonofluoridate (sarin), >95% of purity was kindly provided by Dr H. C. De Bisschop (TDLM-CT, Vilvoorde, Belgium). Dulbecco's modified Eagle medium (DMEM), L-glutamine, sodium pyruvate, lactalbumin hydrolysate, heat inactivated foetal calf

* Abbreviations used: BSS, balanced salt solution; DFP, diisopropylfluorophosphate; DMEM, Dulbecco's modified Eagle medium; FCS, foetal calf serum; OPE, organophosphorus ester; PMA, phorbol 12-myristate 13-acetate; p-NA, para-nitroaniline; pro-u-PA, pro-urokinase; u-PA, urokinase-type plasminogen activator.

serum, and antibiotics were from GIBCO (Paisley, Scotland). Urokinase, phorbol 12-myristate-13 acetate, chicken egg white lysozyme, and thymidine 5′-phosphate *p*-nitrophenol were from Sigma (St. Louis, MO). Human plasminogen and S-2251 chromogenic substrate were from Kabi Vitrum (Brussels, Belgium). All other reagents were from Merck (Darmstadt, F.R.G.).

Culture media. DMEM was always supplemented with L-glutamine 2 mM, sodium pyruvate 1 mM, and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml).

The balanced salt solution (BSS) contained 136.75 mM NaCl, 5.37 mM KCl, 0.44 mM KH_2PO_4, 1.34 mM Na_2HPO_4, 1.26 mM $CaCl_2$, 0.98 mM $MgCl_2$, 0.81 mM $MgSO_4$ and 5.55 mM dextrose, pH 7.4.

Culture procedure. Female OF1 mice (8–10 weeks of age) were obtained from IFFA-CREDO (Brussels, Belgium). Elicited peritoneal macrophages were harvested from animals injected i.p. 3 days before with 1 ml of 6% (w/v) casein in saline. The yield was usually 5.10^6 cells/animal. The pooled cells were plated either in 35 mm Petri-dishes (2.9×10^5 cells/cm^2) or in 16 mm multidishes (3.5×10^5 cells/cm^2); DMEM was supplemented with 10% heat inactivated foetal calf serum (FCS). Incubation was performed at 37° in a humidified incubator in an atmosphere of 8% CO_2. After 3 hr, non-adherent cells were removed by washing with BSS. The cultures were then refed with fresh medium and incubated for an additional 18 hr. On the second day of culture, the cells were washed with BSS and incubated for different times in DMEM supplemented with 0.1% lactalbumin hydrolysate and phorbol 12-myristate 13-acetate (PMA) at a final concentration of 10^{-7} M (stimulation phase).

PA activity in the culture medium. At the end of the stimulation phase, the culture medium was assayed for PA activity. An aliquot (100–400 μl) of the medium was transferred to a tube and its volume was brought to 1 ml with 50 mM Tris–HCl buffer, pH 7.4, containing 38 mM NaCl, 0.3 mM S-2251 as chromogenic substrate of plasmin and 0.165 CU human plasminogen. The tube was incubated at 37° for 3 hr and the amount of *p*-nitroaniline (pNA) released was determined spectrophotometrically (405 mm). Each assay was run in triplicate and a plasminogen-free blank was included in each run.

PA activity of monolayer macrophages. At the end of the stimulation phase, the macrophages were washed and incubated for different times in BSS containing 0.3 mM S-2251 and 0.165 CU/ml human plasminogen (expression phase). At the end of the expression phase, pNA in the medium was determined as above. Each assay was run in triplicate with a plasminogen-free blank.

Subcellular fractionation. At the end of the stimulation phase, the culture medium was poured off and the cells were washed with BSS. The macrophage monolayers were scraped with a rubber policeman and resuspended in ice-cold buffer (imidazole–HCl 3 mM, sucrose 0.25 M, EDTA 1 mM, pH 7.4). Macrophages were recovered by centrifugation at 800 *g* for 10 min. The resulting pellet was resuspended in the same buffer and the cells were disrupted by ten successive slow passages through a G-25 syringe needle (16 × 0.5 mm). After centrifugation for 3 min at 600 *g*, the supernate was removed and saved, the pellet was resuspended in buffer and submitted to another series of ten passages through the needle. This operation was repeated until a total number of 30 passages was performed. The resulting pellet was resuspended and called "nuclear pellet". The pooled supernates were separated in "soluble fraction" and "membranes rich fraction" by centrifugation at 100,000 *g* for 60 min. The whole procedure was carried out at 4° [6]. The different fractions were assayed for PA activity as described for culture medium.

Cell viability. Viability of the macrophages was assessed by measuring lactate dehydrogenase activity (LDH) in the culture medium both after the stimulation phase and after the expression phase. Total LDH activity was determined after cell disruption in 0.1% Triton X-100. LDH activity was measured by monitoring spectrophotometrically the oxidation of NADH (0.13 mM) in the presence of pyruvate (0.84 mM).

Effect of sarin. Adequate dilutions of sarin were prepared in DMEM or BSS immediately before use. Macrophages were incubated with sarin during three different periods, i.e. (a) during the 15 min preceding the stimulation phase (this incubation was performed in BSS), (b) during the 4-hr stimulation phase, and (c) during the 3-hr expression phase. Except in the latter case, the cells were washed with BSS after each incubation period to remove any free OPE remaining in the medium. The controls were incubated in the same conditions without sarin.

Other biochemical assays. Alkaline phosphodiesterase was determined as described by Darte and Beaufay [6]. Proteins were quantified by the method of Lowry *et al.* [7] using lysozyme as a standard.

RESULTS AND DISCUSSION

1. *U-PA activity in culture medium*

No u-PA activity was detected in the culture media of unstimulated macrophages. Very low activities (less than 5% of cell bound activity) was found in the media of macrophages stimulated by PMA for up to 24 hr. As synthesis by mouse peritoneal macrophages of various forms of u-PA inhibitors has been described [8–10], we have tested for the possible secretion of an inhibitor in the culture media by incubating purified urokinase with the culture media of stimulated macrophages. No decrease of the exogenous u-PA activity was observed. The possibility that the enzyme was secreted in the culture medium as an inactive single-chain (pro-u-PA) was also tested. Preincubating the culture media with catalytic amounts of plasmin did not generate any significant u-PA activity. It can therefore be concluded that unstimulated or PMA stimulated casein-elicited macrophages do not secrete any significant amount of u-PA in the culture medium.

2. *U-PA activity of monolayer macrophages*

U-PA activity of monolayer macrophages was directly assayed in the culture dish. After stimulation

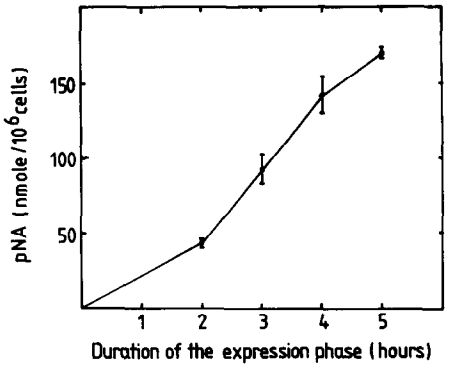

Fig. 1. Kinetics of u-PA assay from PMA-stimulated macrophages (expression phase). Macrophages were stimulated with PMA (10^{-7} M) for 4 hr, washed and incubated in BSS containing plasminogen and S-2251 for various times (expression time). Data represent mean values ± SD of three different experiments (plasminogen-free controls have been subtracted).

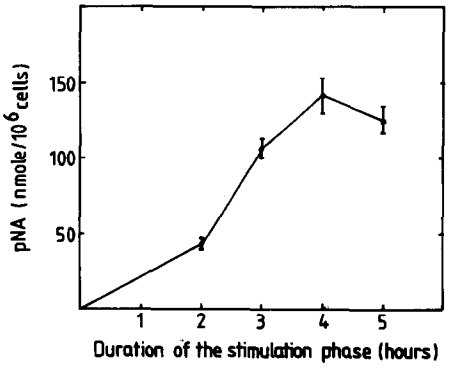

Fig. 2. Influence of the duration of the stimulation phase on u-PA activity of PMA-stimulated macrophages. Macrophages were stimulated with PMA (10^{-7} M) for various times, washed and incubated for 4 hr in BSS containing plasminogen and S-2251. Data represent mean values ± SD of three different experiments (plasminogen-free controls have been subtracted).

by PMA (stimulation phase), the culture medium was replaced by BSS containing plasminogen and a chromogenic substrate of plasmin and the incubation was carried on at 37° (expression phase). Since preliminary results had shown that using DMEM during the expression phase caused a significant plasminogen-independent hydrolysis of the substrate (see also Ref. 11), the incubation was performed in BSS. This assay condition does not significantly impair macrophage viability as LDH released in the culture medium never exceeded 12% of total LDH activity.

The stimulation of macrophages for 4 hr with PMA (10^{-7} M) increased on the average sevenfold the u-PA activity of monolayer macrophages (20.9 ± 2.4 vs 142.1 ± 11.4 nmole/10^6 cells, N = 3). This agrees with the observation of Vassalli *et al.* [12], who has detected u-PA activity in stimulated macrophages with a fibrinolytic assay system.

The present incubation procedure allows to dissociate two different processes, namely, the u-PA production (stimulation phase) and the generation of plasmin activity by the u-PA present on the cell surface of the macrophages at the end of the stimulation phase (expression phase). Kinetics of the two phases in PMA-stimulated cultures are shown in Figs 1 and 2. When the expression phase exceeds 3 hr, the production rate of pNA decreases. An expression time of 3 hr was therefore selected for later experiments. The increase of u-PA activity (stimulation phase) is maximal after 4 hr of incubation with PMA (Fig. 2), and therefore subsequent experiments were performed with a stimulation time of 4 hr. The increase of u-PA activity with the duration of the stimulation phase, despite renewal of the culture medium before the expression phase, indicates that u-PA activity induced by PMA is cell-associated.

3. *U-PA activity in cell lysates*

U-PA specific activities of total cell lysates were 6.8 and 113 nmole pNA/100 μg protein × hr for unstimulated and PMA-stimulated macrophages, respectively. Subcellular fractionation showed that u-PA activity is mainly recovered in the 100,000 *g* fraction, associated with alkaline phosphodiesterase, an enzymatic marker of the plasma membrane [6] (Table 1). This distribution of u-PA activity was

Table 1. Subcellular fractionation of macrophages and distribution of u-PA

	Unstimulated macrophages		PMA stimulated macrophages	
	u-PA (%)	Alkaline phosphodiesterase (%)	u-PA (%)	Alkaline phosphodiesterase (%)
Cell lysate	100	100	100	100
Soluble fraction (S)	4.9	0	7.8	0
Nuclear pellet (N)	1.2	0	0.9	0
Membranes (M)	109.7	105.7	78.1	89.6
Total (S) + (N) + (M)	115.8	105.7	86.8	89.6

Macrophages were incubated without and with PMA for 4 hr and then fractionated as described in Materials and Methods. The u-PA activity of the cell lysate and each fraction was determined.

u-PA specific activities of cell lysates were 6.8 and 113 nmole pNA/100 μg protein × hr for unstimulated and PMA-stimulated macrophages respectively.

Table 2. Effect of sarin on u-PA activity of monolayer macrophages stimulated by PMA

Sarin (mole/l)	Before stimulation (15 min) u-PA (%)	Before stimulation (15 min) LDH* (%)	During stimulation (4 hr) u-PA (%)	During stimulation (4 hr) LDH* (%)	During expression (3 hr) u-PA (%)	During expression (3 hr) LDH† (%)
0	100 ± 12.0	13.5 ± 1.9	100 ± 17.6	13.5 ± 1.3	100 ± 10.9	4.9 ± 1.6
10^{-4}	91.0 ± 28.0	16.0 ± 3.0	95.0 ± 24.2	23.3 ± 1.3	27.1 ± 3.5	10.4 ± 1.4
5.10^{-4}	79.4 ± 14.8	16.0 ± 1.5	33.0 ± 2.0	22.2 ± 2.4	6.0 ± 2.2	9.7 ± 2.1
10^{-3}	80.6 ± 4.4	14.2 ± 3.1	0.5 ± 0.8	23.6 ± 1.1	2.3 ± 1.0	10.1 ± 2.6

The numbers shown are mean values ± SD from three different experiments.
u-PA activities are expressed in percent of the control value.
LDH activities are expressed in percent of total activity determined after cell disruption by Triton X-100.
* Determined on culture medium after the stimulation phase.
† Determined on incubation medium after the expression phase.

similar in unstimulated and PMA-stimulated macrophages. Incubating total cell lysate of unstimulated macrophages with plasmin did not significantly increase u-PA activity. This suggests that PMA-stimulation of u-PA activity of macrophage is not due to the activation of a quiescent pool of pro-u-PA but results from *de novo* synthesis of the enzyme.

4. *Effects of sarin on u-PA activity*

Preliminary experiments (results not shown) demonstrated that sarin irreversibly inhibits purified human urokinase; 50% of the enzymatic activity being inhibited by a concentration of 1.25×10^{-5} M of sarin (30 min incubation at 37°, pH 7.4). The effect of sarin on u-PA activity of monolayer macrophages is summarized in Table 2. Viability of the macrophages, monitored by the release of LDH, was not seriously impaired by the presence of sarin; no dose effect was observed. Incubating the macrophages with sarin for 15 min, just before stimulation by PMA, did not greatly inhibit the enzymatic activity subsequently induced by PMA. Since sarin can easily cross all biological membranes, its lack of effect is not due to its inability to reach a potential critical site within the macrophage. This result also indicates that phosphorylation of various other serine enzymes by sarin does not prevent the subsequent induction of u-PA by PMA. The presence of sarin during the expression phase inhibits the u-PA activity.

The presence of sarin during the stimulation phase results in an inhibition pattern which is intermediate between the two preceding ones. During the stimulation phase, the macrophage is synthesizing pro-u-PA and activating u-PA on the surface membrane; u-PA is thus present in its two different molecular forms and only the double-chain form is inhibited by sarin. The stronger inhibition observed when sarin is added during the expression phase could also result from an effect on plasmin. Plasmin is indeed also a serine enzyme which in the conditions of the experiments might have been inhibited by high concentrations of sarin in spite of the presence of a specific substrate.

The results of the present study on the kinetics of u-PA activity in cultured macrophages, its subcellular localization and the effect of an OPE are consistent with the concept that the development of pericellular proteolysis proceeds through a series of stages [3], namely, (a) synthesis of pro-u-PA, (b) binding to membrane receptors, (c) activation to a double-chain u-PA, and (d) conversion of plasminogen into plasmin. Each of these processes represents a potential target for the action of xenobiotics. The two-phases assay developed in this study provides a sensitive tool to investigate the mechanism of interference of chemicals with these processes.

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