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PARALLEL INDUCTION OF FIBRINOLYSIS AND RECEPTORS FOR PLASMINOGEN AND UROKINASE BY INTERFERON GAMMA ON U937 CELLS

H. Lu*, Mc. Mirshahi, P. Krief, C. Soria, J. Soria, Z. Mishal, O. Bertrand
J.Y. Perrot, H. Li, C. Picot, E. Pujade, A. Bernadou, and J.P. Caen

CNRS UR334 and INSERM U150 Hôpital Lariboisière, Paris
Laboratoire des Prof. Adolphe-Bernadou-Samama, Paris. Hôtel Dieu, Paris
CNRS, service de cytométrie analytique. Villejuif
Université de Rouen, Rouen, France

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SUMMARY : A new cell sorter technique was employed to study the role of interferon gamma (INFγ)in fibrinolysis induced by U937 monocytic cells. INF-γ induced the differen tiation of U937 cells as evidenced by the appearance of CD 14 antigen on the cell surface. Scatchard analysis and dose response curves showed a parallel increase in the number of receptors on U937 cells capable of accepting exogenous plasminogen and urokinase (UPA) synthetized by differentiating U937 monocytic cells. This would favour an activation of plasminogen by UPA.

This adds a new parameter in the regulation of cell-mediated fibrinolysis may be impor tant in a number of biological processes.

INTRODUCTION : Physiological fibrinolysis depends upon the activation of plasminogen into plasmin under the influence of enzymes (plasminogen activator : PA) that catalyse the cleavage of Arg 560/Val561 bond on the plasminogen molecule (1). This is most often shown by the estimation of plasminogen, PA (Urokinase or UPA and tissue type plasminogen activator or tPA) and their inhibitors (PAi) in plasma (2). Less often, the monocytes have also been implicated in this process through the synthesis of UPA (3) which, along with exogenous plasminogen, are bound to specific receptors on the cell surface (4). We present here a new parameter in the regulation of cellular fibrinolysis by interferonγ(INF-γ) (5) employing a new technique developed in our laboratory for this purpose.

MATERIALS AND METHODS : Lysplasminogen (Subtrene-Choay, Paris) was adsorbed on aprotinin-agarose to eliminate plasmin contamination. Glu-plasminogen was prepared accor ding to Deutsch and Mertz (6). Recombinant glycosylated interferonγ(INF-γ) was kindly given by Dr. Fries, Belgium. It was found to be pure on electrophoresis and its concentra tion was estimated on its antiviral activity and expressed in international units. Human fibrinogen (grade L) and S2251 were from Kabi (Stockholm). Fluoresceine isothiocyanate (FITC) was from Sigma.

Cell culture : mycoplasm-free human U937 cell line was kindly given by Dr. Claude Boucheix (Paul Brousse, Villejuif, Paris). The U937 cells were cultured at the initial concen

* From the Medical University II of Shanghaï - R.P. CHINA

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tration of 2 x 10^5 /ml in RPMI 1640 culture medium (Gibco Ltd) complemented with 10 % mycoplasm-free calf serum (Gibco Ltd), 100 U/ml penicillin G, 50 mg/ml streptomycin and 1 % glutamin, for various lengths of time in humidified atmosphere of 4,5 % CO2 at 37°C in plastic culture flasks (Becton Dickson). INF-γ treatment was done by adding various concentrations of INF-γ into cell culture medium. Viability was determined by trypan blue exclusion, and exceded 97 %. Before testing U937 cells were washed 3 times for 30 minutes each time with RPMI and resuspended in RMPI 1640.

Fibrin clot degradation assay : 0.1 ml washed U937 cells (10^7 cells/ml) were added to 0.1 ml fibrinogen (3 mg/ml, Kabi), 0.1 ml glu-plasminogen (2 U/ml) and 0.1 ml RPMI. Clots were then obtained by adding 0.1 ml thrombin (5 U/ml in 0.1 M CaCl2). After 3 hours of incubation at 37° C, the clots were squeezed out and clot degradation was evaluated by determining fibrin degradation products (FbDP) using latex procedure (Diagnostica Stago, Asnières, France).

PA activity expressed by U937 : PA was determined by the extent of plasmin formation in the presence of exogenous lys-plasminogen. Plasmin was determined by the amidolytic activity on a chromogenic substrate S-2251 (Kabi) Val-Leu-Lys-paranitroanilin (PNA). The release of PNA was measured by absorbance at 405 nm with an Elisa reader. For this assay, 50 µl of U937 cell suspension (10^7 /ml) was seeded into the wells of 96 microtiter plates. Then 50 µl RPMI, 50 µl lys-plasminogen (170 µg/ml) or RPMI 1640 (for nonspecific amidolytic activity), 50 µl S-2251 were then added. Change in absorbance of the mixture was measured every 15 minutes after incubation at 37°C. Standard curve using UPA was performed using the same conditions. To express the activity in UPA, the difference between the absorbance obtained in the presence and absence of plasminogen was calculated for each time period.

Preparation of FITC-conjugated glu-plasminogen and urokinase : before coupling, the urokinase was passed on a sephadex G25 column in the presence of coupling buffer, in order to eliminate all preservatives. 0.1 mg FITC per mg of protein was added in 0.5 M carbonate buffer, pH 9.5 for 18 hours at 4° C. Free fluorescein was then removed by gel filtration on sephadex G 25 and centrifuged for 5 minutes at 10 000 g at room temperature. FITC-conjugated glu-plasminogen was tested for plasmin amidolytic activity on S-2251 after activation by adding streptokinae. Our results show that more than 80 % activity was reco vered after coupling. FITC conjugated-urokinase was tested for amidolytic activity on S-2244. Over 40 % of the activity was recovered. The ratio of FITC per protein was in the range of 3 to 7.

Binding assay of glu-plasminogen and urokinase on cells : 5 x 10^5 cells were incubated at room temperature for 1 hour with 50 µl FITC conjugated ligands at various concentrations in the presence or absence of competing unlabelled protein. After 3 washes with RPMI the cells were fixed with 1 % formaldehyde. Background controls were performed first by adding 50 times more of unlabelled ligand to labelled ligand and second with FITC label led albumin. Specificity of the binding was confirmed by no competition with an excess of unrelated proteins.

The quantification of glu-plasminogen and urokinase bound to U937 cell surface was determined by flow cytometry (Facs 440, Becton-Dickinson) which was standardized in respect to the fluorescence emission of fluorescein isothiocyanate (FITC) by FITC conjugated sephadex G25 beads as described by Le Bouteiller et al (7).

The expression of CD 14 antigen on U937 cell surface : was determined by flow cytome try (Facstar, Becton-Dickinson). Results were expressed by the mean value of fluorescence intensity using indirect immunofluorescence technique. Monoclonal antibody RMO-50, given by Dr. Boucheix was used in this test.

RESULTS AND DISCUSSION : Data in fig. 1 show that the introduction of U937 cells in a fibrin clot increased the fibrinolytic activity from the basal level of $< 2\ 000$ ng/ml of fibrin degradation products to approximately 8 000 ng/ml. The fibrinolytic activity increased almost 8 fold if U937 cells were pretreated with INF-γ (400 U/ml for 4 days).

Data in fig. 2a reveal that this increase in fibrinolytic activity (expressed in UPA) is due to the activation of exogenous plasminogen by endogenous PA. In addition, this sort of fibrinolysis seems to be mostly a receptor-mediated event, since no PA could be found in the supernatant of culture medium under these conditions (not shown). Finally, this cellu

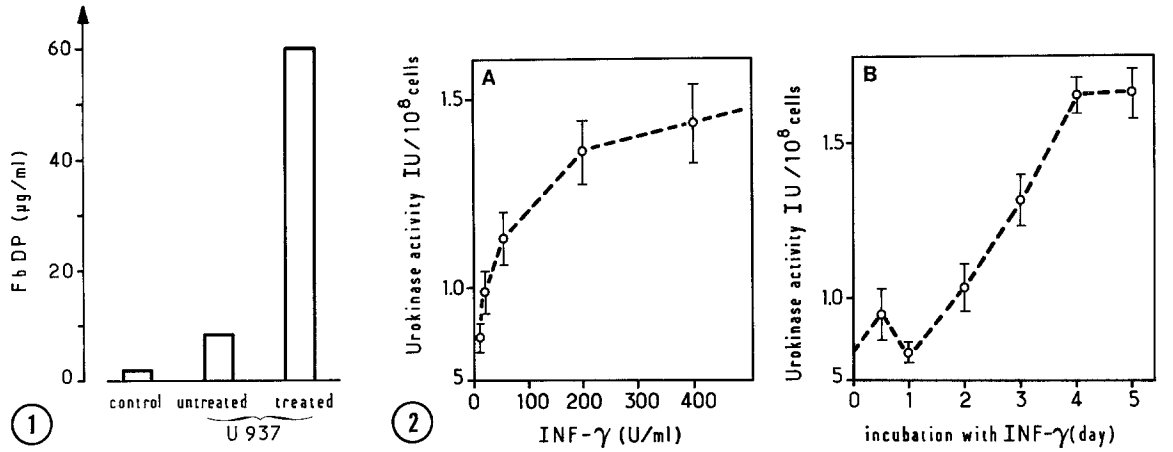


Fig. 1 :
INFγ induces fibrinolytic activity in U937 cells.
0.1 ml U937 cells untreated or INF- γ-treated (10^7 cells/ml) were added to 0.1 ml fibri nogen (3 mg/ml) + 0.1 ml glu-plasminogen (2 CU/ml) + 0.1 ml RPMI. Clotting was obtained with 0.1 ml thrombin (5 U/ml in CaCl2 0.1 M). After 3 hours at 37°C degradation was evaluated by measuring fibrin degradation products.
Control was performed without cells.
Treated U937 : INF- γ treated cells (400 IU/ml for 4 days).

Fig. 2 :
INF- γ in U937 induces plasminogen activation by endogenous PA. 50 μl U937 cells (10^7 /ml) were added to 50 μl lys-plasminogen (170 μg/ml) or RPMI (control) and 50 μl S2251. Mixtures were incubated at 37° C. Absorbance was measured at 405 nm immediately and every 15 minutes for 3 hours. Standard curve was performed using purified urokinase (UPA). To express the results in UPA, the difference between the absorbance obtained in the pre sence and absence of plasminogen was calculated.

2a : U937 were treated by various amounts of INF-γ for 4 days
2b : U937 were treated by 400 IU/ml INF-γ for various lengths of time.

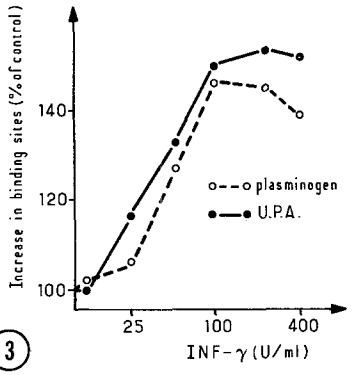
lar fibrinolysis is dependent upon the dose of INF-γ (fig. 2a) and a latent period is required (Fig. 2b) for the maximum effect (4 days).

Since cell surface receptors for plasminogen and PA may provide a mechanism of fibrinolysis regulation, the extent of plasmin formation was compared to the binding of plasminogen and PA on the INF-γ treated and untreated cells. Determination of plasminogen and UPA binding to U937 cells was achieved by using a cell sorter which enabled us to directly assess the number of molecules bound to each cell, and their distribution.

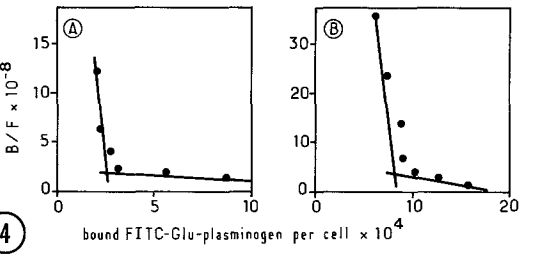
Data in fig. 3 show a dose dependent increase in the binding of plasminogen and UPA in INF-γ treated U937 cells in the presence of an excess of the labelled ligand.

Scatchard analysis (fig. 4) by this method revealed that the binding of plasminogen for the high affinity receptor on the surface of INF-γ treated U937 remains unaltered (KD 0.14/0.10 μM). By contrast, the number of high affinity receptors for plasminogen increased form 36,000 in untreated cells to 104,000 after INF-γ treatment. The contribution of low affinity binding sites appears to remain normal under these conditions and is difficult to establish.

Since the dose response curve in Fig. 2 and 3 are almost parallel, it would seem that the plasmin formation on U937 cells is closely related to binding of both plasminogen and



(3)



(4)

Fig. 3 :
Influence of treatment by INF-γ on plasminogen and urokinase (UPA) receptors on U937 cells.
5 x 10^5 cells were incubated with 50 µl FITC-conjugated ligand (FITC-glu-plasminogen at 200 µg/ml, and(FITC-urokinase at 100 µg/ml) for 1 hour at room temperature. After 3 washes, cells were fixed with glutaraldehylde and quantification of labelled ligand bound to U937 cells was determined by flow cytometry (FACS-440) standardized by FITC-conju gated sephadex G25 beads. Non specific binding was measured in the presence of labelled albumin (500 µg/ml). Each value is the mean of triplicate assays. The percentage of increase in receptors was obtained by comparing treated with nontreated U937 cells level.

Fig. 4 :
Scatchard plot analysis for plasminogen receptors on U937 cells.
A : untreated cells
B : INF-γ treated cells
5 x 10^5 cells were incubated with various concentrations of FITC-plasminogen from 100 to 1.5 µg/ml (50 µl) for 1 hour at room temperature. After 3 washes, quantification of labeled ligand bound to U937 cells was determined as indicated in Fig. 3. Each value is the mean of triplicate determinations.

UPA to the cell membrane, and INF-γ acts by increasing UPA and plasminogen binding sites on U937 cells.

Data in Fig. 5 show a dose dependent increase in CD 14 antigen in presence of INF-γ. Since the same dose levels of INF-γ also induce plasminogen and UPA receptors (fig. 3), it is therefore suggested that the INF-γ induces the process of differentiation in U937 monocytic cells which in turn is responsible for this increased cellular fibrinolysis.

Data reported in this paper reveal new parameters in the regulation of cell fibrinolysis that could be important since the cell surface is generally protected against the interaction with inhibitors of component (UPA, plasmin) responsible for fibrinolysis (4). The induction of cellular fibrinolysis under the action of INF-γ may influence several cellular processes such as cellular migration, tumor lysis, inflammation, fibrin degradation. In addition, degra dation may favour the contact between macrophages and tumor cells which are generally associated by fibrin network (8).

Finally, the increased fibrinolytic activity may favour the dissemination of malignant cells, since U937 cell lines is of leukemic origin. Lastly, the technique established with the cell sorter may be exploited in further studies of the physiological regulation of fibrino lysis and other biological systems.

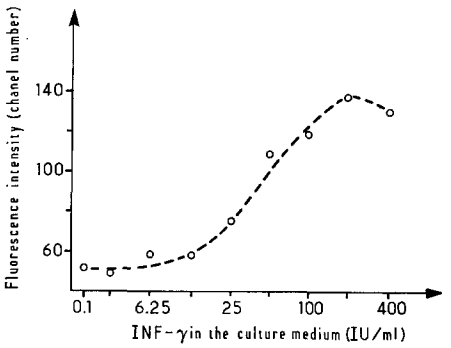


Fig. 5 :
INF-γ induces CD 14 antigen expression on U937 cells. 50 μl U937 cells were incubated with RMO 50 monoclonal antibody diluted 1/100 in PBS. After 1 hour incubation at room temperature, the cells were washed 3 times and then incubated with FITC-anti rabbit immu noglobulin for 30 minutes at room temperature. After 3 washes the cells were fixed with 1 % glutaraldehylde and then passed through FACSTAR cytofluorometer. Numbers indicate relative specific fluorescence, calculated from mean fluorescence channel numbers.

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