Regulation of Plasminogen Receptor Expression on Human Monocytes and Monocytoid Cell Lines

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Abstract. The capacity of human monocytoid cell lines and peripheral blood monocytes to modulate their expression of plasminogen receptors has been assessed. After PMA stimulation, THP-1 or U937 monocytoid cells were separated into adherent and nonadherent populations. Plasminogen bound to adherent cells with similar capacity and affinity as to nonstimulated cells. In contrast, the nonadherent cells bound plasminogen with 5–17-fold higher capacity (without a change in affinity). This increase was selective as urokinase bound with similar affinity and capacity to the adherent and nonadherent populations. Upregulation of plasminogen receptors on the nonadherent monocytoid cells was rapid, detectable within 30 min, and reversible, adhesion of the nonadherent cells resulted in a sixfold decrease in plasminogen binding within 90 min. The increase in plasminogen binding to the nonadherent cells was associated with a marked increase in their capacity to generate plasmin activity from cell-bound plasminogen.

PMA stimulation of human peripheral blood monocytes increased their expression of plasminogen receptors by two- to fourfold. This increase was observed in both adherent and nonadherent monocytes. Freshly isolated monocytes maximally bound 5.0×10^5 plasminogen molecules per cell, whereas monocytes cultured for 18 h or more maximally bound 1.7×10^7 molecules per cell, a 30-fold difference in receptor number. These results indicate that both monocytes and monocytoid cell lines can rapidly and markedly regulate their expression of plasminogen binding sites. As enhanced plasminogen binding is correlated with an increased capacity to generate plasmin, an enzyme with broad substrate recognition, modulation of plasminogen receptors may have profound functional consequences.

CELL-ASSOCIATED proteases participate in a wide variety of physiological and pathophysiologic processes. The enzymes of the fibrinolytic system, the plasminogen activators (urokinase [u-PA][1] and tissue plasminogen activator [t-PA]), and plasmin, together represent one of the most broadly distributed and utilized of these protease systems. Plasmin is a serine protease with broad substrate recognition (49). In addition to its role as the primary enzyme involved in dissolution of fibrin deposits, plasmin also degrades a number of matrix proteins (41). This capability suggests that cell-associated plasmin also may play a key role in regulating cell migration.

Cell surface receptors for plasmin, its zymogen, plasminogen, and for the plasminogen activators provide a mechanism for cells to harness and regulate the activities of these proteases for performance of cellular functions. Cellular binding sites for plasminogen (36, 45), plasmin (8), u-PA (1, 6, 12, 57), and t-PA (2, 3, 19) have been demonstrated on a variety of cells, and evidence for the regulation of specific cellular responses by these receptors is evolving. Plasmin(ogen) receptors are particularly widely distributed [reviewed in reference 36] and play a key role in regulating the activity of plasmin at cell surfaces.

Monocytes/macrophages exhibit a complex interrelationship with the fibrinolytic system. These cells can synthesize both u-PA (56) and t-PA (21, 22) as well as inhibitors of plasminogen activators (10). The biosynthesis of these fibrinolytic components can be modulated by agonists (21, 22, 30, 58, 63). In addition, monocytes and monocytoid cell lines express both u-PA and plasminogen receptors (35, 45, 57). Direct evidence for the occupancy of plasminogen receptors on macrophages within inflammatory regions has been demonstrated recently (51). In addition, monocytoid cells have the capacity to modulate their expression of u-PA receptors in response to agonists (14, 26, 29, 44, 52, and Lu et al. 1989. *Thromb. Haemostasis.* 62:91). Thus, monocytoid cells possess numerous distinct mechanisms for regulating the proteolytic activity of the fibrinolytic system. In this study, we have investigated whether human monocytes and monocytoid cell lines can modulate their expression of plasminogen receptors. In view of the importance of the migratory functions of these cells in inflammatory processes and their

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1. *Abbreviations used in this paper*: PCA, procoagulant activity; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator.

overall capacity to regulate their fibrinolytic activities, the modulation of their plasminogen receptor function would be of central importance. Specific conditions are now identified which result in marked differences in plasminogen receptor expression, and these differences are associated with dramatic changes in the fibrinolytic activity of these cells.

Materials and Methods

Proteins

Glu-plasminogen was isolated from fresh plasma (prepared from blood drawn into 50 μg/ml soybean trypsin inhibitor [Sigma Chemical Co., St. Louis, MO], 100 kallikrein inhibitor units/ml trasylol [FBA Pharmaceuticals, New York, NY], 3 mM benzamidine and 3 mM EDTA), by affinity chromatography on lysine-Sepharose (13) (Pharmacia Fine Chemicals, Piscataway, NJ) in the presence of 1 mM benzamidine, 0.02% NaN_3, and 3 mM EDTA. The protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 16.8 (60). Lys-plasminogen was purchased from National Institute for Biological Standards and Control (London, England). High-molecular weight two-chain urokinase (u-PA) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Glu-plasminogen and u-PA were radiolabeled using a modified chloramine T method (45). After labeling, u-PA was routinely repurified by affinity chromatography on benzamidine-Sepharose (24) and subsequently inactivated by treatment with 5 mM diisopropyl fluorophosphate for 30 min at 4°C. The labeled and unlabeled preparations of the ligands used in this study had the characteristics of previously described preparations from our laboratory (37, 45).

Cells

The human monocytoid THP-1 cell line (55) was cultured in RPMI-1640 containing 100 U/ml penicillin G, 100 μg/ml streptomycin, 1 mM Na pyruvate, 0.05 M Hepes pH 7.35, 5×10^{-4}% 2-mercaptoethanol, and 5% FCS (Mo-Medium). The human monocytoid cell line U937 and the human myeloid cell line HL60 were grown in RPMI-1640 containing 100 U/ml penicillin G, 100 μg/ml streptomycin and 5% FCS.

Human peripheral blood monocytes were prepared from blood drawn into 5 U/ml heparin, 10 mM theophylline, and 10 μg/ml prostaglandin $E_{1\alpha}$. The blood was immediately centrifuged at 500 *g* for 10 min, and the platelet-rich plasma removed. After platelet depletion, two different protocols were used for monocyte isolation. Monocytes, isolated using an adhesion step, were prepared by centrifugation of the platelet-depleted blood cells, resuspended to the original blood volume in RPMI-1640, onto Ficoll-Hypaque (8% Ficoll [Sigma Chemical Co.], 10% Hypaque [Winthrop-Breon, New York, NY], $d = 1.3570$) to obtain the mononuclear cell fraction, and subsequent adherence of these cells ($40–100 \times 10^6$ cells in 10–15 ml RPMI-1640 medium) on fibronectin–gelatin dishes (5, 25) under sterile conditions. Fibronectin-gelatin dishes were prepared by incubation of 2% gelatin (type II; Sigma Chemical Co.) (10 ml/dish) for 1 h at 37°C, washing three times with Dulbecco's PBS (DPBS) and addition of autologous heparinized platelet-poor plasma (10 ml per dish). After 30 min at 22°C and 1 h at 4°C, the plates were washed three times with DPBS and immediately used for adhesion of monocytes. After 1 h of adherence at 22°C, nonattached cells were removed by washing, and adherent monocytes were removed by incubation with 3.5 mM EDTA in DPBS for 10 min at 4°C.

Isolation of monocytes without adhesion was performed using Sephracell-MN (Sephractec Co., Oklahoma City, OK) as described (59). Briefly, Sephracell-MN was added to the platelet-depleted cell pellet at a ratio of 0.6:1 (vol/vol). After mixing by inversion, the suspension was centrifuged for 20 min at 1,500 *g*. The mononuclear cell fraction was removed from the upper phase of the Sephracell-MN and washed once in PBS containing 0.1% BSA (Calbiochem-Behring Corp.) (PBS + BSA). Pellets of mononuclear cells were resuspended in PBS + BSA, mixed again with Sephracell-MN at a ratio of 1:2 (volume/volume), and centrifuged for 20 min at 1,500 *g*. After washing once in PBS + BSA, monocytes were suspended in Mo-medium.

Purity of the monocyte preparations was determined by microscopic examination of cells after Giemsa (Harleco, Gibbstown, NJ) and esterase staining (15). In addition, preparations were subjected to FACS analysis (see below) using an mAb against the Mac-1 alpha chain (MI/70) (kindly provided by Dr. D. Altieri from the Research Institute of Scripps Clinic, La Jolla, CA) (4). Only monocyte preparations containing <20% platelets (mean 13 ± 4% in 6 experiments) and <10% lymphocytes were used in this study. (At a 20% contamination level, platelets would account for <1% of the ^{125}I-plasminogen binding observed with a monocyte preparation). In addition monocyte preparations were routinely assayed for procoagulant activity (PCA) as an index for the absence of stimulation with LPS or other agonists (28), and only preparations expressing <30 mU/10^6 cells were used.

Cell Stimulation and Adhesion

12-*O*-Tetradecanoylphorbol-13-acetate (PMA) (Sigma Chemical Co.), was routinely added at 40 nM to monocytoid cells and 4 nM to monocytes (monocytes were stimulated at a 10-fold lower concentration to minimize cytotoxicity). Unless indicated, stimulation was performed for 18 h in Mo-medium under sterile conditions. Viability was assessed by trypan blue exclusion and recovery by counting of cells in a hemocytometer. After PMA stimulation monocytoid cells become more adherent (54) and the proportion of adherent cells depends on the surface provided. At selected times, nonadherent cells were recovered by gentle decanting and the adherent cells by flushing with a pipette. Several types of vessels were used to obtain varying proportions of adherent and nonadherent cells. Using THP-1 cells, the following percent adherence was obtained after treatment with PMA (40 nM) for 18 h: 150-cm^2 flasks (Costar, Cambridge, MA), 60–75%; 100-mm dishes (Corning Glass Works, Corning, NY), 55–75% (5–10% without PMA stimulation); 30 ml Teflon bottles (Nalge Co., Rochester, NY), 15–25% (3–5% without PMA stimulation); gelatin-fibronectin dishes (85–95%). Unless otherwise indicated, the 100-mm Corning dishes were used in all experiments with the monocytoid cell lines.

Ligand Binding Analyses

Before use in binding analyses, the cells were washed three times in HBSS containing 1.2 mM $CaCl_2$ and 1.6 mM $MgSO_4$ (HBSS) and resuspended in HBSS containing 0.05 M Hepes, pH 7.35, and 0.1% BSA (HBSS-Hepes). Binding analyses were carried out in 1.5 ml polypropylene snap-cap tubes. Radiolabeled ligands, typically 100 nM ^{125}I-plasminogen and 1 nM ^{125}I-u-PA (final concentrations), unlabeled ligands, or buffer were added to a final volume of 200 μl. The cells were at 1.0×10^6 or 1.5×10^7 cells/ml in this 200-μl volume for measuring plasminogen or u-PA binding, respectively. After incubation at 37°C for 1 h, triplicate 50-μl samples were layered over 300 μl 20% sucrose in HBSS-Hepes and centrifuged for 2.5 min in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, CA). The tube tips were cut off and counted in a gamma counter. Molecules of ligand bound per cell were calculated based on the specific activity of each ligand. Specific binding was measured as the difference in the radioactivity bound to the cells in the presence and absence of a 40–100-fold final molar excess of nonlabeled ligand. Dissociation constants (K_d) were determined from specific binding isotherms using a nonlinear curve-fitting computer program, Ligand (42). Linear correlation coefficients were calculated with a TI-55-II calculator (Texas Instruments Inc., Dallas, TX). Statistical comparisons were done using a paired comparison *t* test.

In some experiments, the cells were treated at low pH to remove potential endogenous ligands bound to receptors. The conditions under which >80% of ^{125}I-plasminogen or ^{125}I-u-PA bound to the cells was dissociated were established for each cell type. These conditions were 10 min at pH 3.5 or pH 4.0 for the cell lines or monocytes, respectively. After this treatment the cell lines were >85–90% viable and monocytes were 65–70% viable. After such treatments, each cell type retained >90% of its capacity to bind added ^{125}I-plasminogen or ^{125}I-u-PA.

SDS-PAGE

Cell-bound ligands were extracted with 0.1% Triton-X-100 in 0.1 M Tris, pH 8.1, containing 10 mM EDTA, 1 mM PMSF, 100 U/ml trasylol. Extracts were boiled for 5 min and electrophoresed under reducing conditions on 8% polyacrylamide slab gels in the buffer system of Laemmli (27). Gels were stained with Coomassie blue, dried, and autoradiograms were developed with XRP-1 film (Eastman Kodak, Rochester, NY). The autoradiograms were scanned using a soft laser densitometer (Zeineh; Biomed Instruments Inc., Fullerton, CA). Molecular weights were estimated relative to protein standards obtained from Amersham Corp. (Arlington Heights, IL). Relative mobility (R_m) was calculated by dividing the distance from the top of the gel to the leading front of a protein band by the distance from the top of the gel to the dye front (61).

Ligand Dot Blots

Cells (1.0 x 10^6) were lysed in 1 ml of 0.1% Triton-X-100 in 0.1 M Tris, pH 8.1, containing 10 mM EDTA, 1.0 mM PMSF, 100 U/ml trasylol. The extract, 20 μl, was applied to Immobilon-P filter paper (Millipore Co., Bedford, MA), using a dot-blot apparatus (Bethesda Research Laboratories, Gaithersburg, MD). After 90 min at 37°C, the filters were extensively washed with PBS containing 2% BSA and incubated with ^{125}I-plasminogen (100 nM) in the same buffer for 1 h at 37°C on a shaker. After washing with 2% BSA in PBS for 1 h with several changes, the filters were air dried and subjected to autoradiography.

Cell-associated Fibrinolytic Activity

Plasmin generation by isolated cell populations was determined after preincubating the cells (1.5 x 10^6/ml in HBSS) with 15 μM Glu-plasminogen for 1 h at 37°C and washing the cells twice with HBSS. Plasmin generation was measured at 405 nm as a function of time after addition of u-PA (3 nM) and the chromogenic substrate S-2251 (0.5 mM) (Kabi Vitrum, Malmö, Sweden), in Tris HCl pH 8.8, in a final volume of 200 μl, using an ELISA plate reader (Molecular Devices, Palo Alto, CA).

FACS Analysis of Monocyte Preparations

Fluorescein-conjugated plasminogen (FITC-plasminogen) was prepared by conjugation of Glu-plasminogen with fluorescein (Sigma Chemical Co.) as described (16). Conjugated fluorescein was separated from free fluorescein on Sephadex G-25 in PBS containing 0.1% NaN_3. The fluorescein/protein ratio ranged from two to four (53). To characterize the conjugate, its capacity to inhibit plasminogen binding to cells was assessed. ^{125}I-plasminogen (0.1 μM) binding to THP-1 cells was 80% inhibited by 2 μM unlabeled plasminogen and 74% inhibited by 2 μM FITC-plasminogen. For binding of FITC-plasminogen to monocytes and subsequent FACS analysis, cells (1 x 10^7/ml) were incubated with FITC-plasminogen (10 μM) in HBSS-Hepes containing 0.1% BSA for 1 h at 37°C. After washing once, the resuspended cells were analyzed by flow cytometry in a Becton Dickinson FACS IV/40 (Becton Dickinson Co., Oxnard, CA). Purity of the monocytes was assessed by incubation with a saturating concentration of MI/70 antibody followed by fluorescein-conjugated goat F(ab')^2 anti-mouse IgG (reactive with heavy and light chains) (Tago Corp., Burlingame, CA), and subsequent FACS analysis.

Results

Expression of Fibrinolytic Receptors on THP-1 Cells

The U937 cell line has been used extensively to study the fibrinolytic properties and the interactions of monocytoid cells with fibrinolytic molecules (30, 44, 45, 52, 57). THP-1 cells exhibit a number of the characteristics of monocytes including ones not expressed by U937 cells (55) such as major histocompatibility class-II antigens (64). Therefore, we sought to determine whether THP-1 cells could serve as a second model monocytoid cell line by studying the binding of the fibrinolytic ligands, plasminogen and u-PA, to the cells. In initial experiments, we found that both ^{125}I-plasminogen and ^{125}I-u-PA bound to THP-1 cells in a time dependent manner. At 37°C, apparent equilibrium was achieved at 60 and 30 min for plasminogen and u-PA, respectively, typical of the time courses for binding of these ligands to monocytes, U937 cells, and many other cell types (36, 45). Binding of both ligands was saturable. At a 100-nM input concentration of ^{125}I-plasminogen, binding was 83% inhibited by a 40-fold molar excess of nonlabeled plasminogen (eight experiments). With ^{125}I-u-PA at a 1 nM input concentration, binding was 86% inhibited by a 50-fold molar excess of nonlabeled u-PA (six experiments). The reversibility of plasminogen and u-PA to the THP-1 cells was assessed. Radiolabeled plasminogen (100 nM) or u-PA (1 nM) was incubated with the cells for 1 h at 37°C. At this time, a 90-fold excess of the homologous nonlabeled ligand was added, and the extent of ligand binding was measured after an additional 10 or 30 min. Relative to the ^{125}I-plasminogen specifically bound to the cells at 60 min, the excess nonlabeled plasminogen displaced 70 and 81% of the bound ligand within 10 and 30 min, respectively. Similar results were obtained with u-Pa; the excess nonlabeled u-PA displaced 67 and 71% of the bound ^{125}I-u-PA af-

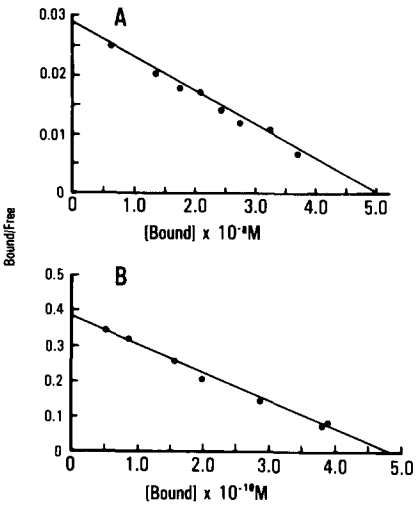

Figure 1. Scatchard plots for the binding of plasminogen (*A*) and urokinase (*B*) to THP-1 cells. Data are derived from specific binding isotherms for the two ligands. The plasminogen binding isotherm was constructed by adding varying amounts of nonlabeled plasminogen (0.1–10 μM) and a constant amount of ^{125}I-plasminogen (100 nM) to cells. The plasminogen binding parameters derived are: K_d = 1.8 μM with 2.4 × 10^7 total binding sites per cell. u-PA binding isotherms (*B*) were constructed by adding varying amounts of ^{125}I-u-PA to the cells (3.75 × 10^6/ml). Incubations were for 60 min at 37°C. The urokinase binding parameters derived are: K_d = 1.3 nM with 7.6 × 10^4 total binding sites.

Table I. Binding of Plasminogen and Urokinase to Nonstimulated and the Adherent and Nonadherent Populations of PMA-stimulated Monocytoid Cell Lines

Plasminogen binding (molecules per cell × 10^6)			
		PMA-stimulated	
Cell type	Nonstimulated	Adherent	Nonadherent
U937	0.4 ± 0.1	0.3 ± 0.1	1.7 ± 0.1
THP-1	0.3 ± 0.1	0.3 ± 0.1	1.9 ± 0.1
HL-60	0.5 ± 0.2	0.2 ± 0.1	1.1 ± 0.1
Urokinase binding (molecules per cell × 10^4)			
Cell type	Nonstimulated	Adherent	Nonadherent
U937	1.8 ± 0.3	3.2 ± 0.6	3.9 ± 0.5
THP-1	2.1 ± 0.1	4.1 ± 1.9	5.3 ± 3.4
HL-60	1.3 ± 0.3	0.6 ± 0.3	1.2 ± 0.2

Specific binding (binding inhibitable by excess nonlabeled ligand) of plasminogen and urokinase were measured with the radiolabeled ligands at 100 and 1 nM, respectively, after a 60-min incubation at 37°C. PMA (40 nM) stimulation was for 18 h, and the adherent and nonadherent cell populations were separated. The means ±SD from three to six experiments are indicated.

ter 10 and 30 min, respectively. The results are similar to the reversibility of the binding of these ligands to U937 cells reported by us (45) and others (51). Representative Scatchard plots for the binding of plasminogen and u-PA to the THP-1 cells are shown in Fig. 1. The data points from each Scatchard plot fit a straight line; the linear correlation coefficients for plasminogen and u-PA were $r = -0.98$ and -0.97, respectively. Scatchard analyses of the data from several experiments gave an average K_d of 1.8 ± 0.5 μM and 2.4 ± 3.0 × 10^7 plasminogen molecules maximally bound per cell (eight experiments). For u-PA, the K_d value was 1.3 ± 1.0 nM and 7.6 ± 2.7 × 10^4 u-PA molecules were maximally bound per cell (six experiments). These values are very similar to those we have obtained for the binding of these two fibrinolytic ligands to U937 cells (45).

Modulation of Plasminogen Receptors on Monocytoid Cells

Expression of u-PA receptors on U937 cells is increased by treatment with PMA, and this change has been reported to be more evident in the population of cells which become adherent after PMA stimulation (44, 52). We sought to determine whether PMA, an agonist that exerts diverse effects on monocytes and monocytoid cells, could differentially modulate plasminogen receptor expression in the adherent and nonadherent populations of THP-1 and U937 cells. THP-1 and U937 cells were cultured for 18 h in either the presence or absence of 40 nM PMA. The nonadherent and adherent cells were recovered separately, and ^{125}I-plasminogen binding to the two populations of each cell type was measured. At an input ligand concentration of 100 nM, marked differences in plasminogen binding to the two populations of cells were observed (Table I). Whereas the adherent population of PMA-stimulated cells bound similar levels of plasminogen as the nonstimulated cells, binding to the nonadherent cells was greatly enhanced. The nonadherent THP-1 cells and U937 cells bound sixfold more plasminogen compared to the adherent populations. Such differences were also observed with a third cell line that acquires macrophage characteristics upon PMA stimulation, the human promyeloid leukemic HL60 cell line. The nonadherent population of HL60 cells bound five times more ^{125}I-plasminogen than the adherent HL60 cells (Table I). In contrast to these marked changes in plasminogen binding, u-PA binding was less dramatically altered. PMA stimulation increased u-PA binding by two- to threefold, and the adherent and nonadherent THP-1 and U937 cells showed no significant differences in binding ($P > 0.05$) based upon a paired comparison t-test.

To determine whether the observed differences in plasminogen receptor expression reflected an altered affinity or capacity of the cells for the ligand, Scatchard analyses were performed with separated adherent and nonadherent populations of PMA-stimulated THP-1 and U937 cells. Straight lines were obtained suggesting that the differences were not due to induction of a second class of receptors with respect to affinity. The binding parameters summarized in Table II indicate that the affinity of plasminogen for the adherent and nonadherent PMA-stimulated cells and for the nonstimulated cells was very similar. The number of binding sites, however, increased markedly in the nonadherent cells. The difference between the adherent and nonadherent cells was 17-fold with the THP-1 cells and 5-fold with the U937 cells.

Table II. Summary of the Binding Parameters for the Interaction of Plasminogen and Urokinase with Nonstimulated and the Adherent and Nonadherent Populations of PMA-stimulated Monocytoid Cells

	Plasminogen binding		
	THP-1 cells		
		PMA-stimulated	
Binding parameter	Nonstimulated	Adherent	Nonadherent
K_d (μM)	2.1 ± 0.4	2.7 ± 2.4	3.5 ± 3.0
Sites (× 10^7)	1.2 ± 0.3	1.0 ± 0.6	16.9 ± 0.8
	U937 cells		
K_d (μM)	1.2 ± 0.2	2.0 ± 0.1	2.2 ± 0.4
Sites (× 10^7)	3.2 ± 0.8	1.9 ± 0.2	10.2 ± 3.0
	Urokinase binding		
	THP-1 cells		
		PMA-stimulated	
Binding parameter	Nonstimulated	Adherent	Nonadherent
K_d (nM)	1.3 ± 1.0	1.0 ± 0.7	1.0 ± 0.9
Sites (× 10^4)	7.6 ± 2.7	13.8 ± 1.5	14.9 ± 1.3
	U937 cells		
K_d (nM)	0.7 ± 0.3	1.2 ± 1.0	1.1 ± 1.2
Sites (× 10^4)	2.3 ± 1.5	6.0 ± 4.0	5.3 ± 4.8

The binding parameters are derived from nonlinear curve-fitting analyses in the ligand computer program of specific binding isotherms for the two ligands to the monocytoid cells. The results are the means ±SD from four to six experiments.

To assure ourselves that such large differences in number of plasminogen binding sites on the adherent and nonadherent cells could be accurately quantitated in the assay configuration used, we measured the plasminogen binding at final nonstimulated THP-1 cell concentrations of 5.6 × 10^6/ml and 0.5 × 10^6/ml. This 11-fold difference in cell number approximates the difference in the plasminogen binding capacity of the adherent and nonadherent cells. At a 100 nM ^{125}I-plasminogen input concentration, 4.4 ± 0.4 × 10^5 and 3.0 ± 1.19 × 10^5 plasminogen molecules were bound per cell (mean ±SD of three experiments) at the higher and lower input concentration, respectively. As these differences are not statistically significant ($P > 0.05$), the 5- to 17-fold differences in the binding capacity of the nonadherent and adherent monocytoid cells appear to be appropriate estimates.

The changes in u-PA binding in these same cell populations were more modest and also arose from an increase in the number of binding sites and not from an increase in affinity. The differences in u-PA binding to the adherent and nonadherent populations of PMA-stimulated cells were not statistically significant ($P > 0.05$), for either the U937 or the THP-1 cells. Stimulation of the two cell lines for up to 5 d with 120 nM PMA failed to induce greater changes in u-PA receptor expression. Acidification to remove any receptor associated endogenous u-PA did not enhance the capacity of the stimulated adherent or nonadherent cells to bind u-PA.

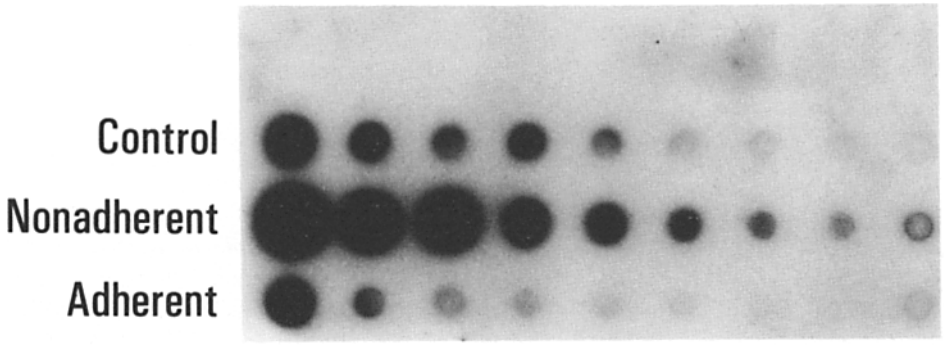

Figure 2. Ligand dot-blot analysis of plasminogen binding to nonstimulated and the adherent and nonadherent populations of PMA-stimulated THP-1 cells. Twofold dilutions of detergent extracts of the indicated populations of 1.0×10^6 THP-1 cells/ml were dotted onto Immobilon-P filters and then reacted ^{125}I-plasminogen (100 nM). The blots were then washed after 90 min at 37°C, and autoradiograms developed.

For example, acid-treated, nonadherent THP-1 cells bound $6.2 \pm 1.5 \times 10^4$ molecules/cell compared with $5.3 \pm 3.4 \times 10^4$ molecules/cell for the non-acid-treated cells (1 nM ^{125}I-u-PA added). Acidification also did not enhance the binding capacity of the cells for ^{125}I-plasminogen, suggesting that the plasminogen binding sites were not occupied by endogenous ligands.

The effect of varying doses of PMA on the upregulation of plasminogen receptors in the nonadherent cells was explored. With a 100-nM input concentration of ^{125}I-plasminogen, the ratio of plasminogen molecules bound to the nonadherent and adherent cells ranged from 9:1 to 5:1 using doses of PMA in the 10–120-nM range. Lower PMA doses (2–6 nM) induced variable responses. As an independent index of the response of the cells to PMA, the procoagulant activity (PCA) of the cell lysates was measured. After an 18-h exposure to 40 nM PMA, U937 cells expressed 4,000 mU/10^6 cells compared with 350 mU/10^6 cells in the nonstimulated U937 cells. The procoagulant activity (PCA) response in the THP-1 cells reached a maximum at an earlier time point. At 5 h, the PMA stimulated THP-1 cells expressed 5,000 mU/10^6 cells compared with 300 mU/10^6 nonstimulated cells. These data are in agreement with published reports (17, 47) on the PCA of these monocytoid cell lines after PMA stimulation.

The enhanced binding of plasminogen to the nonadherent cells was also demonstrable in ligand dot-blot analyses. Serial dilutions of detergent extracts of the same number of adherent and nonadherent PMA stimulated and nonstimulated THP-1 cells were applied to an Immobilon filter. After thorough washing, the filter was incubated with ^{125}I-plasminogen and then thoroughly washed again. An autoradiogram of the filter is shown in Fig. 2. Reactivity with the ^{125}I-plasminogen is clearly observed at higher dilutions of the nonadherent cells than of the adherent or the nonstimulated cells. All binding to the filter was abrogated (reviewed in reference 36) by 0.2 M 6-AHA (not shown), which prevents plasminogen binding to cells. Thus, this ligand dot-blot system provides a facile means to detect and compare the plasminogen binding capacity of cells and corroborates the increase in plasminogen binding to the nonadherent THP-1 cells.

Plasmin has been reported to bind to cells with a higher affinity than plasminogen (8). To determine whether differential activation of plasminogen to plasmin could account for the differences in ligand binding, the ligands bound to the adherent and nonadherent cells were analyzed on SDS-PAGE gels. Under reducing conditions, plasminogen (M_r = 92,000) can be distinguished from the plasmin heavy chain (M_r = 62,000). Autoradiograms of the gels were developed and then analyzed by densitometric scanning. At 1 h, the radiolabeled ligand incubated in the absence of cells was 93% plasminogen and 7% plasmin. The ligand bound to nonadherent and adherent cells as 83 and 81% plasminogen, respectively. Thus, the cells caused a moderate conversion of plasminogen to plasmin, but no difference in the amount of plasmin bound to the stimulated nonadherent and adherent cells was noted. Moreover, the extent of plasmin formation was similar in the bound and unbound ligand. This modest conversion of plasminogen to plasmin by the cells (10–12%) is in agreement with previous published data (19, 45, 51) and with other observations suggesting a similar affinity of plasminogen and plasmin for cells (20, 34, 39). To exclude that the traces of plasmin generated by cells could give rise to the differences in plasminogen binding to the adherent and nonadherent monocytoid cells, THP-1 cells were stimulated with PMA (40 nM) for 90 min, and plasminogen binding to the nonadherent and adherent cells determined in the presence or absence of 100 U/ml trasylol. At a 100-nM input concentration of ^{125}I-plasminogen, the nonadherent cells bound 6.3- and 5.3-fold more molecules of plasminogen per cell than the adherent cells, in the presence or absence of trasylol, respectively.

It also has been reported that Lys-plasminogen has a higher affinity for cells than native Glu-plasminogen (18, 38,

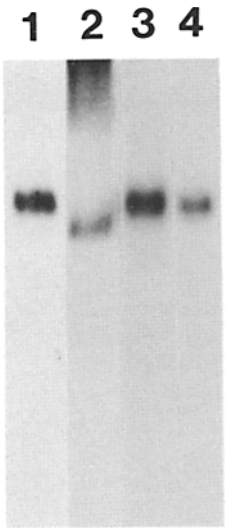

Figure 3. Molecular identity of plasminogen bound to THP-1 cells. ^{125}I-glu-plasminogen (100 nM input concentration) was incubated with adherent and nonadherent PMA-stimulated THP-1 cells (1.5×10^6/ml) for 1 h at 37°C and the bound ligand was extracted using a 0.1% Triton solution as described in Materials and Methods. Cell bound material as well as ligand added (^{125}I-Glu-plasminogen or ^{125}I-Lys-plasminogen) were analyzed by SDS-PAGE using 8% polyacrylamide gels under nonreducing conditions. In the autoradiogram shown, lane *1* is the added Glu-plasminogen; lane *2* is Lys-plasminogen; and lanes *3* and *4* are the ligands bound to the nonadherent and adherent cells, respectively.

Table III. Plasminogen and Urokinase Binding to Adherent and Nonadherent Populations of PMA-stimulated and Nonstimulated THP-1 Cells

Cell population	Plasminogen (molecules × 10^6/cell)		Urokinase (molecules × 10^4/cell)	
	Nonstimulated	PMA-stimulated	Nonstimulated	PMA-stimulated
Adherent cells	0.2 ± 0.1	0.3 ± 0.2	3.4 ± 0.1	4.2 ± 0.3
Nonadherent cells	0.3 ± 0.1	4.2 ± 0.3	3.3 ± 0.4	5.3 ± 0.3

Gelatin-fibronectin was used to separate an adherent and a nonadherent population of nonstimulated THP-1 cells and of cells stimulated with 40 nM PMA for 18 h. The specific binding of plasminogen and u-PA was measured with the radiolabeled ligands at 100 and 1 nM concentrations, respectively. The means ±SD from three experiments, are indicated.

51). To determine whether the increase in ligand binding to the nonadherent cells was due to this alteration, SDS-PAGE analyses were performed on the cell-bound ligands under nonreducing conditions under which Glu and Lys-plasminogen are distinguished. An autoradiogram of the gel is shown in Fig. 3. After 1 h of binding to the cells, the material bound to the nonadherent and adherent cells had similar mobilities and remained predominantly Glu-plasminogen (R_m = 0.33 for both cell types). Thus, the ligands bound to the adherent and nonadherent cells were indistinguishable. (The mobilities of the bound ligands were similar to but not precisely identical to isolated Glu-plasminogen [R_m = 0.32] and clearly distinct from the Lys-plasminogen standard [R_m = 0.37]).

Requirements for the Modulation of Plasminogen Receptor Expression on Monocytoid Cells

The relationship between PMA stimulation and cell adhesion in regulating plasminogen receptor expression in the monocytoid cells was examined. Nonstimulated THP-1 cells can adhere to fibronectin-gelatin; and this substratum was used to separate an adherent and a nonadherent population of nonstimulated as well as of PMA-stimulated cells. When ^{125}I-plasminogen binding was measured, the stimulated and nonstimulated adherent cells bound similar levels of the ligand (Table III). In contrast, plasminogen binding to the two nonadherent cell populations differed markedly. The nonstimulated, nonadherent cells showed no significant difference in plasminogen binding compared with the adherent nonstimulated cells. The stimulated nonadherent cells, however, bound 14 times more plasminogen compared to the stimulated adherent population. Thus, stimulation of the cells was necessary for augmented plasminogen binding to the nonadherent cell population. u-PA binding increased slightly, but not significantly, in both the adherent and nonadherent populations of stimulated cells.

To determine the role of cell adhesion in plasminogen receptor modulation, THP-1 cells were stimulated with PMA in tissue culture flasks which allow only minimal cell adhesion. Under conditions where <0.1% of the cells were adherent after 18 h of stimulation with PMA, no increase in plasminogen binding to the cells was observed (2.8 ± 1.2 and 3.1 ± 0.8 × 10^5 molecules bound per cell for stimulated and nonstimulated cells respectively, at an input concentration of 100 nM ^{125}I-plasminogen). This result suggests that an adherent population of cells is required for modulation of plasminogen receptor expression in the nonadherent cells, and this possibility was further assessed. THP-1 cells were stimulated with PMA in vessels with varying surface areas and adhesive properties such that the ratio of adherent to nonadherent cells differed. Plasminogen binding to the adherent and nonadherent populations was then measured. As shown in Fig. 4, under conditions where <40% of the cells were adherent, no difference in plasminogen binding to the adherent and nonadherent populations was detected. When the population of adherent cells exceeded 60%, the nonadherent cells exhibited more extensive binding of plasminogen compared to the adherent population.[2] Thus, to upregulate plasminogen receptor expression in the nonadherent cells, a significant proportion of the cells had to become adherent. These data also imply that the extent of surface available for adherence and not the presence of a distinct subpopulation of nonadherent cells is responsible for the separation of the two populations of cells and the modulation of receptor expression in the nonadherent cells. Corroboration of this point was obtained by exposing an isolated population of stimulated nonadherent cells to a second culture flask and again separating adherent and nonadherent populations (Table IV). The nonadherent cell population became 78% adherent after 90 min in a new culture dish. Adherence induced downregulation of receptors in the previously nonadherent population and the new population of nonadherent cells bound eightfold more plasminogen molecules than the adherent cells. This eightfold difference was observed in either the presence or absence of PMA added during the second adherence.

2. As noted in Materials and Methods, some variation was observed in the distribution of adherent and nonadherent cells under similar experimental conditions. This difference in distribution could account for the variability in the increment in plasminogen binding to the nonadherent versus the adherent cells. In the experiments with THP-1 cells reported in the study, the ratio of plasminogen binding to the nonadherent versus the adherent cells ranged from 5.8 to 16.9, a 2.9-fold variation.

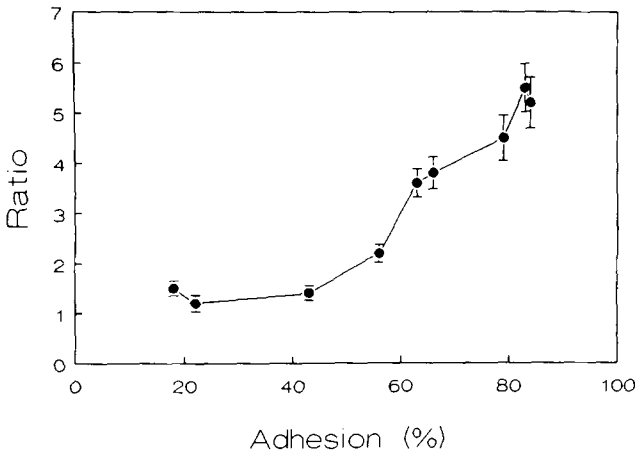

Figure 4. Influence of cell adhesion on plasminogen receptor expression. THP-1 cells were stimulated with 40 nM PMA for 18 h in different culture vessels to allow varying proportions of adherent and nonadherent populations to develop. (*Ratio*) Number of ^{125}I-plasminogen (at a 100-nM input concentration) molecules specifically bound to nonadherent cells divided by the number of molecules bound to the adherent cells. The ratio is plotted versus the percent of adherent cells in the cultures.

Table IV. Modulation of Plasminogen Binding to the Adherent and Nonadherent Populations of THP-1 Cells

Cell population	^{125}I-Plasminogen bound (molecules/cell $\times 10^6$)	
	First adhesion	Second adhesion
Adherent	0.25 ± 0.04	0.28 ± 0.06
Nonadherent	1.22 ± 0.25	2.35 ± 0.64

THP-1 cells were stimulated with 40 nM PMA. After 18 h, the adherent and nonadherent cells from the 1st adhesion were isolated and specific ^{125}I-plasminogen (at a 100-nM input concentration) binding was measured. The nonadherent cells were exposed to a fresh culture flask for 90 min, the adherent and nonadherent cells from the second adhesion were separated, and ^{125}I-plasminogen binding was again measured. The percent of adherent cells in the first and second adhesion was 75 and 80%, respectively. The results are the means ±SD of two separate experiments in which the nonstimulated cells bound 0.26 ± 0.03 × 10^6 plasminogen molecules per cell.

Upregulation of plasminogen receptors in the nonadherent THP-1 cells is a rapid phenomenon. In 30 min, the nonadherent cells bound 3.5-fold more plasminogen than the adherent cells (Fig. 5). Maximal modulation of plasminogen receptors occurred in 90 min. At this time, 75–90% of cells were adherent and an eightfold increase in ^{125}I-plasminogen binding to the nonadherent population was seen. An initial set of experiments was undertaken to determine if the adherent cells released soluble factors that induced plasminogen binding sites in the nonadherent cells. THP-1 cells were stimulated with 40 nM PMA in RPMI with or without 10% FCS, and the conditioned media derived after 1.5 or 18 h was added to nonstimulated THP-1 cells grown under conditions where adherence was prevented (in polypropylene tubes). ^{125}I-plasminogen binding was measured after culturing the cells in the conditioned media for either 1.5 or 3 h. These cells showed no increase in plasminogen binding relative to cells cultured in fresh media with or without FCS. For example, cells cultured for 3 h in the conditioned FCS or fresh media specifically bound 2.8 ± 0.2 and 2.4 ± 0.3 × 10^5 molecules of ^{125}I-plasminogen per cell, respectively (100 nM ^{125}I-plasminogen, 60 min incubation at 37°C).

Finally, we sought to determine whether PMA was unique among agonists in modulating plasminogen receptor expression (Table V). After 40 h stimulation of THP-1 cells with 5 ng/ml vitamin D-3, 55–65% of the THP-1 cells became adherent. The nonadherent population bound 1.3 ± 0.3 × 10^6 molecules of plasminogen per cell, whereas adherent cells bound 0.3 ± 0.2 × 10^6 molecules of plasminogen (input concentration 100 nM), a fourfold difference. After treatment of U937 cells with vitamin D-3 under the same conditions, the nonadherent cells bound 21.7 ± 13.6 × 10^6 molecules of plasminogen per cell and adherent cells bound 0.5 ± 0.09 × 10^6 molecules per cell. Several other agonists were also tested, but no modulation of plasminogen binding was observed (Table V). These other agonists also failed to induce an adherent population of cells. Nevertheless, stimulation of the cells by the representative agonist, LPS, could be documented by increased expression of PCA of the cells. Thus, of the agonists tested, only the two that induced adherence influenced plasminogen receptor expression.

Functional Consequence of the Modulation of Plasminogen Receptors on Monocytoid Cells

We sought to determine if differences in plasminogen receptor expression were associated with changes in fibrinolytic activity of the cells. PMA-stimulated THP-1 cells were separated into adherent and nonadherent populations. These cells, as well as nonstimulated cells, were incubated with 15 μM plasminogen for 60 min at 37°C, and then the cells were washed by centrifugation to remove unbound plasminogen. Plasmin generation was measured as a function of time upon addition of u-PA and the plasmin substrate, S-2251. As

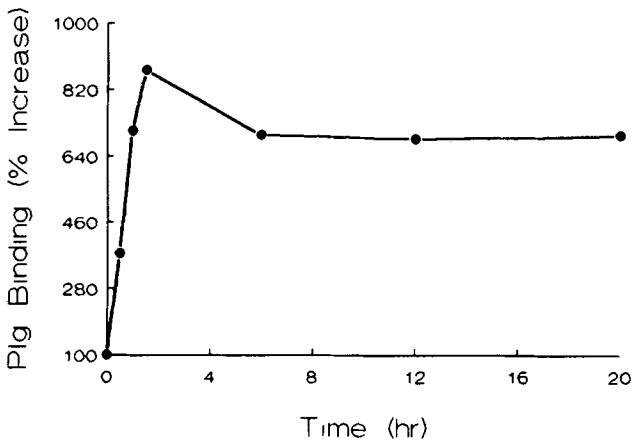

Figure 5. Time course of the upregulation of plasminogen receptors in the nonadherent population of THP-1 cells. The increase in ^{125}I-plasminogen (*Plg*) binding to nonadherent THP-1 cells was measured as a function of time after stimulation with 40 nM PMA. At a 100-nM input concentration the number of molecules of ^{125}I-plasminogen specifically bound to the adherent cells at each time was assigned a value of 100%, and the increase in plasminogen binding to the nonadherent cells is calculated as the percent increase relative to its binding to the adherent cells.

Table V. Effects of Monocyte Agonists on Plasminogen and Urokinase Receptor Expression

Agonist	Plasminogen binding (molecules/cell × 10^6)	Urokinase binding (molecules/cell × 10^4)
None	0.29	2.0
r-TNFα (*200 U/ml*)	0.28	2.8
r-IL-1α (*5 U/ml*)	0.31	ND
LPS (*10 μg/ml*)	0.32	ND
Vitamin D-3 (*5 ng/ml*)		
Adherent	0.31 ± 0.2	0.8 ± 0.4
Nonadherent	1.3 ± 0.3	1.2 ± 0.7
Aldosterone (*3 μM*)	0.38	2.8
Progesterone (*3 μM*)	0.38	3.1
Retinoic acid (*3 μM*)	0.15	4.7
Testosterone (*3 μM*)	0.71	2.6
Progesterone (*3 μM*) retinoic acid (*3 μM*)	0.40	3.5
Dexamethasone (*3 μM*)	0.41	3.0
Dexamethasone (*3 μM*) retinoic acid (*3 μM*)	0.47	3.0

THP-1 cells were stimulated for 18 h with recombinant tumor necrosis factor α (TNFα; Genzyme Corp., Boston, MA), interleukin 1α (IL-1α; Genzyme Corp.), lipopolysaccharide (LPS; Calbiochem-Behring Corp.), vitamin D-3 (Hoffmann-La Roche Inc., Montclair, NJ) or the other indicated agonists (Sigma Chemical Co.). The selected concentrations of agonists were based upon those reported to affect monocyte functions (7, 33, 43, 46–48, 50, 62). Only vitamin D-3-induced separatable adherent and nonadherent cell populations. ^{125}I-plasminogen and ^{125}I-urokinase binding was measured with 100 and 1 nM input concentrations, respectively.

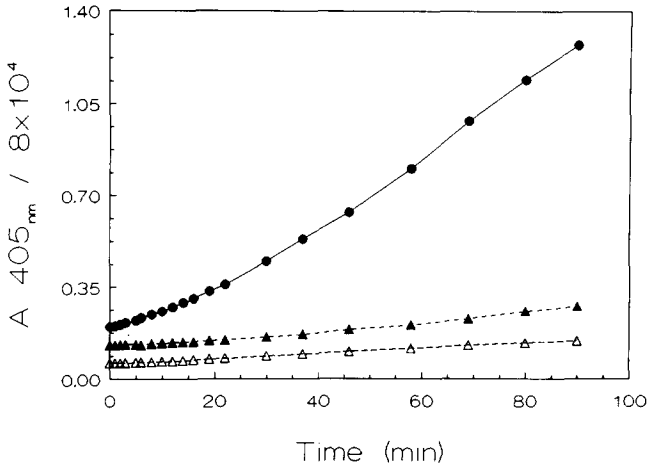

Figure 6. Plasmin generation by THP-1 cells after PMA stimulation. Nonstimulated (△) and the adherent (▲) and nonadherent populations (●) of PMA-stimulated cells were incubated with 15 μM plasminogen for 1 h at 37°C (in polypropylene tubes to prevent adherence). After washing, 3 nM u-PA was added to the system and plasmin generation measured as the hydrolysis of the chromogenic substrate S-2251 at 405 nm as described in Materials and Methods.

Table VI. Plasminogen and Urokinase Binding Parameters for Peripheral Blood Monocytes

Cell population	Plasminogen binding			
	Nonstimulated		PMA-stimulated	
	K_d	Sites	K_d	Sites
	μM	×10⁷	μM	×10⁷
Nonadherent	1.2 ± 0.4	1.4 ± 0.4	1.5 ± 0.7	3.5 ± 1.3
Adherent	1.7 ± 1.2	1.6 ± 0.9	2.2 ± 0.6	7.1 ± 1.7
Suspension	2.0 ± 1.7	1.7 ± 0.4	1.2 ± 0.2	4.1 ± 0.7

	Urokinase binding (molecules bound per cell ×10⁴)	
	Nonstimulated	PMA-stimulated
Nonadherent	3.1 ± 1.3	2.8 ± 1.4
Adherent	2.6 ± 1.5	2.6 ± 0.7
Suspension	2.4 ± 0.8	3.2 ± 0.9

Peripheral blood monocytes were isolated by a protocol without an adherence step and cultured for 18 h in Mo-Medium (see Materials and Methods), in the presence or absence of 4 nM PMA. Adherent and nonadherent populations were separated. At the same time, cells were maintained in suspension for the 18 h by culturing in polypropylene tubes. Plasminogen binding parameters were determined from specific binding isotherms using the Ligand program. ^{125}I-urokinase binding was measured using a 1-nM input ligand concentration. The means ±SD from three experiments are reported.

shown in Fig. 6, the nonadherent cells were much more efficient in generating plasmin activity. After 60 min, eightfold more plasmin activity was generated by the nonadherent population of stimulated cells than by the nonstimulated cells. The adherent population of cells showed only a twofold increase in plasmin-generating activity.

Modulation of Plasminogen Receptor Expression in Monocytes

Experiments were undertaken to determine whether peripheral blood monocytes could also modulate their expression of plasminogen binding sites. In view of the influence of adherence on plasminogen receptor expression in cells of the monocytoid lineage, blood monocytes were isolated by an adherence-independent protocol using centrifugation of blood through Sephracell-MN (59). Monocytes were recovered by this method at high yield (~2.8×10^7 cells from 200 ml of peripheral blood cells) and purity (>90%). The cells were cultured for 18 h in the presence or absence of 4 nM PMA and then separated into adherent and nonadherent populations. Binding isotherms were constructed at 37°C, and the K_d and number of plasminogen binding sites derived from Scatchard plots are summarized in Table VI. The affinities of all populations of monocytes were very similar and consistent with the value that we have previously reported for monocytes isolated using adherence to fibronectin-gelatin (35). The number of plasminogen binding sites was altered by PMA stimulation. Both the adherent and the nonadherent populations of PMA stimulated cells had significantly ($P < 0.02$) higher plasminogen binding capacities than the nonstimulated cells; the extent of the increase was 2.5- to 4.4-fold. Differences in plasminogen binding to the adherent and nonadherent cells were not significantly different ($P > 0.5$) for either the stimulated or nonstimulated cells. When monocytes were maintained in suspension for the same time (18 h) in polypropylene tubes, PMA stimulation again induced a similar increase in plasminogen binding (Table VI), indicating that a population of adherent cells was not required for the upregulation of receptor expression in the stimulated cells. The limited yield of monocytes precluded detailed studies of u-PA binding to the same cell populations; however, a sufficient number of cells were obtained to permit evaluation of ^{125}I-u-PA binding at a single ligand dose. At this dose, 1 nM, no significant differences in u-PA binding was observed (Table VI). Neither stimulation nor adherence altered the extent of u-PA binding.

The above data indicate that monocytes are similar to the monocytoid cells with respect to showing a selective increase in plasminogen binding upon PMA stimulation. In contrast to the monocytoid cells, however, the expression of plasminogen binding sites was not different in the adherent and nonadherent populations of stimulated monocytes. In addition, the monocytes required a longer exposure to PMA to alter their plasminogen binding capacity. As noted in Fig. 5, a marked increase in plasminogen binding to the monocytoid cells was observed within 30 min after exposure to PMA. With monocytes, a 6-h exposure to the stimulus was required to observe a statistically significant ($P < 0.05$) difference in plasminogen binding to the PMA stimulated versus nonstimulated cells (based on six experiments). The values for the plasminogen binding capacity of the nonstimulated monocytes are 30–80-fold higher than in our previous study (35). In the present study, the monocytes were cultured for 18 h to allow time for PMA stimulation, whereas previously the studies were performed with freshly isolated monocytes. Therefore, a direct comparison of plasminogen binding to fresh and cultured monocytes was performed. The K_d values for the fresh and the cultured monocytes were similar: 0.9 ± 0.1 and 2.0 ± 1.7 μM, respectively (three experiments). The number of binding sites in the two preparations was vastly different: $5.0 \pm 0.1 \times 10^5$ molecules per cell with the fresh monocytes and $1.7 \pm 0.4 \times 10^7$ molecules per cell in the cultured monocytes. Similar increases were observed when the monocytes were cultured in either the

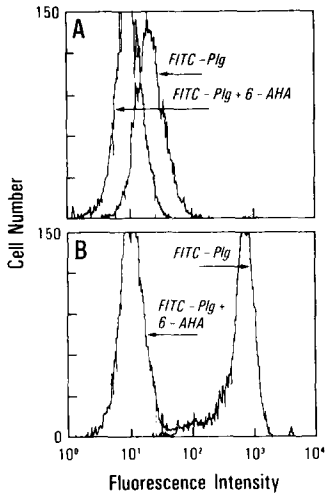

Figure 7. FACS analysis of plasminogen receptor expression on monocytes. Monocytes were isolated on Sephracell-MN (59). Freshly isolated monocytes (*A*) or monocytes cultured for 18 h (*B*) were incubated with 100 nM FITC-plasminogen. The background controls in each panel were established in the presence of 0.15 M 6-AHA that blocks plasminogen binding to its receptors.

presence or absence of 10% FCS or when monocytes were isolated by Ficoll-Hypaque centrifugation and adherence to fibronectin-gelatin. An increase was observed as early as 6 h and remained at a maximally elevated level from 24 to 84 h. The cultured monocytes were not stimulated on the basis of their expression of PCA; typically, they expressed <30 mU per 10^6 cells, similar to the level expressed by the fresh monocytes and the level reported for nonstimulated monocyte preparations (28). In parallel experiments, no difference in u-PA binding to the fresh and cultured monocytes was noted; at a 1 nM ^{125}I-u-PA input concentration, the fresh monocytes bound $2.0 \pm 0.5 \times 10^4$ molecules per cell compared to $3.5 \pm 1.2 \times 10^4$ molecules per cell ($P > 0.5$), (three experiments) in the cells cultured for 18 h.

This dramatic difference in plasminogen binding to the fresh and cultured monocytes could also be observed by FACS analysis. Plasminogen was labeled with FITC and was shown to retain its capacity to bind to THP-1 cells. As shown in Fig. 7, the fluorescence associated with the cultured monocytes was 1.5–2.0 orders of magnitude greater than with the same preparation of fresh cells. Addition of 0.15 M 6-AHA, reduced the fluorescence of both populations to the level in the absence of added FITC-plasminogen. The FACS analyses further indicate that the entire population of monocytes, rather than a subpopulation, increased its plasminogen binding capacity.

Discussion

In response to a variety of agonists, monocytes, and monocytoid cells undergo extensive phenotypic alterations including marked changes in their fibrinolytic properties (reviewed in reference 11). This study shows that plasminogen receptor expression can be dramatically altered in these cells, thus demonstrating an additional mechanism for the regulation of the fibrinolytic function of monocytoid cells. Indeed, increased receptor expression was associated with an enhanced capacity of the cells to generate plasmin, consistent with this functional consequence of plasminogen binding to cell surfaces (20, 34). Changes in plasminogen receptor expression were noted with isolated peripheral blood monocytes and for the monocytoid cell lines tested although the conditions for attaining modulation may not be identical.

With the monocytoid cells, stimulation and an adhesive event were both required to elicit the increase in plasminogen receptor expression. Agonists which stimulate monocytoid cells but do not alter their adhesive properties failed to change receptor expression; and, conversely, cell adhesion in the absence of a stimulus also failed to induce a change. Thus, PMA and vitamin D-3, which induced both adhesion and stimulation of monocytoid cells, were unique among the agonists tested in enhancing plasminogen receptor expression.

Other workers have previously reported the modulation of u-PA receptors on monocytoid cells by PMA or vitamin D-3 (14, 29, 44; Lu et al. 1989. *Thromb. Haemostasis.* 61:91). Changes in the affinity of u-PA for its receptors on U937 cells stimulated with these agonists as well as on the increase in the number of u-PA receptors have been noted (14, 44, 52). These changes were most pronounced after extended modulation of the cells with the agonists (4–5 d), suggesting that receptor modulation was associated with differentiation of the cells to a more macrophage-like phenotype. In our study, we were only able to detect modest changes in the number of u-PA receptors on the stimulated monocytoid cells that we tested. Differences among U937 cell lines in various laboratories have been reported, including differences in their fibrinolytic functions (32), and these disparities could simply reflect differences in the cells used or in the conditions of culture. From a broader perspective, our data clearly do show that plasminogen and u-PA binding sites can be independently modulated on cells. Moreover, as regulation of plasminogen receptor expression is a very rapid event, differentiation of the monocytoid cells to a more macrophage-like phenotype does not appear to be required for these changes to occur.

The role of cell adhesion in induction of plasminogen binding sites is complicated. The possibility that adhesion prevents the upregulation of plasminogen receptors induced by stimulation seems unlikely since the development of a population of adherent cells was required to see the increase in plasminogen binding to the nonadherent cells. As the number of adherent and nonadherent cells was determined by the available surface and the nonadherent populations could rapidly downregulate receptor expression upon adherence, the differential binding of plasminogen to clonally distinct subpopulations of cells can be excluded also. A plausible explanation for these observations is that the adherent cells release components that influence the plasminogen binding properties of the nonadherent but not the adherent cells. Proteolysis of cells has been recently shown to markedly augment their ability to bind plasmin (9). The reversibility of plasminogen receptor expression argues against a role of released proteases from the adherent cells in the process. The communication between the adherent and nonadherent cells is rapid and must induce major changes in the cell-surface properties to account for the magnitude of the increase in receptor number. Cell adhesion is, in fact, known to induce rapid changes in the properties of monocytoid cells. For example, changes in oncogene expression are initiated within 30 min after cell adhesion (23). We have provided initial evidence for a role of both proteinaceous and nonproteinaceous (gangliosides) cell surface molecules as plasminogen binding sites on cells (40). In view of the magnitude of the increase in plasminogen binding observed, a major alteration in the cell-surface properties of the nonadherent cells, rather than a change in the density of a single protein receptor, seems most likely.

Human peripheral blood monocytes shared the capacity to dramatically upregulate their expression of plasminogen binding sites with the monocytoid cell lines. As with the monocytoid cells, PMA stimulation induced an increase in plasminogen binding to the monocytes. In contrast to the monocytoid cells, no difference in plasminogen binding to the adherent and nonadherent monocytes was observed. This distinction in receptor expression may not represent a genuine difference between these cell types. As culture of the monocytes alone caused a marked increase in the number of plasminogen binding sites, this change might obscure differences among the adherent and the nonadherent populations. The changes in receptor expression associated with culture of the monocytes do distinguish these cells from the monocytoid cell lines. No difference has been observed in plasminogen receptor expression by U937 cells or THP-1 cells as a function of days in culture (not shown). As the number of binding sites on the cultured monocytes approaches that on the monocytoid cell lines, it may be that the plasminogen binding sites on these cell lines are permanently in a partially upregulated state. Accordingly, one might speculate as to the existence of blood factors that maintain plasminogen receptors in a downregulated state on circulating monocytes. With the increase in plasminogen binding observed with culture and with PMA stimulation, monocytes have an intrinsic capacity to augment their plasminogen binding by as much as 300-fold.

Two new technical procedures have been developed in this manuscript that could greatly facilitate analysis of plasminogen binding sites. First, a dot-blot analysis has been described that permits detection of plasminogen binding to cell extracts. This protocol permits semiquantitative analysis of plasminogen binding capacities of cells and suggests that plasminogen binding sites can be detergent solubilized, an important initial step for receptor isolation. Second, plasminogen binding to cells could be detected by FACS analysis using fluoresceinated plasminogen. FACS analysis should prove particularly useful for examining the distribution of plasminogen binding sites on different cell types and subpopulations and for the detection of abnormal binding of plasminogen to cells in pathological circumstances.

From a biological perspective, our data suggest that the monocyte can upregulate its number of plasminogen receptors in a very short period of time. Associated with this increase in receptor number is an increase in the capacity of the cells to generate plasmin. Plasmin on the cell surface may perform a variety of functions including degradation of substratum. This would permit the cells to migrate in a directed fashion. After migration, adhesion to a new surface would be associated with a rapid downregulation of plasminogen receptor expression, and the cell:substratum contacts would become stabilized. This scenario could be of particular importance in inflammatory reactions and for cell invasion such as during tumor cell metastasis.

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References

1. Bajpai, A., and J. B. Baker. 1985. Cryptic urokinase binding sites on human foreskin fibroblasts. *Biochem. Biophys. Res. Commun.* 133:475–482.
2. Barnathan, E. S., A. Kuo, H. van der Keyl, K. R. McCrae, G. R. Larsen, and D. B. Cines. 1988. Tissue-type plasminogen activator binding to human endothelial cells: evidence for two distinct binding sites. *J. Biol. Chem.* 263:7792–7799.
3. Beebe, D. P. 1987. Binding of tissue plasminogen activator to human umbilical vein endothelial cells. *Thromb. Res.* 46:241–254.
4. Beller, D. E., T. A. Springer, and R. D. Schreiber. 1982. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. *J. Exp. Med.* 156:1000–1010.
5. Bevilacqua, M. P., D. Amrani, M. W. Mosesson, and C. Bianco. 1981. Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes. *J. Exp. Med.* 153:42–60.
6. Blasi, F. 1988. Surface receptors for urokinase plasminogen activator. *Fibrinolysis.* 2:73–84.
7. Breitman, T. R., S. E. Selonick, and S. J. Collins. 1980. Induction of differentiation of human promyelocytic leukemia cell line (HL60) by retinoic acid. *Proc. Natl. Acad. Sci. USA.* 77:2936–2940.
8. Burtin, P., and M.-C. Fondaneche. 1988. Receptor for plasmin on human carcinoma cells. *J. Natl. Cancer Inst.* 80:762–765.
9. Camacho, M., M.-C. Fondaneche, and P. Burtin. 1989. Limited proteolysis of tumor cells increase their plasmin-binding ability. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 245:21–24.
10. Chapman, H. A., and O. L. Stone. 1985. Characterization of a macrophage-derived plasminogen-activator inhibitor. *Biochem. J.* 230:109–116.
11. Dano, K., P. A. Andreasen, J. Grondahl-Hansen, P. Kristensen, L. S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* 44:139–267.
12. Del Rosso, M., G. Dini, and G. Fibbi. 1985. Receptors for plasminogen activator, urokinase, in normal and Rous Sarcoma virus-transformed mouse fibroblasts. *Cancer Res.* 45:630–636.
13. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen: purification from human plasma by affinity chromatography. *Science (Wash. DC).* 170: 1095–1096.
14. Estreicher, A., A. Wohlwend, D. Belin, W.-D. Scleuning, and J. D. Vassalli. 1989. Characterization of the cellular binding site for the urokinase-type plasminogen activator. *J. Biol. Chem.* 264:1180–1189.
15. Ferrarini, M., A. Cadoni, A. T. Franzi, C. Ghigliotti, A. Leprini, A. Zicca, and C. E. Grossi. 1980. Ultrastructure and cytochemistry of human peripheral blood lymphocytes. Similarities between the cells of the third population of TG lymphocytes. *Eur. J. Immunol.* 10:562–570.
16. Goding, J. W. 1976. Conjugation of antibodies with fluorochromes: modification to the standard methods. *J. Immunol. Methods.* 13:215–226.
17. Gregory, S. A., and T. S. Edgington. 1985. Tissue factor induction in human monocytes. *J. Clin. Invest.* 76:2440–2445.
18. Hajjar, K. A., and R. L. Nachman. 1988. Endothelial cell-mediated conversion of glu-plasminogen to lys-plasminogen. *J. Clin. Invest.* 82:1769–1778.
19. Hajjar, K. A., N. M. Hamel, P. C. Harpel, and R. L. Nachman. 1987. Binding of tissue plasminogen activator to cultured human endothelial cells. *J. Clin. Invest.* 80:1712–1719.
20. Hajjar, K. A., P. C. Harpel, E. A. Jaffe, and R. L. Nachman. 1986. Binding of plasminogen to cultured human endothelial cells. *J. Biol. Chem.* 261:11656–11662.
21. Hart, P. H., D. R. Burgess, G. F. Vitti, and J. A. Hamilton. 1989. Interleukin-4 stimulates human monocytes to produce tissue-type plasminogen activator. *Blood.* 74:1222–1225.
22. Hart, P. H., G. F. Vitti, D. R. Burgess, D. K. Singleton, and J. A. Hamilton. 1989. Human monocytes can produce tissue-type plasminogen activator. *J. Exp. Med.* 169:1509–1514.
23. Haskill, S., C. Johnson, D. Eierman, S. Becker, and K. Warren. 1988. Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. *J. Immunol.* 140:1690–1694.
24. Holmberg, L., B. Bladh, and B. Astedt. 1976. Purification of urokinase by affinity chromatography. *Biochem. Biophys. Acta.* 445:215–222.
25. Johnston, P. A., S. D. Somers, and T. A. Hamilton. 1987. Expression of a 120,000 dalton protein during tumoricidal activation in murine peritoneal macrophages. *J. Immunol.* 138:2739–2744.
26. Kirchheimer, J. C., Y.-H. Nong, and H. Remold. 1988. IFN-gamma, tumor necrosis factor-alpha, and urokinase regulate the expression of urokinase receptors on human monocytes. *J. Immunol.* 141:4229–4234.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:280–282.
28. Levy, G., and T. S. Edgington. 1980. Lymphocyte cooperation is required for amplification of macrophage procoagulant activity. *J. Exp. Med.* 151:1232–1244.
29. Lu, H., M. 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. 1988. Parallel induction of fibrinolysis and receptors for plasminogen and urokinase by interferon gamma on U937 cells. *Biochem. Biophys. Res. Commun.* 155:418–422.
30. Lu, H., M. Mirshahi, C. Soria, J. Soria, H. Li, C. Dosquet-Bernard, and

J. P. Caen. 1988. Enhanced expression of urokinase activity on U937 cell line by 1,25-dihydroxyvitamin D3 induction. *Thromb. Res. Supp.* 8: 57–63.
31. Deleted in proof.
32. Deleted in proof.
33. Matsushima, K., M. Taguchi, E. J. Kovacs, H. A. Young, and J. J. Oppenheim. 1986. Intracellular localization of human monocyte associated interleukin 1 (IL1) activity and release of biologically active IL1 from monocytes by trypsin and plasmin. *J. Immunol.* 136:2883–2891.
34. Miles, L. A., and E. F. Plow. 1985. Binding and activation of plasminogen on the platelet surface. *J. Biol. Chem.* 260:4303–4311.
35. Miles, L. A., and E. F. Plow. 1987. Receptor mediated binding of the fibrinolytic components, plasminogen and urokinase, to peripheral blood cells. *Thromb. Haemost.* 58:936–942.
36. Miles, L. A., and E. F. Plow. 1988. Plasminogen receptors: Ubiquitous sites for cellular regulation of fibrinolysis. *Fibrinolysis.* 2:61–71.
37. Miles, L. A., M. H. Ginsberg, J. G. White, and E. F. Plow. 1986. Plasminogen interacts with human platelets through two distinct mechanisms. *J. Clin. Invest.* 77:2001–2009.
38. Miles, L. A., C. M. Dahlberg, and E. F. Plow. 1988. The cell-binding domains of plasminogen and their function in plasma. *J. Biol. Chem.* 263:11928–11934.
39. Miles, L. A., E. G. Levin, J. Plescia, D. Collen, and E. F. Plow. 1988. Plasminogen receptors, urokinase receptors and their modulation on human endothelial cells. *Blood.* 72:628–635.
40. Miles, L. A., C. M. Dahlberg, E. G. Levin, and E. F. Plow. 1989. Gangliosides interact directly with plasminogen and urokinase and may mediate binding of these components to cells. *Biochemistry.* 280:9337–9343.
41. Moscatelli, D., and D. B. Rifkin. 1988. Membrane and matrix localization of proteinases: a common theme in tumor cell invasion and angiogenesis. *Biochim. Biophys. Acta.* 948:67–85.
42. Mundson, P. J., and D. Rodbard. 1980. Ligand a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* 107:220–239.
43. Okazaki, T., Y. Kato, M. Tashima, H. Sawada, and H. Uchino. 1989. Evidence of intracellular and trans-acting differentiation-inducing activity in human promyelocytic leukemia HL-60 cells: its possible involvement in process of cell differentiation from a commitment step to a phenotype-expression step. *J. Cell. Physiol.* 134:261–268.
44. Picone, R., E. L. Kajitaniak, L. S. Nielsen, N. Behrendt, M. R. Mastronicola, M. V. Cubellis, M. P. Stoppelli, S. Pedersen, K. Dano, and F. Blasi. 1989. Regulation of urokinase receptors in monocytelike U937 cells by phorbol ester phorbol myristate acetate. *J. Cell Biol.* 108:693–702.
45. Plow, E. F., D. E. Freaney, J. Plescia, and L. A. Miles. 1986. The plasminogen system and cell surfaces: Evidence for plasminogen and urokinase receptors on the same cell type. *J. Cell Biol.* 103:2411–2420.
46. Polla, B. S., A. M. Healy, M. Byrme, and S. M. Krane. 1987. 1,25-Dihydroxyvitamin D3 induces collagen binding to the human monocyte line U937. *J. Clin. Invest.* 80:962–969.
47. Rana, S. V., H. J. Reimers, M. S. Pathikonda, and S. P. Bajaj. 1988. Expression of tissue factor and factor viia/tissue factor inhibitor activity in endotoxin or phorbol ester stimulated U937 monocyte-like cells. *Blood.* 71:259–262.
48. Rao, K. M. K., M. A. Misukonis, H. J. Cohen, and J. B. Weinberg. 1988. Cooperative effect of tumor necrosis factor and gammainterferon on chemotactic peptide receptor expression and stimulus-induced actin polymerization in HL-60 cells. *Blood.* 71:1062–1067.
49. Reich, E. 1989. Activation of plasminogen. A general mechanism for producing localized extracellular proteolysis. *In* Molecular Basis of Biological Degradative Processes. R. D. Berlin, L. Herrman, I. H. Lepow, and J. M. Tanzer, editors. Academic Press Inc., New York. 155–169.
50. Reilly, T. M., M. D. Whitfield, D. S. Taylor, and P. B. M. W. M. Timmermans. 1989. Binding of tissue plasminogen activator to cultured human fibroblasts. *Thromb. Haemostasis.* 61:454–458.
51. Silverstein, R. L., R. J. Friedlander, Jr., R. L. Nicholas, and R. L. Nachman. 1988. Binding of lys-plasminogen to monocytes/macrophages. *J. Clin. Invest.* 82:1948–1955.
52. Stoppelli, P., A. Corti, A. Soffientini, G. Cassani, F. Blasi, and R. K. Assoian. 1985. Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. *Proc. Natl. Acad. Sci. USA.* 82:4939–4943.
53. The, T. H., and T. E. W. Feltkamp. 1970. Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. *Immunology.* 18:865–873.
54. Tsuchiya, S., Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, and K. Tada. 1982. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.* 42:1530–1536.
55. Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kabayashi, T. Konno, and K. Tada. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer.* 26:171–176.
56. Vassalli, J., J. Dayer, A. Wohlwend, and D. Belin. 1984. Concomitant secretion of prourokinase and of a plasminogen activator specific inhibitor by cultured human monocytes-macrophages. *J. Exp. Med.* 159:1653.
57. Vassalli, J.-D., D. Baccino, and D. Belin. 1985. A cellular binding site for the M_r 55,000 form of the human plasminogen activator, urokinase. *J. Cell Biol.* 100:86–92.
58. Vassalli, J. D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors. *Cell.* 8:271–281.
59. Vissers, M. C. M., S. A. Jester, and J. C. Fantone. 1988. Rapid purification of human peripheral blood monocytes by centrifugation through Ficoll-Hypaque and Sepracell-mn. *J. Immunol. Methods.* 110:203–207.
60. Wallen, P., and B. Wiman. 1972. Characterization of human plasminogen. II. Separation and partial characterization of different molecular forms of human plasminogen. *Biochim. Biophys. Acta.* 257:122–134.
61. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.
62. Weinberg, J. B., and J. W. Larrik. 1987. Receptor-mediated monocytoid differentiation of human promyelocytic cells by tumor necrosis factor: Synergistic actions with gamma interferon and 1,25 dihydroxyvitamin D3. *Blood.* 70:994–998.
63. Wohlwend, A., D. Belin, and J. D. Vassalli. 1987. Plasminogen activator-specific inhibitors in mouse macrophages: in vivo and in vitro modulation of their synthesis and secretion. *J. Immunol.* 139:1278–1284.
64. Ziegler-Heitbrock, H. W. L., E. Thiel, A. Futterer, V. Herzog, A. Wirtz, and G. Riethmuller. 1988. Establishment of a human cell line (mono mac 6) with characteristics of mature monocytes. *Int. J. Cancer.* 41:456–461.