CONCOMITANT SECRETION OF PROUROKINASE AND OF A PLASMINOGEN ACTIVATOR–SPECIFIC INHIBITOR BY CULTURED HUMAN MONOCYTES-MACROPHAGES

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Plasminogen activators (PA)[1] are thought to play a role in the proteolytic events associated with cell migration and tissue destruction and remodeling (1), in particular in the context of inflammation (2). Indeed, monocytes and macrophages from both human and murine origin produce PA (3, 4), and the secretion of these enzymes has been linked with the participation of mononuclear phagocytes in inflammatory reactions: peritoneal macrophages collected from inflammatory exudates synthesize and secrete such enzymes (reference 3, D. Belin et al., manuscript in preparation); furthermore, PA production is enhanced by inflammatory agents (5, 6) and suppressed by antiinflammatory glucocorticoids (2). However, little is known at the molecular level about the mechanisms by which PA activity is regulated.

Recently progress has been made in the characterization of PA. Two different enzymes, urokinase (Uk) and tissue activator (TA), have been identified. Their primary structures have been elucidated at the protein or mRNA level (7–9), and it is now clear that they are the products of different genes. Aspects of their biosynthesis have been investigated. Uk has been shown to be secreted by certain human and murine cells as a one-chain inactive zymogen, with a M_r of 55,000 for the human protein (10–12). Active M_r 55,000 human Uk is generated through a single intramolecular proteolytic cleavage, and consists of two disulfide-linked polypeptide chains. M_r 33,000 human Uk is a proteolytic product of the M_r 55,000 enzyme with similar specific catalytic activity. The cellular production of PA inhibitors has also been described: in particular the secretion of protease-nexin (13) by human fibroblasts has been suggested to play a role in the regulation of extracellular PA activity (14); mouse peritoneal macrophages have been reported to be a source of such inhibitors (15, 16).

In view of these developments, it was of interest to determine the type of PA produced in cultures of human monocytes-macrophages, as well as to investigate

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DFP, diisopropyl-fluorophosphate; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; PA, plasminogen activator; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate; SDS, sodium dodecyl sulfate; TA, tissue activator; Uk, urokinase.

the possible secretion of PA inhibitors by these cells. In this communication we show that both freshly isolated human peripheral blood monocytes and cells from the monocytic line U 937 (17) secrete Uk, mostly in the form of the inactive zymogen. Concomitantly, these cells also release a PA-specific protease inhibitor.

Materials and Methods

Materials. Rabbit antibodies to human urinary Uk (18) and to human HeLa cells TA, as well as conditioned medium from phorbol myristate acetate (PMA)–treated HeLa cells, were the kind gift of Dr. W.-D. Schleuning. Controls for the specificity of these antibodies with respect to the two different types of PA are included in the experiment of Fig. 1. IgG were prepared by Protein A–Sepharose chromatography (19). M_r 55,000 Uk (1.5×10^5 U/mg) was from the Green Cross Corporation, Osaka, Japan; M_r 33,000 Uk was generously provided by Serono, Denens, Switzerland. Thrombin from human plasma (T 6759) was from Sigma Chemical Co. (St. Louis, MO), and bovine serum albumin (BSA, 11920) from Serva Feinbiochemica (Heidelberg, Federal Republic of Germany). Percoll was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Plasminogen was purified from human plasma (20); plasmin was prepared by Uk activation in the presence of glycerol as described (21). RPMI 1640, fetal bovine serum (FBS), and Ca^{++}-Mg^{++} free Hank's balanced salt solution (HBSS) were from Grand Island Biological Co. (Grand Island, NY), diisopropyl-fluoro-phosphate (DFP) and Triton X-100 from Merck & Co., Inc. (Rahway, NJ), sodium dodecyl sulfate (SDS) from Bio-Rad Laboratories (Richmond, CA), Iodogen from Pierce Chemical Co. (Rockford, IL), acrylamide, bisacrylamide, *N,N,N′,N′*,-tetramethylethylenediamine and ammonium persulfate from Eastman Kodak Co. (Rochester, NY), and Na [^{125}I] from Amersham Corp. (Arlington Heights, IL). Tissue culture plasticware was obtained from Falcon Labware (Oxnard, CA).

Cell Culture. U 937 cells were obtained from Dr. R. Snyderman (Duke University Medical Center, Durham, NC), grown in RPMI 1640 supplemented with 5% FBS, and cultures were split 1 to 3, every 4 d. To prepare conditioned media the cells were collected by centrifugation, washed, and incubated at 2×10^6/ml as described below. Monocytes were prepared from freshly drawn citrated human blood by Ficoll-Hypaque sedimentation (22) after dilution with 2 volumes of HBSS; the blood mononuclear cells were layered on a preformed Percoll gradient (12,900 *g* for 4 min 30 s at 4°C in a Beckman superspeed J2-21 centrifuge using the JA 20.1 rotor). The gradient was centrifuged for 4 min 30 s at 8,240 *g* in the same rotor. The monocytes (upper cell layer) were washed and plated at 2×10^6/ml in RPMI 1640 supplemented with 10% heat-inactivated (56°C, 45 min) FBS. After 2 h at 37°C, adherent cells were washed and incubated as described below. Purified cells were analyzed by staining for nonspecific esterase (23) and peroxidase (24) and found to be >98% monocytes. Human foreskin fibroblasts were grown in RPMI 1640 plus 5% FBS, and used at passage 10; confluent cultures in 35-mm tissue culture dishes were washed and incubated with 1 ml serum-free medium as described below. To prepare conditioned media, cells were washed 3 times in RPMI 1640 with 1 mg/ml BSA and cultured in RPMI 1640 plus 1 mg/ml BSA for 24–48 h at 37°C in a 95% air–5% CO_2 atmosphere. At the end of the culture period the media were collected, spun twice for 10 min at 500 *g* to remove cells and debris, and stored at −20°C. Cell viability was assessed by trypan blue exclusion; 98% of the cells excluded the dye after 24 h, and 92–95% after 48 h of culture in RPMI 1640 plus 1 mg/ml BSA.

Immunoprecipitation. Samples (20 μl) were mixed with 2 μl of anti-human Uk (0.2 mg/ml), anti-human TA (1 mg/ml), or irrelevant (1 mg/ml) IgG, and incubated for 2 h at 4°C. Fixed *S. aureus* (15 μl), prepared according to Kessler (25), were added; after 30 min at 20°C, the samples were centrifuged (5 min, Beckman Microfuge; Beckman Instruments, Inc., Palo Alto, CA). Supernatants were mixed with an equal volume of double strength electrophoresis sample buffer (0.1 M Tris-Cl, pH 6.8, 2% SDS, 20% glycerol). Pellets of the Uk, monocyte, and U 937 medium immunoprecipitations were washed twice with PBS containing 1 mg/ml BSA, and eluted in 50 μl electrophoresis sample buffer. Pellets of the TA immunoprecipitations were washed 3 times with NET-TS (NaCl 0.5 M, EDTA

0.001 M, Tris-Cl 0.05 M pH 8.1, Triton X-100 1%, SDS 0.2%) and once with NT-S (NaCl 0.5 M, Tris-Cl 0.05 M pH 8.1, SDS 0.1%) before elution in sample buffer; this modification was introduced to overcome nonspecific adsorption of TA to *S. aureus*.

SDS Polyacrylamide Gel Electrophoresis. Samples (15 μl) were electrophoresed at 20 mA constant current in 10% polyacrylamide slab gels (0.8 mm thick and 5.5 cm long), using the buffer system of Laemmli (26); all gels were run under nonreducing conditions.

Zymographic Assay for PA. The method described by Granelli-Piperno and Reich (21) was used; the electrophoretic gels were shaken for 20 min in 2.5% Triton X-100 in H_2O followed by 20 min in PBS before being layered on the substrate gels. Substrate gels consisted of a mixture of 0.8% agar, 1.3% Carnation instant non-fat dry milk, 40 μg/ml plasminogen in PBS; the mixture was prepared at 45°C, cast onto a flat glass or plastic surface, and allowed to solidify at 20°C before use. Zymograms were allowed to develop at 37°C for 5–30 h. Photographs were taken using dark-ground illumination.

[^{125}I]-Fibrin Plate Assay. This was performed exactly as described (27).

[^{125}I]-Labeling of Uk and Thrombin. These proteins were labeled using Iodogen as described (13), except that spun Sephadex G-50 columns (28) were used to separate the proteins from unreacted [^{125}I]. The specific activities of the [^{125}I]-proteins were as follows: M_r 55,000 Uk, 1–2 × 10^7 cpm/μg; M_r 33,000 Uk, 0.8–1.7 × 10^7 cpm/μg; thrombin, 3 × 10^6 cpm/μg. Two radioactive polypeptides were present in the preparation of [^{125}I]-thrombin (Fig. 8, track *1*): an unidentified contaminant of M_r 20,000, and thrombin itself, with an M_r of 34,000.

Autoradiography. Fixed and stained gels (0.2% Coomassie Brilliant Blue R250 in 30% methanol–7.5% acetic acid) were placed in Saran wrap, and exposed for 16–40 h to Kodak XAR-5 film at −80°C between DuPont Cronex Par-Speed intensifying screens (DuPont Co., Wilmington, DE).

Results

PA Secreted by Monocytes-Macrophages and U 937 Cells Is of the Uk Type. Human monocytes-macrophages and U 937 cells cultured in serum-free medium secrete PA. To determine the type of enzyme released by these cells, samples of conditioned media were mixed with specific anti-Uk and anti-TA IgG, and the immune complexes precipitated by the addition of fixed *S. aureus*. Aliquots of the supernatants and immune precipitates were subjected to SDS-PAGE, and the gels analyzed by zymography (Fig. 1). Monocyte-macrophage–conditioned medium contained one major form of PA activity (track *b1*); the migration of this PA in the electrophoretic gel was identical to that of a standard of M_r 55,000 human Uk (track *a1*), and different from that of HeLa cells TA (track *d1*). Monocyte-macrophage PA was quantitatively recovered in the immune precipitate in the presence of anti-Uk IgG (tracks *b2*), but remained in the supernatants of the immunoprecipitation reactions in presence of irrelevant IgG (tracks *b1*) or of anti-TA IgG (tracks *b3*). Identical results were obtained with conditioned medium from U 937 cells (tracks *c*). In addition, the increased levels of PA present in conditioned media from PMA (100 ng/ml)– or concanavalin A (10 μg/ml)–treated monocytes-macrophages and U 937 cells could be entirely accounted for by an increase in the production of the same enzyme. Thus, all the PA produced by monocytes-macrophages and U 937 cells is immunologically related to Uk, but not to TA.

The nature of the M_r 100,000 lytic zones that developed in the zymograms of monocyte-macrophage and U 937–conditioned media is not entirely clear; although these were not recovered in the anti-Uk immune precipitates, the fact

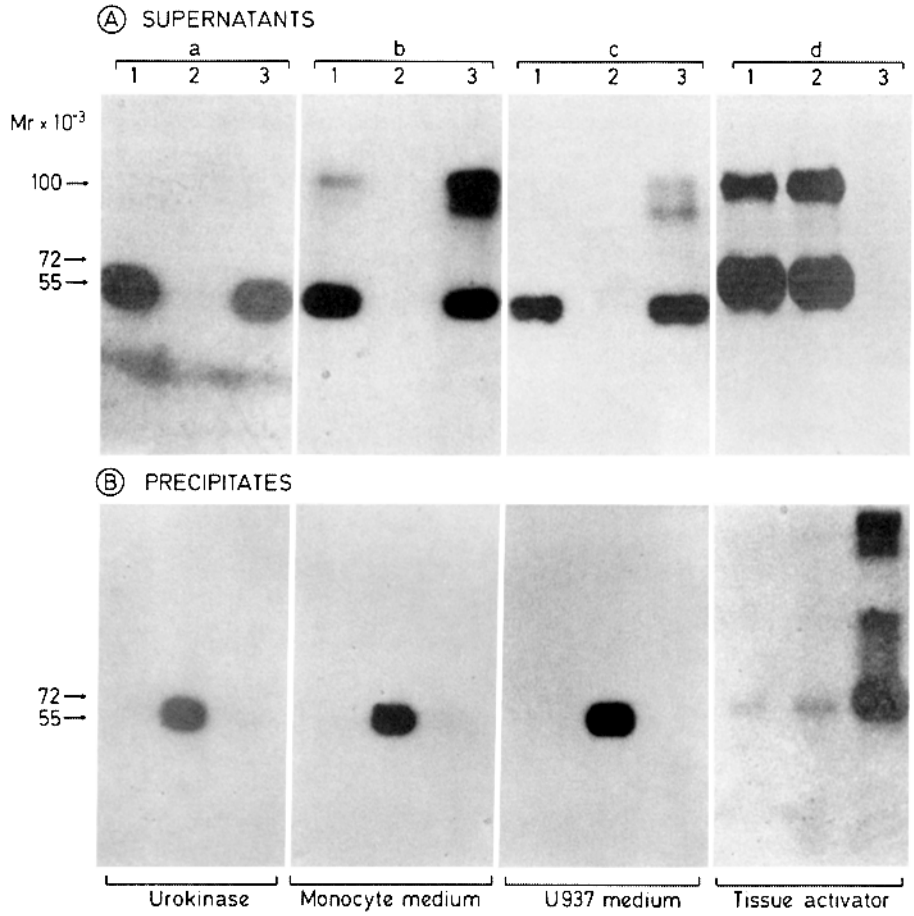

FIGURE 1. Immunoprecipitation of PA secreted by monocytes-macrophages and U 937 cells. Samples (20 μl) of M_r 55,000 human Uk (0.2 U/ml) (tracks *a*), of conditioned medium from human monocytes-macrophages (tracks *b*), from U 937 cells (tracks *c*), or from PMA-treated HeLa cells (a source of human TA[2]) (tracks *d*), were mixed with IgG from uninjected rabbits (2 μg, tracks *1*), or from rabbits immunized against human Uk (0.4 μg, tracks *2*) or against human TA (2 μg, tracks *3*). After SDS-PAGE of the supernatants and eluted pellets from the immunoprecipitations, the gels were analyzed by zymography. Zones of lysis did not develop when plasminogen was omitted from the zymogram. Molecular weights were calculated from the position of markers (Pharmacia, low molecular weight calibration kit) electrophoresed in parallel lanes and stained with Coomassie Brilliant Blue R 250.

that they were absent from the anti-Uk supernatants, but remained in the anti-TA supernatants, suggested that they were due to Uk-related forms of PA. In view of the results reported in later sections of this paper, we believe that these lytic zones are due to the activity of covalent complexes between Uk and a PA

[2] Human TA has a M_r of 72,000. A TA-related form of PA with a M_r of 110,000 was also present in conditioned medium from HeLa cells (A, tracks *d1* and *2*); it probably consists of a complex between the enzyme and a cell-derived ligand, as discussed further in the case of Uk and U 937 cells or monocytes. TA-containing immune complexes did not dissociate completely before electrophoresis (the samples being neither boiled nor reduced), resulting in the presence of high M_r species of PA in this sample (B, track *d3*).

inhibitor; different levels of plasmin contamination in the IgG preparations could explain the variable amounts of such complexes.

U 937 Cells Secrete an Inhibitor of Fibrinolysis. To assay for the presence of inhibitor(s) of Uk-catalyzed fibrinolysis in the conditioned medium of U 937 cells, a constant amount of M_r 55,000 Uk was added to increasing amounts of medium. After 30 min of incubation at 4°C, the samples were assayed in [^{125}I]-fibrin–coated wells, in the presence of plasminogen. Fibrinolysis was completely prevented when Uk had been preincubated with as little as 1.5 μl of conditioned medium (Fig. 2). This suggested that U 937 cells released an inhibitor(s) of Uk and/or plasmin. Further experiments were designed to investigate the possible interaction of Uk with this inhibitor.

The Inhibitor Released by U 937 Cells Forms a Covalent Complex with Uk, and This Complex Is Enzymatically Inactive. Covalent complexes between proteolytic enzymes and their inhibitors have been observed in many systems (29). The formation of such complexes can be used to assay for the presence of protease inhibitors. M_r 55,000 [^{125}I]-Uk was added to U 937–conditioned medium; after 60 min at 4°C, SDS-containing electrophoresis sample buffer was added and the boiled sample subjected to SDS-PAGE. Autoradiography of the fixed gel (Fig. 3) revealed that almost all the radioactivity migrated with an apparent M_r of 94,000 (track *2*). This indicated that [^{125}I]-Uk had formed an SDS-resistant complex with a component present in U 937 medium. Addition of unlabeled Uk prevented formation of the radioactive M_r 94,000 complex (track *3*); thus, complex formation with [^{125}I]-enzyme could be competed by unlabeled enzyme, suggesting that interaction of Uk with the U 937–secreted ligand was not an exclusive property of the iodinated molecule.

To determine whether binding of Uk to this ligand was associated with inhibition of enzymatic activity, we used M_r 33,000 Uk, a proteolytic product of M_r 55,000 Uk with similar specific activity; this allowed us to discriminate

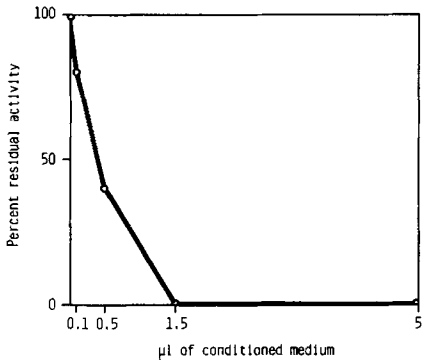

FIGURE 2. Inhibition of Uk-catalyzed fibrinolysis by conditioned medium from U 937 cells. Uk (3 mU) was added to dilutions of U 937–conditioned medium (0.1 to 5 μl in a total volume of 20 μl RPMI 1640 plus 1 mg/ml BSA). After 30 min at 4°C, the samples were assayed in [^{125}I]-fibrin–coated wells in presence of plasminogen. Enzymatic activity remaining in the samples was determined by comparison with a standard curve of Uk (0.3–3 mU). U 937 medium without added Uk did not catalyze any fibrinolysis under the conditions of this experiment; in the absence of U 937 medium, 3 mU Uk catalyzed the lysis of 15% of the available substrate.

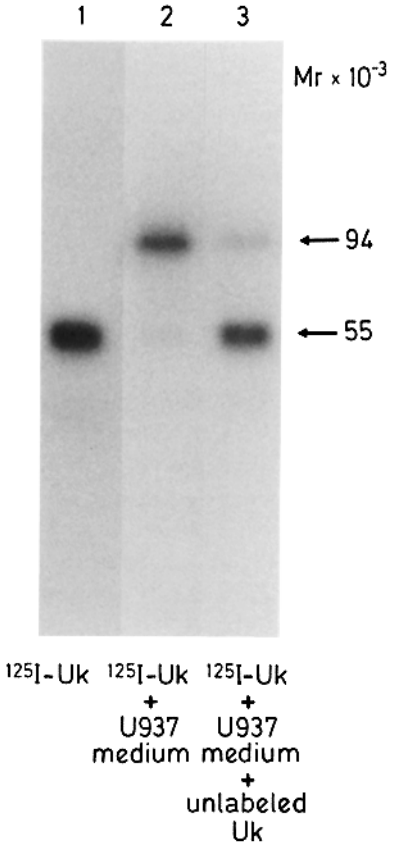

FIGURE 3. Formation of a complex between [^{125}I]-Uk and a U 937–produced ligand. M_r 55,000 [^{125}I]-Uk (0.5 ng) was added to 20-μl samples of fresh medium (track *1*), of U 937–conditioned medium (track *2*), or of U 937–conditioned medium containing 10 ng unlabeled Uk (track *3*). After 1 h at 4°C an equal volume of double-strength sample buffer was added and the samples were boiled and subjected to SDS-PAGE. The fixed and stained gel was analyzed by autoradiography. Molecular weights were calculated from the position of markers in the stained gel.

between the endogenous M_r 55,000 enzyme and the exogenously added Uk. M_r 33,000 [^{125}I]-Uk was mixed with U 937 medium, and aliquots of the sample were subjected to SDS-PAGE. Part of the gel was processed for autoradiography (Fig. 4*A*), and part for zymography (Fig. 4*B*). Whereas 60% of the [^{125}I]-label was recovered at M_r 72,000, in the form of the enzyme-ligand complex, no M_r 72,000 PA activity could be detected by the zymogram. Since the free M_r 33,000 [^{125}I]-Uk remaining after incubation with U 937 medium was mostly inactive, almost all the active enzyme present in our [^{125}I]-Uk preparation had been inactivated upon binding to the ligand. With longer incubation of the zymogram, enzymatic activity of the M_r 72,000 complex did become detectable (data not shown); this low activity may reflect the lability of the inhibitor in SDS (unpublished observations).

Thus, culture medium from U 937 cells contained a ligand for human Uk; the enzyme-ligand covalent complex had an apparent M_r of 94,000 with M_r 55,000

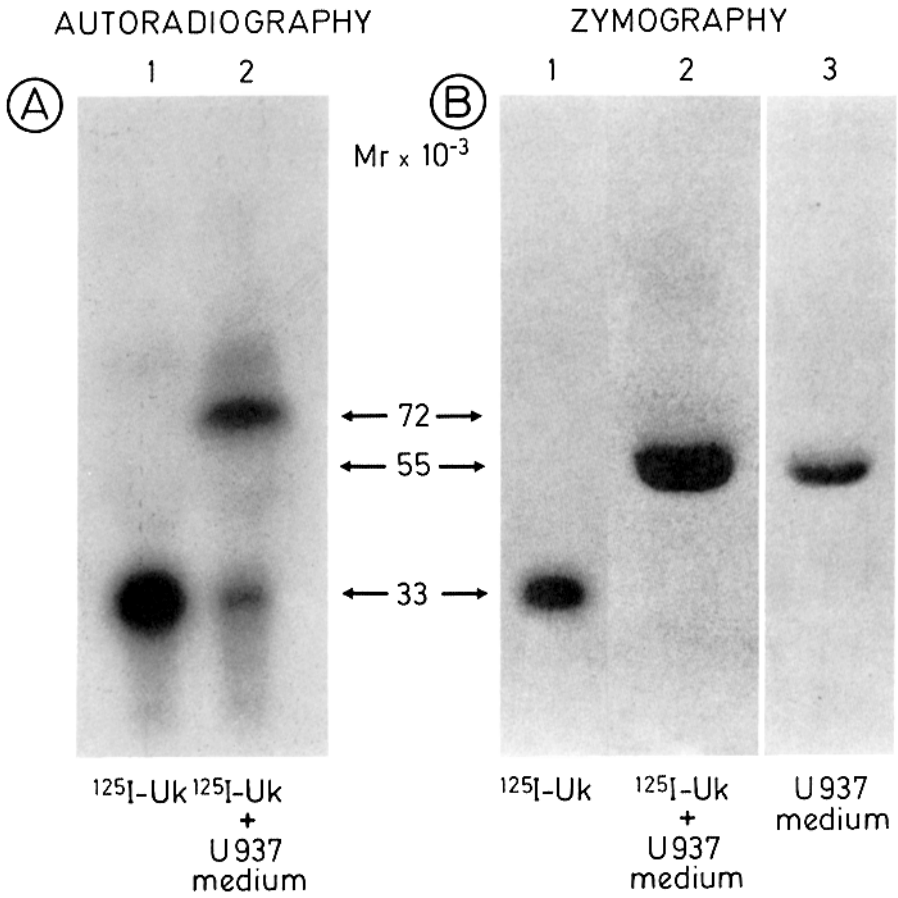

FIGURE 4. The complex between Uk and the U 937–produced ligand is enzymatically inactive. M_r 33,000 [^{125}I]-Uk was added to fresh medium (tracks *1*) or to U 937–conditioned medium (tracks *2*). After 1 h at 4°C, double-strength sample buffer was added and duplicate aliquots of the samples were electrophoresed in a 10% polyacrylamide SDS–containing slab gel; U 937 medium without [^{125}I]-Uk added was also electrophoresed in the same gel (track *3*). The gel was cut in two parts. Part *A* was processed for autoradiography; for quantitation, the gel was cut and slices counted in a gamma counter. Part *B* was processed for zymography.

Uk, and 72,000 with M_r 33,000 Uk, indicating that the ligand, when bound to Uk, had a M_r of 39,000. Since the complexed enzyme was functionally inactivated, this ligand can thereby be considered as an inhibitor of Uk. We can calculate (from the data of Fig. 3) that the concentration of this inhibitor in U 937 medium was sufficient to totally account for the inhibition of fibrinolysis illustrated in Fig. 2.

The Uk Secreted by U 937 Cells Is a Proenzyme. The results reported in the preceding sections demonstrated the apparently paradoxical coexistence, in U 937 culture medium, of a M_r 55,000 form of Uk that could be detected catalytically by zymography (Fig. 1) and of a component that reacted rapidly with exogenously added [^{125}I]-Uk to form a stable, inactive complex of higher M_r (Figs. 3 and 4). Such a result would in fact be expected if the Uk activity

detected in the culture medium was due to a form of the enzyme that was inactive before zymography, and therefore that could not have reacted with the available ligand. Uk has been shown to be secreted by certain cell types as an inactive zymogen (proUk), which can become activated under the effect of trace amounts of plasmin present in the plasminogen used for zymographic detection of Uk activity (10–12, 14). ProUk consists of a single polypeptide chain; it does not activate plasminogen, nor does it react with DFP, an inhibitor of serine esterases. Plasmin cleaves proUk to generate the two-chain active enzyme, which can be irreversibly inhibited by reaction with DFP (10–12, 14).

We have used the DFP inhibition and plasmin activation reactions to characterize the form of Uk present in U 937 medium (Fig. 5). Active M_r 55,000 Uk (track *c1*) was totally inactive after reaction with DFP (track *c3*); by contrast, U 937–secreted Uk (track *a1*) was unaffected by DFP (track *a3*). The activity of Uk was not altered by exposure to plasmin (track *c2*), whereas the U 937–secreted enzyme was undetectable after such treatment (track *a2*); this result would be expected if plasmin converted the U 937–secreted molecule into an active form, which could react with and be inactivated by the available ligand. To further substantiate this hypothesis, we took advantage of the lability of the inhibitor in presence of propanol (unpublished observations). Propanol did not alter the activity of Uk, nor its susceptibility to DFP (tracks *d* 1–*4*). In propanol-treated U 937 medium Uk activity remained resistant to DFP inactivation (track *b3*); plasmin did not cause the disappearance of Uk activity (track *b2*), but

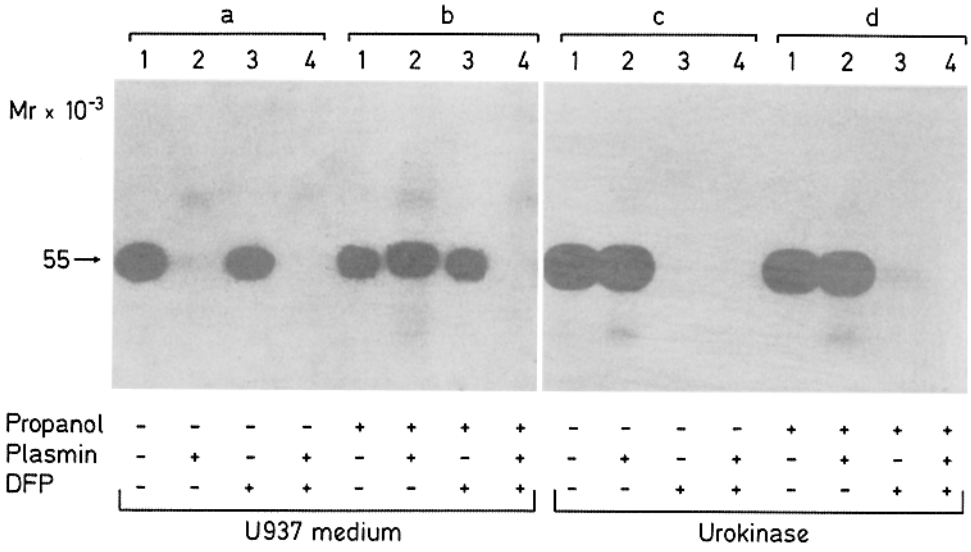

FIGURE 5. Zymographic analysis of U 937–produced Uk: effects of propanol, plasmin, and DFP. U 937–conditioned medium and M_r 55,000 Uk (0.6 U/ml in fresh medium) were treated in parallel. Samples were exposed first to propanol, second to plasmin, and third to DFP, as indicated. Propanol was added to a final concentration of 40% (tracks *b* and *d*); both propanol-treated and untreated samples were lyophilized in a Savant SpeedVac apparatus and reconstituted to the original volume with H_2O. Plasmin treatment (1 μg/ml) was for 1 h at 37°C (tracks *2* and *4*). DFP treatment (10 mM) was for 1 h at 20°C (tracks *3* and *4*). Samples were mixed with an equal volume of double-strength sample buffer, and subjected to SDS-PAGE. The gel was processed for zymography.

rendered it totally susceptible to inactivation by DFP (track *b4*). Furthermore, exposure of propanol-treated medium to plasmin accelerated the kinetics of substrate lysis catalyzed by the U 937–secreted form of Uk (Fig. 6).

Taken together, these results suggest that U 937 cells secrete Uk as the inactive, DFP-resistant, proenzyme. Plasmin converts this proUk into active Uk, which is rapidly complexed to and inhibited by the U 937–secreted ligand. In the absence of functional inhibitor, i.e., after exposure to propanol, active Uk accumulates in U 937 medium supplemented with plasmin, and can be inactivated by reaction with DFP.

Human Monocytes-macrophages in Culture Secrete ProUk and a Ligand for Uk. Freshly isolated human monocytes-macrophages secreted a M_r 55,000 form of Uk (Fig. 1), which was also resistant to inactivation by DFP (data not shown). Hence, like U 937 cells, monocytes-macrophages secrete proUk.

Addition of M_r 33,000 [^{125}I]-Uk to monocyte-macrophage conditioned medium resulted in the formation of a M_r 72,000 radioactive complex (Fig. 7, track *5*), that co-migrated with the complex formed with the U 937–produced Uk inhibitor (track *2*). As shown in Fig. 4 for the U 937 complex, Uk present in the monocyte complex was also functionally inactivated (data not shown). Thus, freshly prepared human monocytes-macrophages release a Uk ligand comparable to that of U 937 cells.

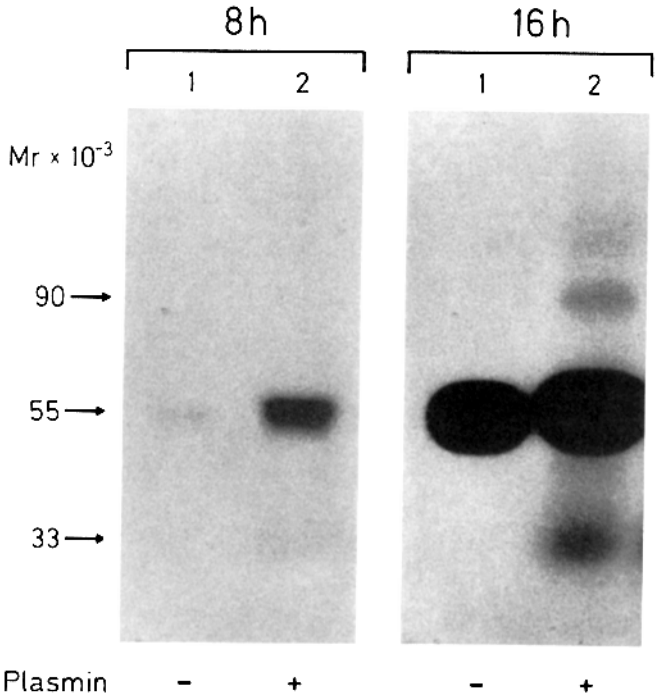

FIGURE 6. Plasmin-catalyzed activation of U 937–secreted proUk. U 937–conditioned medium was treated with propanol as in Fig. 5, and incubated for 1 h at 37°C without (tracks *1*) or with addition of plasmin (1 μg/ml) (tracks *2*). The samples were electrophoresed and the gel processed for zymography. Photographs were taken after 8 and 16 h of incubation of the zymogram.

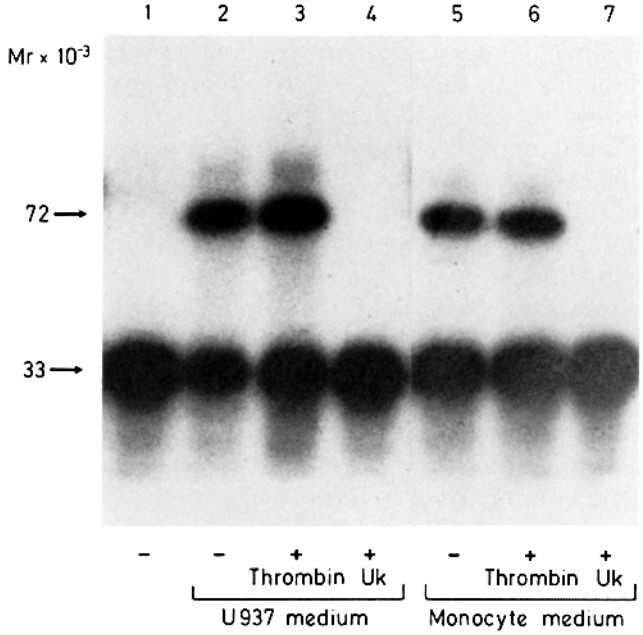

FIGURE 7. Formation of complexes between [^{125}I]-Uk and U 937- or monocyte-macrophage-produced ligand; lack of competition by thrombin. M_r 33,000 [^{125}I]-Uk (2 ng, 10^6 cpm/μg) was added to 10-μl samples of fresh medium (track *1*), U 937–conditioned medium (tracks *2–4*), or monocyte-conditioned medium (tracks *5–7*), in the absence of (tracks *1*, *2*, *5*) or 15 min after addition of unlabeled thrombin (0.2 μg) (tracks *3*, *6*) or unlabeled M_r 33,000 Uk (0.2 μg) (tracks *4*, *7*). After 1 h at 4°C, the samples were subjected to SDS-PAGE, and analyzed by autoradiography.

The Uk Inhibitor Secreted by Monocytes-Macrophages and U 937 Cells Is not a Ligand for Thrombin. Cultured human fibroblasts secrete protease-nexin, an inhibitor of Uk that forms SDS-resistant complexes with either Uk or thrombin (13). The reported M_r of Uk complexed with protease-nexin is comparable to that obtained with the monocyte-released ligand. It was therefore of interest to determine whether the ligands secreted by these different cell types shared additional properties.

In contrast to Uk (Fig. 7, tracks *4* and *7*), thrombin did not prevent the binding of [^{125}I]-Uk to the ligand from U 937 cells or monocytes-macrophages (tracks *3* and *6*). Furthermore, no complex formation was observed when [^{125}I]-thrombin was added to U 937 medium (Fig. 8, track *3*) or monocyte-macrophage medium (data not shown), whereas fibroblast culture medium did contain ligand(s) for both thrombin and Uk (Fig. 8, tracks *2* and *5*). Heparin (2 U/ml), which accelerates the reaction of thrombin with protease-nexin (13), had no effect on the outcome of these experiments (data not shown). Thus, the monocyte-secreted inhibitor of Uk did not react with thrombin. Finally, the complex formed between [^{125}I]-Uk and the U 937 ligand was clearly resolved from that formed with the fibroblast ligand (Fig. 8, tracks *5* and *6*). In conclusion, the monocyte ligand and fibroblast-produced protease-nexin are different inhibitors.

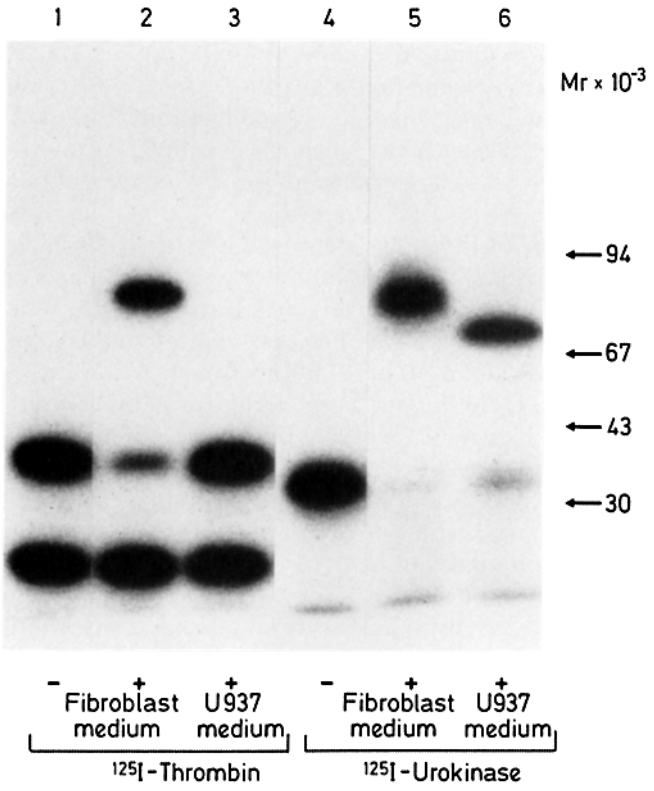

FIGURE 8. Formation of complexes between [^{125}I]-thrombin or [^{125}I]-Uk and fibroblast- or U 937–produced ligands. [^{125}I]-thrombin (2 ng, 10^6 cpm/μg) (tracks *1–3*) or M_r 33,000 [^{125}I]-Uk (2 ng, 10^6 cpm/μg) (tracks *4–6*) were added to 10 μl samples of fresh medium (tracks *1* and *4*), fibroblast-conditioned medium (tracks *2* and *5*), or U 937–conditioned medium (tracks *3* and *6*). After 1 h at 4°C the samples were subjected to SDS-PAGE, and analyzed by autoradiography.

Discussion

The studies reported in this paper establish some of the characteristics of PA secretion by cultured human monocytes and by cells from the histiocytic lymphoma line U 937.

—First, the enzyme produced by these cells has an apparent M_r of 55,000 by SDS-PAGE, and co-migrates with Uk purified from human urine. Moreover, it is recognized by antibodies raised against human Uk, but not by antibodies to human TA; we can therefore exclude the contribution of a form of PA recently described in certain acute myeloblastic leukemia cells that is immunologically related to TA and not to Uk, but that co-migrates electrophoretically with Uk (30). Thus, monocytes-macrophages purified from human peripheral blood, like granulocytes (30, 31), secrete Uk as their only type of PA.

—Second, the form of Uk that accumulates in the serum-free culture medium is not inhibitable by DFP, but it can be converted to a DFP-sensitive form by plasmin. By these criteria, it behaves like the proUk secreted by human carcinoma and glioblastoma cell lines (10, 11), and by cultured fibroblasts (14). Whether

monocytes-macrophages also secrete active Uk, in addition to proUk, cannot be decided at this time: indeed, active enzyme would be rapidly inhibited by the co-secreted ligand, and such enzyme-inhibitor complexes may be catabolized by the cells (32), or, if remaining in the medium, would have only low specific enzymatic activity in our assays. Biosynthetic labeling of cellular proteins followed by immunoprecipitation of Uk-related polypeptides will be necessary to resolve this issue.

—Third, an inhibitor of fibrinolysis is released by the cells in culture. Exogenously added active Uk forms with this inhibitor a complex that is not dissociated by boiling in SDS, and that is functionally inactivated as a PA. The apparent M_r of the complex is ~39,000 daltons higher than that of free Uk; gel filtration of the ligand-inhibitor indicates a M_r of 40,000 before reaction with Uk (in preparation). Taken together, these data suggest the formation of a 1:1 Uk-ligand complex. The ligand does not react with thrombin, nor with plasmin (unpublished observations); thus it is different from protease-nexin, a protease inhibitor released by human fibroblasts that forms similar complexes with Uk and thrombin (13). A further characterization of the monocyte-macrophage inhibitor is under way: since the inhibitor can be detected amongst biosynthetically labeled products of U 937 cells, it must be synthesized during the culture; the currently available data allow it to be distinguished from all characterized inhibitors, with the possible exception of the fibrinolytic inhibitor originally described in human placenta (33, 34).

—Fourth, proUk and the Uk inhibitor are released concomitantly by monocyte-macrophage cultures, although we have not determined whether both proteins are secreted by the same cells at the same time. From the data presented here, we can estimate that the free inhibitor is in large (10–100-fold) excess over proUk. Since proUk is catalytically inactive and does not react with the inhibitor, both proteins can coexist separately in the same media. In the presence of trace amounts of plasmin, or perhaps of some other proteases capable of converting proUk to the active enzyme, Uk is generated and reacts with the available inhibitor. Thus, the presence of Uk and its inhibitor may appear to be mutually exclusive, as has been reported for mouse macrophages (15, 16), when plasminogen was added to the unfractionated sample; alternatively, both can be detected as shown in our study, in which the proenzyme was separated from the inhibitor before exposure to plasminogen.

—Fifth, U 937 cells do not differ from cultured monocytes-macrophages with respect to proUk and inhibitor production. The use of this cell line overcomes possible problems that could arise from the contamination of freshly prepared monocyte preparations by other cell types. The U 937 line therefore provides a convenient source for the further study of these processes.

The concomitant secretion of proUk and of an inhibitor of Uk has recently been reported for cultured human fibroblasts (14). Our results extend these observations to another cell type. We have also shown that the monocyte-produced inhibitor is different from the fibroblast protease-nexin. Thus, the regulation of extracellular PA activity in both fibroblasts and monocytes-macrophages, and possibly in other cell types, may involve, in addition to transcriptional events (35), controls at the posttranslational level as well as through the produc-

tion of enzyme inhibitors. Furthermore, these inhibitors appear to differ between different cell types. The secretion of Uk inhibitors by murine macrophages, which also synthesize a Uk-type PA (15, 16, manuscript in preparation), as well as by hepatoma tissue culture cells (36), endothelial cells (37–39), and other cell types (40–42), further underlines the possible generality of these phenomena.

The physiological role of monocyte-macrophage PA remains conjectural. Studies on the modulation of enzyme production suggest that it may be involved in the proteolytic events that accompany cell migration (1, 2), and macrophage-mediated digestion of certain extracellular matrices has been shown to require PA activity (43). Whether proUk activation and/or Uk inhibitor secretion are modulated as a function of mononuclear phagocyte differentiation can be determined by further studies. The DFP-inactivation assay that we have used to distinguish between proUk and Uk allows easy evaluation of the zymogen/enzyme ratio in conditioned media. Also, formation of SDS-resistant [^{125}I]-Uk-inhibitor complexes can be used to quantitate the available inhibitor; using this approach we have observed that production of a comparable Uk ligand by murine peritoneal macrophages varies with the inflammatory status of the animal (A. Wohlwend et al., in preparation). We have recently described the presence of high affinity binding sites specific for M_r 55,000 Uk on the surface of U 937 cells and human peripheral blood mononuclear cells.[3] DFP-inactivated M_r 55,000 Uk also binds to these cells, whereas M_r 33,000 Uk does not; we have therefore proposed that determinants of the A chain of M_r 55,000 Uk are involved in this binding, and that the enzyme need not be active for binding to occur. In view of the findings described in this paper, it will be of interest to determine whether proUk also can bind to U 937 and other cells, and whether cell-bound enzyme is susceptible to inhibition by the secreted ligand. The results of such experiments should allow us to better evaluate a potential role for Uk in the biology of mononuclear phagocytes.

Summary

The plasminogen activator (PA) produced by freshly purified human monocytes-macrophages and histiocytic, lymphoma-derived U 937 cells was analyzed by zymography after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and found to migrate with an apparent M_r of 55,000, identical to that of urokinase (Uk). By immunoprecipitation with antibodies specific for the two different types of PA, the enzyme was shown to be immunologically related to urokinase, and not to tissue PA. Urokinase was secreted in the form of the inactive M_r 55,000 zymogen prourokinase, and could be converted to the active M_r 55,000 enzyme by limited proteolysis with plasmin.

Conditioned media from cultures of U 937 cells and monocytes-macrophages inhibited the fibrinolytic activity of exogenously added urokinase. Using [^{125}I]-labeled urokinase we observed the formation of an enzyme-ligand complex, which was not dissociated by boiling in SDS and migrated with an apparent M_r 40,000 daltons higher than the free enzyme; since complexed urokinase was functionally inactivated as a PA, the ligand is an inhibitor of urokinase. This

[3] J.-D. Vassalli, D. Baccino, and D. Belin, A cellular binding site for the M_r 55,000 form of human urokinase; manuscript submitted for publication.

inhibitor is different from fibroblast-produced protease-nexin, in that it did not interact with thrombin.

These results suggest that plasminogen activation by mononuclear phagocytes can be modulated through the secretion of both (pro)enzyme and a specific inhibitor.

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References

1. Reich, E. 1978. Activation of plasminogen: a general mechanism for producing localized extracellular proteolysis. *In* Molecular Basis of Biological Degradative Processes. R. D Berlin, H. Herrmann, I. H. Lepow, and J. M. Tanzer, editors. Academic Press, New York. pp. 155–169.
2. Vassalli, J.-D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nucleotides. *Cell.* 8:271.
3. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* 139:834.
4. Gordon, S., J. C. Unkeless, and Z. A. Cohn. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis, evidence for a two-stage process. *J. Exp. Med.* 140:995.
5. Vassalli, J.-D., J. Hamilton, and E. Reich. 1977. Macrophage plasminogen activator: induction by concanavalin A and phorbol myristate acetate. *Cell.* 11:695.
6. Vassalli, J.-D., and E. Reich. 1977. Macrophage plasminogen activator: induction by products of activated lymphoid cells. *J. Exp. Med.* 145:429.
7. Steffens, G. J., W. A. Gunzler, F. Otting, E. Frankus, and L. Flohé. 1982. The complete amino acid sequence of low molecular mass urokinase from human urine. *Hoppe-Seyler's Z. Physiol. Chem.* 363:1043.
8. Gunzler, W. A., G. J. Steffens, F. Otting, S.-M. A. Kim, E. Frankus, and L. Flohé. 1982. The primary structure of high molecular mass urokinase from human urine. The complete amino acid sequence of the A chain. *Hoppe-Seyler's Z. Physiol. Chem.* 363:1155.
9. Pennica, D., W. E. Holmes, W. J. Kohr, R. N. Harkins, G. A. Vehar, C. A. Ward, W. F. Bennett, E. Yelverton, P. H. Seeburg, H. L. Heyneker, D. V. Goeddel, and D. Collen. 1983. Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli. Nature (Lond.)* 301:214.
10. Wun, T.-C., L. Ossowski, and E. Reich. 1982. A proenzyme form of human urokinase. *J. Biol. Chem.* 257:7262.
11. Nielsen, L. S., J. G. Hansen, L. Skriver, E. L. Wilson, K. Kaltoft, J. Zeuthen, and K. Danö. 1982. Purification of zymogen to plasminogen activator from human glioblastoma cells by affinity chromatography with monoclonal antibody. *Biochemistry.* 21:6410.
12. Skriver, L., L. S. Nielsen, R. Stephens, and K. Danö. 1982. Plasminogen activator released as inactive proenzyme from murine cells transformed by sarcoma virus. *Eur. J. Biochem.* 124:409.
13. Baker, J. B., D. A. Low, R. L. Simmer, and D. D. Cunningham. 1980. Protease-nexin: a cellular component that links thrombin and plasminogen activator and mediates their binding to cells. *Cell.* 21:37.

14. Scott, R. W., D. L. Eaton, N. Duran, and J. B. Baker. 1983. Regulation of extracellular plasminogen activator by human fibroblasts: the role of protease-nexin. *J. Biol. Chem.* 258:4397.
15. Klimetzek, V., and C. Sorg. 1979. The production of fibrinolysis inhibitors as a parameter of the activation state in murine macrophages. *Eur. J. Immunol.* 9:613.
16. Chapman, H. A., Z. Vavrin, and J. B. Hibbs, Jr. 1982. Macrophage fibrinolytic activity: identification of two pathways of plasmin formation by intact cells and of a plasminogen activator inhibitor. *Cell.* 28:653.
17. Sundström, C., and K. Nilson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line. *Int. J. Cancer.* 17:565.
18. Wun, T. C., W.-D. Schleuning, and E. Reich. 1982. Isolation and characterization of urokinase from human plasma. *J. Biol. Chem.* 257:3276.
19. Hjelm, H., K. Hjelm, and J. Sjöquist. 1972. Protein A from *Staphylococcus aureus*. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 28:73.
20. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen purification from human plasma by affinity chromatography. *Science (Wash. DC).* 170:1095.
21. Granelli-Piperno, A., and E. Reich. 1978. A study of proteases and protease-inhibitor complexes in biological fluids. *J. Exp. Med.* 148:223.
22. Böyum, A. 1976. Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* 5(suppl. 5):9.
23. Manconi, P. E., M. G. Marrosu, L. Paghi, G. Correale, and D. Zaccheo. 1979. Alpha-naphthyl acetate esterase activity in human lymphocytes: distribution in lymphocyte subpopulations and in mitogen-activated cells. *Scand. J. Immunol.* 9:99.
24. Preud'homme, J.-L., and G. Flandrin. 1974. Identification by peroxidase staining of monocytes in surface immunofluorescence tests. *J. Immunol.* 113:1650.
25. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* 115:1617.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
27. Strickland, S., and W. H. Beers. 1976. Studies on the role of plasminogen activator in ovulation. In vitro response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. *J. Biol. Chem.* 251:5694.
28. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *In* Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. pp. 466–467.
29. Travis, J., and G. S. Salvesen. 1983. Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* 52:655.
30. Wilson, E. L., P. Jacobs, and E. B. Dowdle. 1983. The secretion of plasminogen activators by human myeloid leukemic cells in vitro. *Blood.* 61:568.
31. Granelli-Piperno, A., and J.-D. Vassalli, and E. Reich. 1977. Secretion of plasminogen activator by human polymorphonuclear leukocytes: modulation by glucocorticoids and other effectors. *J. Exp. Med.* 146:1693.
32. Low, D. A., J. B. Baker, W. C. Koonce, and D. D. Cunningham. 1981. Released protease-nexin regulates cellular binding, internalization, and degradation of serine proteases. *Proc. Natl. Acad. Sci. USA.* 78:2340.
33. Kawano, T., K. Morimoto, and Y. Uemura. 1970. Partial purification and properties of urokinase inhibitor from human placenta. *J. Biochem.* 67:333.
34. Holmberg, L., I. Lecander, B. Persson, and B. Astedt. 1978. An inhibitor from placenta specifically binds urokinase and inhibits plasminogen activator released from ovarian carcinoma in tissue culture. *Biochim. Biophys. Acta.* 544:128.

35. Nagamine, Y., M. Sudol, and E. Reich. 1983. Hormonal regulation of plasminogen activator mRNA production in porcine kidney cells. *Cell.* 32:1181.
36. Coleman, P. L., P. A. Barouski, and T. D. Gelehrter. 1982. The dexamethasone-induced inhibitor of fibrinolytic activity in hepatoma cells. A cellular product which specifically inhibits plasminogen activation. *J. Biol. Chem.* 257:4260.
37. Dosne, A. M., E. Dupuy, and E. Bodevin. 1978. Production of a fibrinolytic inhibitor by cultured endothelial cells derived from human umbilical vein. *Thromb. Res.* 12:377.
38. Smariga, P. E., and J. R. Maynard. 1982. Platelet effects on tissue factor and fibrinolytic inhibition of cultured human fibroblasts and vascular cells. *Blood.* 60:140.
39. Loskutoff, D. J., J. A. van Mourik, L. A. Erickson, and D. Lawrence. 1983. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proc. Natl. Acad. Sci. USA.* 80:2956.
40. Roblin, R. O., P. L. Young, and T. E. Bell. 1978. Concomitant secretion by transformed SVWI38-VA13-2RA cells of plasminogen activator(s) and substance(s) which prevent their detection. *Biochem. Biophys. Res. Commun.* 82:165.
41. Crutchley, D. J., L. B. Conanan, and J. R. Maynard. 1981. Human fibroblasts produce inhibitor directed against plasminogen activator when treated with glucocorticoids. *Ann. N. Y. Acad. Sci.* 370:609.
42. Birkedal-Hansen, H., and R. E. Taylor. 1983. Production of three plasminogen activators and an inhibitor in keratinocyte cultures. *Biochim. Biophys. Acta.* 756:308.
43. Jones, P. A., and Z. Werb. 1980. Degradation of connective tissue matrices by macrophages. II. Influence of matrix composition on proteolysis of glycoproteins, elastin, and collagen by macrophages in culture. *J. Exp. Med.* 152:1527.