Brief Definitive Report

HUMAN MONOCYTES CAN PRODUCE TISSUE-TYPE PLASMINOGEN ACTIVATOR

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The two types of plasminogen activator (PA), tissue-type PA (t-PA) and urokinase-type PA (u-PA), are serine proteases that cleave plasminogen to plasmin and are the products of separate genes (1). t-PA, which has a high affinity for fibrin, is likely to be the major PA involved in fibrinolysis; u-PA probably mediates extracellular proteolytic events, such as tissue destruction and remodeling (1). With the exception of a distinctly different but uncharacterized 52-kD PA-like species (2), evidence has previously been presented that monocytes/macrophages produce only u-PA (3–5). Furthermore, previous investigators have detected PA activity in the supernatants from both unstimulated and stimulated monocytes only after inhibitors of PA activity have been removed from the supernatants by treatment with SDS (3), by separation on SDS-PAGE (4), or by dialysis (2). We show here for the first time that human monocytes, when stimulated appropriately in vitro, can produce t-PA of 70 kD; this activity is detectable in culture supernatants without prior biochemical treatment to remove inhibitors.

Materials and Methods

Monocyte Isolation and Culture. As before (6), monocytes were isolated from peripheral venous blood by countercurrent centrifugal elutriation; extensive precautions were taken to limit LPS contamination. Cell fractions containing ≥95% monocytes, identified by morphological criteria and by nonspecific esterase staining, were pooled and cultured for 18 h ($0.8–1.0 \times 10^6$ in 1 ml α-modified Eagle's medium containing 1% FCS (6). Where indicated, LPS from *Escherichia coli* 0111:B4 (Difco Laboratories, Inc., Detroit, MI) and human rIFN-γ (Dr. E. Hochuli, Hoffmann-La Roche, Basel, Switzerland) were added at 100 ng/ml and 100 U/ml, respectively. To terminate the cultures, the supernatants were centrifuged to remove nonadherent cells; adherent and nonadherent cells were pooled, washed, and lysed with 0.2% Triton X-100 in PBS.

Assay for PA Activity. Monocyte supernatants or lysates (50 μl) and human plasminogen (0.8 μg), dissolved in 100 μl 0.1 M Tris-HCl, pH 8.1, were added to 0.28-cm^2 wells previously coated with [^{125}I]fibrin (7). After 2–3 h, soluble [^{125}I]fibrin degradation products were measured. PA activity was expressed according to the activity of u-PA standards (Leo Pharmaceutical Products, Denmark). Monocyte-derived plasminogen-independent fibrinolytic activity was always ≤5% of the plasminogen-dependent activity.

IgGs. IgGs were isolated from rabbit antisera to human u-PA and to human t-PA (Dr. W.-D. Schleuning, CHUV, Lausanne, Switzerland) by standard methods using protein

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A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The mouse myeloma IgG, HOPCY (Dr. A. Burgess, Ludwig Institute for Cancer Research, Melbourne), was used as an irrelevant antibody. Culture supernatants, cell lysates, or PA standards (u-PA as above, t-PA as a culture supernatant from the MM138 melanoma cell line [8, 9]) (Dr. R. Whitehead, Ludwig Institute for Cancer Research, Melbourne) (0.2 IU/ml) were incubated with IgGs (1 μg/ml final concentration) for 1 h at 37°C before assay of residual PA activity. In the immunoprecipitation experiments, protein A-Sepharose CL-4B was used (4).

SDS-casein Zymography. SDS-PAGE zymography was carried out essentially as published (10). The resolving gel (10%) contained casein (2 mg/ml; Sigma Chemical Co., St. Louis, MO) and human plasminogen (6 μg/ml). After electrophoresis and washing, the gels were stained as previously described (10).

Detection of t-PA mRNA. Total monocyte RNA (11) (5 μg/lane of a formaldehyde-containing 1% agarose gel) was transferred to Genescreen Plus nylon membrane (DuPont Co., Wilmington, DE) and hybridized overnight at 60°C in a standard hybridization buffer (7) containing >2 × 10^6 cpm/ml of ^{32}P-labeled t-PA cRNA. The cRNA probe was prepared from a human t-PA-containing pGEM-4 blue vector (Promega Biotec, Madison, WI) (12). After hybridization, the filter was washed three times with 2× SSC before treatment with 1 μg/ml RNase A (Boehringer Mannheim Biochemicals, Mannheim, FRG) for 20 min at 37°C.

Results

PA Activity in Monocyte Culture Supernatants. As previously reported for unstimulated cells (2–5), PA activity was not detected in the supernatants of untreated human monocytes. However, culture with LPS (100 ng/ml) resulted in measurable PA activities in the supernatants of monocytes from 90% of donors examined (Fig. 1; after 18 h, mean PA activity ± SEM = 0.14 ± 0.03 IU/10^6 cells). Levels of LPS as low as 10 ng/ml were sufficient to stimulate the expression of PA activity by monocytes from many individuals. IFN-γ (100 U/ml) did not stimulate monocytes for detectable PA activity, but consistently synergized with LPS to increase PA activity of monocytes from all donors (Fig. 1; after 18 h, mean PA activity ± SEM = 0.43 ± 0.08 IU/10^6 cells). PA activity could be detected just 6 h after adding LPS with IFN-γ.

Characterization of PA activity in Monocyte Culture Supernatants. All of the PA activity

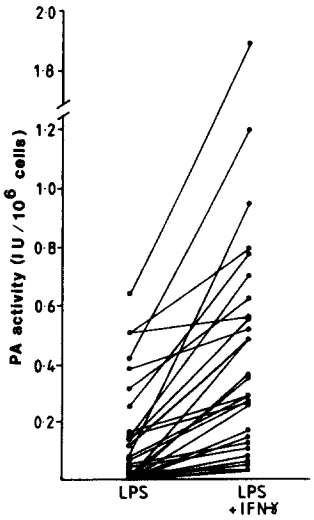

FIGURE 1. PA activity in the supernatants of cultured human monocytes from 29 donors. Monocytes were cultured for 18 h with LPS ± IFN-γ, and PA activity was assayed as described in Materials and Methods. Each point represents the mean PA activity detected in supernatants from triplicate monocyte cultures. The activity is expressed according to the number of cells initially plated; no significant change was seen in the number of monocytes in any treatment group after 18 h in culture.

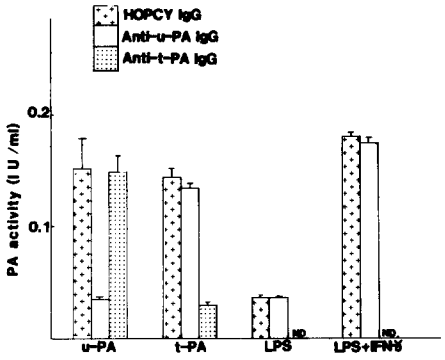

FIGURE 2. Immunological characterization of the PA activity in monocyte culture supernatants. PA standards and culture supernatants from monocytes incubated for 18 h with LPS or with LPS + IFN-γ (pooled aliquots from duplicate cultures) were treated with HOPCY IgG, anti-u-PA IgG, or anti-t-PA IgG, as described in Materials and Methods. Mean ± SEM (n = 3).

detected in the monocyte culture supernatants was blocked by anti-t-PA IgG but not by anti-u-PA IgG (Fig. 2). By SDS-casein zymography, a technique that determines the apparent molecular weight of PA and that can also detect PA in the presence of inhibitors (4, 10), a band of 52 kD corresponding to the molecular weight of u-PA was seen from both unstimulated and stimulated monocytes (Fig. 3 *A*, lanes *4–7*). This result agreed with previous studies (3, 4). However, unlike the previous

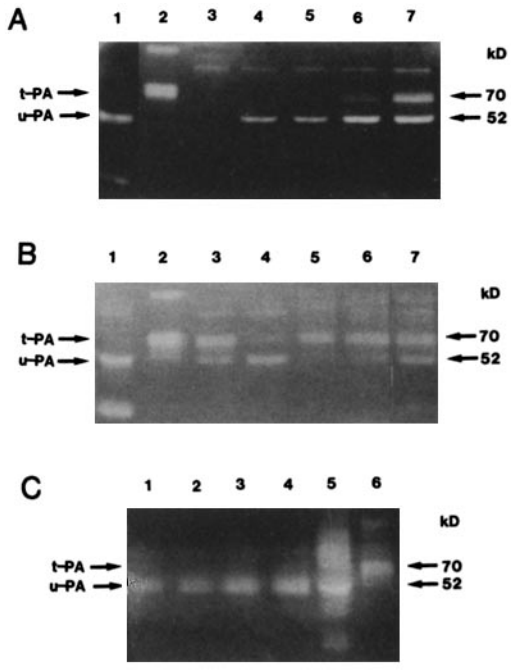

FIGURE 3. Characterization of monocyte PA activity by SDS-casein zymography. Clear bands of lysis appeared where PA had converted plasminogen to plasmin, which then degraded the casein. The main caseinolytic bands for u-PA and t-PA standards (0.2 IU/ml) were detected at 52 and 70 kD, respectively, as defined by the migration pattern of low molecular weight markers (Pharmacia Fine Chemicals). For the u-PA standard, but not the monocyte supernatants or lysates, a 36-kD breakdown product (4) was detected. (*A*) Supernatants from monocytes of a representative donor after culture for 18 h. Lane *1*, u-PA standard; lane *2*, t-PA standard; lane *3*, medium used for monocyte culture supplemented with 1% FCS (showing the caseinolytic bands due to high molecular weight serum components); lane *4*, pooled supernatants from duplicate cultures of control monocytes; lanes *5–7*, pooled supernatants from duplicate cultures of monocytes incubated with IFN-γ, LPS, and LPS + IFN-γ, respectively. (*B*) Identification of the lytic species in a monocyte culture supernatant by immunoprecipitation. Lane *1*, u-PA standard; lane *2*, t-PA standard; lane *3*, pooled supernatant from monocytes stimulated with LPS and IFN-γ before incubation with IgG; lanes *4* and *5*, unprecipitated material and pellet, respectively, after incubation with anti-t-PA IgG; lane *6*, unprecipitated material after incubation with anti-u-PA IgG; lane *7*, unprecipitated material after incubation with HOPCY IgG. (*C*) Characterization of PA activity in monocyte lysates. Lane *1*, control monocytes incubated for 18 h; lanes *2–4*, monocytes incubated for 18 h with IFN-γ, LPS, and LPS + IFN-γ, respectively; lane *5*, u-PA standard; lane *6*, t-PA standard.

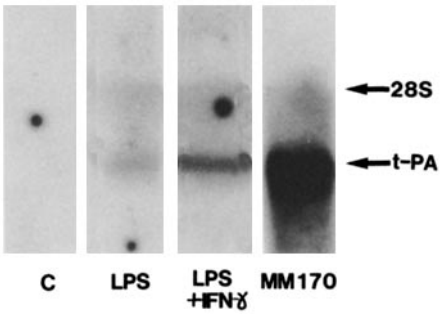

FIGURE 4. Expression of t-PA mRNA in human monocytes. Cultures were with no added stimuli (*C*), with LPS, or with LPS and IFN-γ. Northern analysis was carried out as described in Materials and Methods. Levels of mRNA in t-PA-producing melanoma cells, MM170 (8, 9), were also examined.

studies, a 70-kD band migrating in a manner characteristic of the t-PA standard was found, as well as the u-PA band in the supernatants of monocytes incubated with either LPS or LPS with IFN-γ (Fig. 3 *A*, lanes *6* and *7*). By precipitation with anti-t-PA IgG, this band of PA activity was confirmed as t-PA (Fig. 3 *B*, lanes *4* and *5*).

Monocyte-associated PA Activity. PA activities in monocyte lysates were consistently <0.01 IU/10^6 cells and hence were considerably lower than those found in culture supernatants (Fig. 1). When mean cell-associated PA activities were calculated for nine cell donors, there were no significant differences between control cells and cells treated with IFN-γ or with LPS ± IFN-γ. Regardless of the treatment protocol, this cell-associated PA activity was of the u-PA type, based on zymography (Fig. 3*C*) and on its removal by incubation with anti-u-PA IgG (data not shown).

Detection of Monocyte t-PA mRNA. Northern analysis showed the presence of t-PA mRNA in monocytes treated with LPS and even greater levels in cells incubated with LPS and IFN-γ together (Fig. 4). Actinomycin D (2 μg/ml; Sigma Chemical Co.) inhibited the appearance of monocyte t-PA activity, suggesting enhanced t-PA gene transcription. Northern blots probed with a ^{32}P-labeled u-PA cDNA (7) showed that LPS, but not IFN-γ, elevated the basal u-PA mRNA level; IFN-γ did not synergise with LPS for further enhancement of the LPS-induced u-PA mRNA (data not shown).

Discussion

We report here that human monocytes can secrete into the culture medium a species of PA that is readily detected without prior electrophoresis, SDS treatment, or dialysis. This PA activity was identified immunologically to be t-PA (Fig. 2). SDS-casein zymography revealed two bands of PA activity, corresponding to enzymes of 52 and 70 kD, confirming the production of u-PA (as previously reported) (3–5) and t-PA (previously unreported), respectively (Fig. 3). Monocytes secrete predominantly PA inhibitor type 2, which has been reported to inhibit preferentially u-PA (13), and which could account for the detection of t-PA but not u-PA activity in monocyte culture supernatants (Fig. 2). Detection of t-PA mRNA was consistent with the biochemical and immunological characterization of t-PA produced by human monocytes. Deliberate addition of lymphocytes to the highly purified monocyte cultures did not increase the t-PA activity, suggesting that the t-PA detected was indeed a monocyte product. LPS was necessary in our studies to induce t-PA activity; however, there may be other biological stimuli that similarly enhance the expression of monocyte t-PA activity.

It is significant that t-PA activity was observed even in SDS-casein gels where fibrin, which potentiates t-PA activity (1), is absent; the casein in these gels is more selective for u-PA (14). Cell-associated PA activity was of the u-PA type (Fig. 3 *C*) and suggested that t-PA is secreted rather than stored by stimulated monocytes or that stored t-PA cannot be detected until it is released from the cells. Precise determination of the relative amounts of intracellular and extracellular PA is difficult because of the complicating influences of monocyte-derived PA inhibitor(s) (4, 13), of u-PA receptors on the monocyte surface (1), and of the differential enhancement of t-PA and u-PA activities by fibrin and casein (1, 14), respectively.

Fibrin deposition occurs at sites of tissue damage, including chronic inflammatory lesions, and the stimulation of monocytes to express t-PA activity could provide a mechanism to hasten dissolution of fibrin at such sites. In addition, there is evidence for a significant cellular-phase component of blood fibrinolytic activity both under normal resting conditions, and in physiologically or pharmacologically altered states (15). We suggest the possible involvement of monocytes through their expression of t-PA activity.

The clinical use of t-PA as a thrombolytic agent for the treatment of myocardial infarcts, for example, is becoming established (16). However, because of its short half-life, large quantities of t-PA are administered intravenously over several hours and this may cause unwanted bleeding (16). Pharmacological induction of local t-PA activity at a thrombus might be extremely useful, and controlled stimulation of local monocytes for t-PA activity could be one approach. In this context, it should be noted that since foam cells in atherosclerotic plaques are derived from circulating monocytes (17), the level of t-PA activity produced by these monocytes/foam cells may determine in part the persistence or resolution of the fibrin in early lesions.

Summary

Evidence has previously been presented that monocytes and macrophages produce urokinase-type plasminogen activator. We have shown for the first time that human monocytes, when stimulated appropriately in vitro, can produce tissue type-plasminogen activator (t-PA) of 70 kD. Detection of t-PA mRNA was consistent with the biochemical and immunological characterization of t-PA produced by human monocytes.

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