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Minactivin expression in human monocyte and macrophage populations

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Minactivin Expression in Human Monocyte and Macrophage Populations

By Ross W. Stephens, Jeffrey P. Golder, David R.H. Fayle, David A. Hume, Andrew J. Hapel, William Allan, Carol J. Fordham, and William F. Doe

Adherent monolayer cultures of human blood monocytes, peritoneal macrophages, bone marrow macrophages, and colonic mucosa macrophages were examined for their ability to produce and secrete minactivin, a specific inactivator of urokinase-type plasminogen activator. All except colonic mucosa macrophages produced and secreted appreciable amounts of minactivin, but only blood monocytes were stimulated by muramyl dipeptide (adjuvant peptide) to increase production. The minactivin from each of these populations could be shown to preferentially inhibit urokinase-type plasminogen activator and not trypsin, plasmin, or "tissue"-type plasminogen activator (HPA68). A plasminogen-activating enzyme present in monocyte cultures appeared unaffected by the presence of minactivin and could be shown to be regulated independently by dexamethasone.

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Materials and Methods

Monocyte Preparations

Human blood monocytes. Human mononuclear leukocytes were isolated on Ficoll-Paque (Pharmacia Fine Chemicals, Sydney, Australia) gradients from buffy coats provided by the Red Cross Blood Service of the Woden Valley Hospital. After four washes in phosphate-buffered saline (Pi/NaCl) at 4 °C to remove contaminating platelets, the cell pellet was resuspended in RPMI 1640 (GIBCO, Grand Island, NY) containing 60 U/mL gentamicin. Purified monocyte monolayers were obtained by plating 10^7 mononuclear cells in RPMI 1640 with 10% AB serum into 30-mm wells (Linbro multiwell plates, Flow Labs, McLean, Va; catalog No. 7605805) that had been precoated for 30 minutes with 2 mL of human AB serum. Monocytes were allowed to adhere for 60 minutes at 37 °C in an atmosphere 5% CO2 in air. Nonadherent cells were then removed by washing monolayers six times with RPMI 1640 prewarmed at 37 °C.

Peritoneal macrophages. Two liters of dialysate fluid from patients undergoing routine peritoneal dialysis for chronic renal failure were centrifuged at 300 g for ten minutes at 20 °C. The cell pellet was resuspended in 50 mL of RPMI 1640 and recentrifuged for ten minutes at 200 g at 20 °C. The cells were then resuspended in RPMI 1640 to a final concentration of approximately 2 × 10^6/mL, and 6-mL aliquots of the peritoneal cell suspension were underlayed with 3 mL of Ficoll-Paque and centrifuged for 20 minutes at 400 g at 20 °C. The cells were resuspended to a concentration of 1 × 10^6 cells per milliliter in RPMI 1640 containing 10% human AB serum, and macrophage monolayers were prepared by adherence to plastic culture trays as described previously for monocytes.

Bone marrow-derived macrophages. Fresh pieces of human iliac crest and rib marrow were obtained during orthopedic procedures and dispersed in RPMI 1640 media containing 10% giant cell tumor (GCT) culture supernatant (GIBCO-Biocult, Grand Island, NY) and 10% fetal bovine serum. This primary culture was maintained for seven days on gelatin-coated flasks. After this period, monocyte/macrophages were purified by adherence to culture dishes prepared as for blood monocytes and the secondary adherent culture used for minactivin measurements. The cells obtained were greater than 90% positive for nonspecific esterase. Details of the method are published elsewhere.

Colonic mucosa macrophages. These cells were obtained from resected colons by enzymatic disaggregation of the colonic mucosa according to the method of Golder and Doe. They were grown as adherent monolayers in RPMI 1640 as for monocytes.

Monocyte and Macrophage Cell Culture

Monocyte or macrophage monolayers were cultured in RPMI 1640 media containing 1% human serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia). Limulus amebocyte lysate assays of this culture media showed that the endotoxin level was less than 1 ng/mL. Cultures of cells from sources (a) through (d) above were kept as close as possible to a density of 3 × 10^6 cells per milliliter of media.

The following agents were used in cell culture experiments: (a) muramyl dipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamin, Peninsula Laboratories, San Carlos, Calif), at 5 μg/mL; (b) Salmonella minnesota R595 cell wall lipopolysaccharide. This was prepared by sonication of the water-insoluble extract in 0.1% triethylamine followed by extensive dialysis against Pi/NaCl. The final concentration in cultures was 0.1 μg/mL. Dexamethasone (Sigma Chemical Co, St Louis) was used at a final concentration of 0.1 μmol/L.

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Morphology and Cytochemistry

For phase-contrast microscopy, adherent monocyte or macrophage monolayers were washed twice and fixed in 1.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for at least 30 minutes at room temperature and examined.

Cytocentrifuge preparations were prepared in a Shandon Southern centrifuge. Briefly, 0.2-mL aliquots containing 50,000 cells in 10% fetal bovine serum were spun for three minutes at 300 rpm. The preparation was then dried rapidly with a hair dryer, and the preparations stained with May-Grunwald Giems.

The presence of nonspecific esterase was assayed using α-naphthyl acetate as substrate. Phagocytic activity was assayed by incubating adherent monolayers or cell suspensions in RPMI 1640 containing 10% human AB serum and 1.1 μm latex beads (Sigma) at a concentration of 100 beads per cell for 60 minutes at 37°C. Noningested beads were removed by washing monolayers or cell suspensions, 150 g for ten minutes at 20°C, three times with RPMI 1640. Cells ingesting two or more latex beads were judged to be phagocytic.

Colorimetric Assays of Minactivin and Plasminogen Activator

Minactivin produced in cultures was quantified by the inhibition produced in colorimetric assays of human urokinase reference standard (Calbiochem, Behring Corp, La Jolla, Calif). Interference by endogenous plasminogen activator was prevented by the ionic strength of culture media. When it was required to measure endogenous plasminogen activator, the preparation was dialyzed against 50 mmol/L glycine, pH 7.8, which allowed direct assay despite the presence of the minactivin still present. The assay method for urokinase and for endogenous plasminogen activator was based on the coupled assay of Coleman and Green. For assay of minactivin in cell culture supernatant or 0.5% Triton lysate, 20 μL of the sample was first precubated with urokinase (4 milli-Ploug unit [mPU] in 20 μL) for 90 minutes at 23°C, and then affinity-purified human plasminogen (4 μg in 20 μL) was added and the mixture incubated for 45 minutes at 37°C. The assay buffer contained 50 mmol/L glycine, pH 7.8, 0.1% Triton X-100 (Sigma Chemical Co, St Louis), and 0.1% gelatin. The plasmin produced was then assayed by the addition of the thioesterase substrate (Z-lysine-thiobenzyl ester, pH 7.8, 0.1% Triton x-100, and 0.1% gelatin). The color was developed at 350 nm by the addition of Trasylol (Bayer Australia Ltd, Botany) (16 μg in 20 μL) and the absorbance read at 412 nm. Checks were made to verify that inhibition was at the level of plasminogen activation and not simply inhibition of plasmin, by direct addition of the minactivin source to the assay of preformed plasmin.

Radial Diffusion Assays for Specificity Studies

Fibrinogen (Sigma, type X), human plasminogen, and thrombin (Sigma, grade 1) were cast in a 1.25% agarose (Sea Plaque, FMC Corp, Rockland, Me) gel matrix 1.2 mm thick. After clotting at 37°C, the gel was hardened at 4°C, and 3-mm wells were cut for the application of protease/minactivin mixtures (5 μL).

The following proteases were used to determine the specificity of the supposed minactivin from the different cell populations: plasmin (300 ng), trypsin (50 ng), human urokinase (25 mPU), and culture supernatant from human melanoma (undiluted). Each of these enzymes were preincubated for 90 minutes at 23°C with cell culture supernatant or lysate (total volume 10 μL). The gels were incubated in a humidified box for 20 hours at 37°C, and the lysis produced was enhanced by extensive washing in saline, followed by staining with amido black.

RESULTS

Macrophage Populations and Minactivin Production

Human blood monocytes. Adherent monolayers were shown to be greater than 85% monocytes by several criteria. Polychromat staining of the monolayer cells showed 94% ± 6% of the cells to be monocytes having a large round or indented nucleus with basophilic cytoplasm. Staining for the cytoplasmic enzyme nonspecific esterase showed monolayers to be 85% ± 2% positive for this monocyte marker. Ninety-one percent of the adherent cells phagocytosed latex beads, confirming that the adherent cell population that showed the morphological and histochemical appearances of mononuclear phagocytes also exhibited this characteristic functional property.

During in vitro culture, monocytes underwent pronounced changes in cell size and morphology. Cytocentrifuge preparations of cultured monocytes showed that monocytes continued to increase in size throughout the culture period, and by the end of seven days, many cells had more than doubled in apparent size. Occasional binucleated cells were also seen. As monocytes increased in size, their cytoplasm became heavily vacuolated, and cytoplasmic granules also became evident. Further culture resulted in increased formation of multinucleated giant cells.

We have shown previously that serum-free conditioned media from 24-hour cultures of human peripheral blood monocytes contains a specific inactivator of human urokinase-type plasminogen activators (Fig 1), which we have characterized and named minactivin. Minactivin was shown to be a product of washed, adherent monocytes and was not produced by cultures of the nonadherent mononuclear cells. Activation of monocytes in vitro by muramyl dipeptide or bacterial lipopolysaccharide led to sustained production and secretion of minactivin (Fig 2A and B).

Human peritoneal macrophages. High yields of mononuclear cells were obtained from Ficoll-Paque gradients of peritoneal washings. Forty-seven percent of the cells were macrophages as judged by morphological criteria. Cytocentrifuge preparations of the mononuclear cell fraction showed that the macrophage population was heterogeneous and included large mature cells with abundant cytoplasm and
occasionally two nuclei, as well as smaller cells more characteristic of monocytes, with indented nuclei and a higher nucleus-cytoplasm ratio.

Peritoneal macrophages were further purified by adherence to plastic culture trays. After removal of the nonadherent cell layer, viable peritoneal macrophages could be maintained in culture for up to three weeks, provided that the culture media was changed every two to three days. Peritoneal monolayers were routinely greater than 87% macrophages, as determined by nonspecific esterase staining, and produced high specific activities of the macrophage marker enzymes N-acetyl β-D glucosaminidase, acid phosphatase, and β-glucuronidase, thus confirming their purity and viability.

Although lysates of freshly isolated blood monocytes did not contain minactivin (see Fig 2B), all the lysates of freshly isolated adherent peritoneal macrophages studied contained significant levels of minactivin (approximately 30% inhibition), which subsequently increased during culture (Fig 3). High levels of production of minactivin were also reflected in the output of secreted minactivin found in culture supernatants. Unlike control monocyte cultures, peritoneal macrophage lysate minactivin levels remained high during the three-day culture period. Moreover, addition of muramyl dipeptide to peritoneal macrophage cultures did not further increase minactivin activities in either the lysates or the supernatants (Fig 3).

**Bone marrow-derived macrophages.** Bone marrow macrophages were obtained as the adherent cells after seven days of GCT (giant cell tumor culture supernatant) stimulated growth of primary marrow cultures. In this population, 90% of the cells phagocytosed opsonized zymosan. Because these cells could only be studied as secondary cultures after the seven-day primary culture of the tissue, their initial minactivin production and secretion could not be compared directly with freshly isolated blood monocytes or peritoneal macrophages.

Not unexpectedly, after primary culture for seven days, the lysates of adherent bone marrow macrophages obtained during secondary culture showed high activities of minactivin, which declined regardless of the presence or absence of muramyl dipeptide (Fig 4).

The secondary cultures of bone marrow cells also secreted high activities of minactivin into supernatants (Fig 4), but as with lysates, this level was not affected by muramyl dipeptide.

**Colonic mucosal macrophages.** Macrophages isolated from enzymically disaggregated mucosa of resected colon were greater than 85% nonspecific esterase positive, phago-
cytosed sheep red blood cells, and, as shown previously, produce the constitutive macrophage marker enzyme lysozyme. These cells had only limited ability to produce minactivin in culture, however, and the levels were insignificant compared with those from the three sources above (mean value of 4% inhibition for 24-hour supernatants in four experiments). These low levels were not increased when S. minnesota lipopolysaccharide (0.1 μg/mL) was added to cultures.

To determine whether this lack of responsiveness of intestinal macrophages could be attributed to the prolonged exposure to the degradative enzymes (collagenase and DNase) used in their isolation, some normal human blood monocytes were subjected to several hours incubation in the supernatant medium from an intestinal macrophage preparation. The enzyme-treated blood monocytes retained their ability to produce and secrete minactivin in subsequent culture (see Table I).

### Specificity of the Products From Cell Cultures

The specificity of the supposed minactivin product from the cells of the different tissue compartments was compared with that of monocyte minactivin, which we have previously shown to have a high specificity for human urokinase-type plasminogen activators. A fibrin radial diffusion assay was used to test for inhibition of a selection of proteases and activators, including plasmin, trypsin, HPA66 ("tissue-type" activator from human melanoma culture supernatant), and human urokinase (containing HPA52 and HPA36).

Culture supernatants from peritoneal macrophages and blood monocytes showed specific inhibition of urokinase activity (Fig 5) but did not inhibit the activities of plasmin, trypsin, or HPA66.

Triton lysates of peritoneal macrophages, blood monocytes, and bone marrow macrophages all produced inhibition of urokinase, without inhibition of plasmin or trypsin. Lysates of blood monocytes and peritoneal macrophages also showed some weak inhibition of HPA66 (see Fig 5).

### Independence of Minactivin and Plasminogen Activator Production

By making use of the salt sensitivity of the endogenous plasminogen activator, minactivin and plasminogen activator could be assayed independently in culture supernatants and in Triton lysates from blood monocytes, peritoneal macrophages, and bone marrow. Their simultaneous occurrence was inconsistent with minactivin serving as a direct regulator of plasminogen activator from cells of the macrophage lineage. Further evidence to support this view was obtained when blood monocyte cultures were treated with dexamethasone (10^{-7} mol/L). While minactivin activities in dialyzed culture supernatants and lysates did not change significantly (Fig 6A), plasminogen activator activity was abolished (see Fig 6B), as earlier reported for human neutrophils.

### DISCUSSION

The results extend our previous findings on the human monocyte product, minactivin, which we have characterized from normal human blood monocytes. Minactivin is able to inactivate specifically human urokinase-type plasminogen activators and may have significant actions in regulating extracellular proteolysis during physiologic or pathologic events of tissue remodeling and invasion.

Human peritoneal macrophages were found to contain minactivin when freshly isolated and to go on producing and secreting it in vitro in appreciable quantities. This cell population appeared from its morphology to consist largely of newly recruited monocytes, indicating that the in vivo stimulus of mild inflammation induced by repeated dialysis was sufficient to recruit blood monocytes into the peritoneum and activate them to produce minactivin within the perito-
Mature tissue macrophages obtained after seven-day primary culture were not responsive to further activation, since muramyl dipeptide did not increase minactivin production or secretion in vitro.

Peripheral macrophages, in contrast, were responsive to further activation, since muramyl dipeptide increased minactivin production in vitro.

Bone marrow-derived macrophages were also able to produce appreciable levels of minactivin, although the cells obtained after seven-day primary culture were unresponsive to muramyl dipeptide. Mature tissue macrophages obtained from human colonic mucosa appeared incapable of producing minactivin in vitro.

Putting these results together, blood monocytes can be seen to be at the optimal stage of development for potential secretion of minactivin, a potential that is realized when they are activated with muramyl dipeptide in vitro or actively recruited to an inflammatory site in vivo, such as the peritoneal macrophages above. Mature tissue macrophages, however, isolated from intestinal mucosa contained little or no minactivin on isolation and could not be stimulated to produce or secrete it in vitro.

The relationship between minactivin and the other human monocyte/macrophage product, plasminogen activator, was also of interest. In these studies and previously reported work on blood monocytes, it was shown that, under conditions of low salt, monocyte plasminogen activator can be assayed in the presence of minactivin, which is inhibitory when the sample is added to an assay of urokinase. Minactivin, therefore, does not appear to function as a direct regulator of monocyte plasminogen activator. This was also suggested by experiments with dexamethasone, which indicated that steroid repression of monocyte plasminogen activator was mediated by a minactivin-independent mechanism, so that the synthesis of these two products may be regulated independently.

On the basis of these results we propose that minactivin may be a useful indicator of inflammatory activity in human tissues and possibly an active participant in the host-v-tumor reaction.

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