Acid Phosphatase Activity in Mononuclear Phagocytes and the U937 Cell Line: Monocyte-Derived Macrophages Express Tartrate-Resistant Acid Phosphatase

By Rose G. Snipes, K.-W. Lam, Richard C. Dodd, T. Kenney Gray, and Myron S. Cohen

Tartrate-resistant acid phosphatase (TRAcP) is used as a marker for osteoclasts, which are believed to be derived from phagocytic cells or phagocyte stem cell precursors. To further investigate the relationship between mononuclear phagocytes and osteoclasts, acid phosphatase (AcP) activity was measured by three different techniques in human peripheral blood monocytes, monocyte-derived macrophages, and the U937 cell line. We found that cytochemistry and gel electrophoresis led to similar results, but that the colorimetric assay was inconsistent. Normal human peripheral monocytes expressed both tartrate-sensitive and -resistant AcP. In culture these cells formed polykaryons and expressed TRAcP activity that was further identified as an isoenzyme associated with bone tissue. In contrast, the U937 cells did not express TRAcP activity as measured by gel electrophoresis. Both U937 cells and monocytes possess material that interferes with interpretation of the colorimetric assay of AcP. The presence of TRAcP in monocyte-derived macrophages further supports the relationship between phagocytic cells and bone osteoclasts.

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Isolation and Preparation of Cells

Human monocytes. Venous blood was obtained from normal volunteers consistent with the guidelines of the Human Rights Committee of the University of North Carolina at Chapel Hill. Human monocytes were prepared as previously described by placing heparinized whole blood on a Plasmagel (Cellular Products, Buffalo) sedimentation gradient. The white cell layer was removed, centrifuged, and the pellet diluted in phosphate-buffered saline and placed on a Ficoll-Hypaque gradient (specific gravity 1.077, Sigma Chemical Co, St Louis, and Winthrop Laboratories, New York). Cells were centrifuged at 150 g for 35 minutes. The mononuclear layer was removed, centrifuged at 200 g for 10 minutes, and subsequently washed three times in alpha-modified minimal essential medium (alpha MEM, Gibco, Grand Island, NY). Cells were enumerated in a Coulter Counter (Coulter Electronics, Hialeah, Fla). As judged by nonspecific esterase staining, greater than 90% of the cells were monocytes. The cells were plated in 35 × 100-mm Falcon 3001 plastic Petri dishes (Falcon Labware, Oakland, Calif) at a density of 3.5 × 10⁶ monocytes/mL. Cell viability was >90% as determined by trypan blue exclusion. Cell culture media consisted of alpha MEM supplemented with 10% autologous human serum, 15 mmol/L HEPES, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL). After two hours of incubation at 37 °C in humidified 5% CO₂, the nonadherent cells and overlying media were removed and the plates washed three times with Hank’s balanced salt solution (HBSS). Fresh media containing variables to be examined were then added to the monolayer, and cells were incubated at 37 °C for three to seven days. Media were changed after three days of incubation.

U937 cells. U937 cells obtained from the tissue culture facility of the Lineberger Cancer Research Center (UNC-CH) were plated at 2 × 10⁶ cells/mL in Dulbecco’s modified MEM/Ham’s F-12

Materials and Methods

Reagents

1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) was obtained as a gift from Milan Uskokovic, PhD, Hoffmann-LaRoche, Nutley, NJ. The 1,25(OH)₂D₃ was dissolved in 100% ethanol and added to cell cultures to give a final ethanol concentration of 0.1%. In all experiments, appropriate controls were performed to exclude an ethanol effect.

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medium (GIBCO) that was supplemented with 5% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL).

Acid Phosphatase Determinations

Cytchemistry. Human monocytes were isolated as previously described, placed on tissue culture slides (LAB-TEK, Miles Scientific, Naperville, Ill), and incubated for the desired length of time. At the end of the incubation the media were removed. The slides were washed and air dried. U937 cells were suspended in culture media as previously described. At the end of the desired incubation period, cytopreparations were made of the cell suspensions by centrifugation at 1,500 rpm for five minutes (Shandon-Elliott Cyto spin, Sewickley, Pa). Cytopreparations of U937 cells and adherent monocytes were air dried, fixed, and stained as described by Janckila et al.

Colorimetric determination. Human monocytes were incubated the desired length of time, and the culture media were removed. Plates were washed twice with HBSS. The adherent monolayer was then disrupted with a rubber policeman in a 1-mL vol of 10 mmol/L N-tris(2-hydroxymethyl)methyl 2-aminoethanesulfonic acid (TES) buffer. Cells disrupted by sonication and suspended in buffer were used in the biochemical assay. U937 cells were cultured for the desired period of time and washed three times in HBSS. The cell pellet was diluted to a concentration of 5 x 10⁶ cells/mL in 10 mmol/L TES (pH 7.4). This suspension was sonicated for 30 seconds to form an extract. The extract was then centrifuged at 150 g for ten minutes. The supernatant was removed and used in the biochemical analysis. U937 cells treated with 1,25(OH)₂D₃ required removal with a rubber policeman. The cells were then counted and prepared as described for the immature U937 cells.

Cell extracts (0.1 mL) were mixed with an assay mixture containing 9.2 mmol/L para-nitrophenylphosphate (P-NPP, Sigma) and 0.1 mol/L acetate buffer (pH 6) in a final volume of 0.60 mL. For the determination of TRAcP, the reaction mixture included 10 mmol/L l-sodium tartrate (Mallinkrodt, Inc). The reaction was stopped by the addition of 2.5 mL 0.05 mol/L NaOH. The color was measured at 410 nm in a Beckman Acta V spectrophotometer (Beckman Instruments, Inc, Fullerton, Calif). A Lowry protein assay was run simultaneously on cell extracts. The amount of hydrolyzed P-NPP was then calculated by comparing the absorbance of the sample to a standard solution of P-NPP. Enzyme activity is defined as nanomoles of substrate hydrolyzed per minute (milli-units). L-ascorbic acid (10⁻³ mol/L) and Fe²⁺ (10⁻⁴ mol/L) were also added to some of the cell extracts containing l-sodium tartrate.

Isoenzymes determination. Human mononuclear phagocytes and U937 cells were cultured for the desired period of time. Media from phagocytes and U937 cells were removed and culture dishes were washed vigorously with HBSS to remove residual serum. Cell extracts were analyzed by electrophoresis on an acidic acrylamide gel as previously described by Lam et al.

RESULTS

Histochemistry of AcP Activity

Monocytes isolated from the peripheral blood of normal human volunteers were separated from lymphocytes by two

Fig 1. Light microscopic examination of phagocytic cells. TRAcP activity is represented by red stain. (A) Peripheral blood monocytes at two hours of culture have no tartrate-resistant activity (original magnification × 10). (B) After three days of culture, human monocytes demonstrate TRAcP activity. The activity is most intense in multinucleated giant cells (original magnification × 10). (C) Close-up of three-day giant cell with strong TRAcP activity (original magnification × 100). (D) Replicating U937 cells demonstrate no TRAcP activity (original magnification × 40). (E) In the presence of 1,25(OH)₂D₃, a speckled pattern is seen that appears to represent extracellular enzyme activity (original magnification × 40).
ACID PHOSPHATASE EXPRESSION IN MONOCYTES

hours of adherence and examined using standard staining technique for total and TRACP activity. As shown in Fig 1A, AcP expressed by these cells was inhibited by Na tartrate. After three days of incubation, monocytes developed the appearance of monocyte-derived macrophages, and an increased number of multinucleated giant cells was noted. All cells demonstrated TRACP activity, which was most intense in multinucleated giant cells (Fig 1B and C). Cells cultured over a seven-day period continued to express this activity.

We and others have recently demonstrated a variety of effects of 1,25(OH)2D3 on the U937 cell line. This cell line also resorbs devitalized bone chips in vitro. We examined the cytochemical AcP activity of this cell line under conditions similar to normal monocytes. Replicating (immature) U937 cells demonstrated tartrate-sensitive AcP activity (Fig 1D). Cells incubated for three days with 1,25(OH)2D3 (10⁻⁸ mol/L) showed increased maturation, as has been previously reported. However, tartrate-sensitive AcP activity continued to be the primary enzyme expressed. A few cells demonstrated TRACP activity in a speckled pattern near the cell membrane (Fig 1E).

Colorimetric Analysis

AcPs hydrolyze p-nitrophenol phosphate rapidly at a pH of 5 to 5.5. Peripheral blood monocytes demonstrated both tartrate-sensitive and tartrate-resistant AcP activity (Table 1). Monocytes in culture for three to seven days developed an increase in this activity (Table 1). Most of the activity was resistant to tartrate. Immature U937 cells demonstrated less AcP than human monocytes. U937 cells were differentiated by exposure to 1,25(OH)2D3, but only small differences were noted in AcP activity (Table 1). These results conflicted with the cytochemical examination and prompted further analysis of this enzyme activity.

Isoenzyme Expression of AcP

AcPs isolated from human tissues migrate as five distinct bands on gel electrophoresis, and these bands most likely represent distinct isoenzymes. TRACP can be isolated in high concentration from bone. Although the cell of origin of this enzyme has not been as yet identified.

Table 1. AcP Expression by Phagocytic Cells (μU P-NPP Hydrolyzed/mg Protein)

<table>
<thead>
<tr>
<th></th>
<th>AcP</th>
<th>TRAcP</th>
<th>Percentage of TRAcP</th>
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<tbody>
<tr>
<td>U937 cells</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(replicating)</td>
<td>9.6 ± 0.34</td>
<td>8.3 ± 0.3</td>
<td>86</td>
</tr>
<tr>
<td>U937 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(differentiated)</td>
<td>13.7 ± 0.36</td>
<td>10.6 ± 0.22</td>
<td>77</td>
</tr>
<tr>
<td>Monocytes</td>
<td>16.7 ± 5.9</td>
<td>13.9 ± 6.6</td>
<td>83</td>
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<tr>
<td>Monocyte-derived</td>
<td></td>
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<tr>
<td>macrophages</td>
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<tr>
<td>(three days in culture)</td>
<td>36.2 ± 3.8</td>
<td>25.3 ± 2.4</td>
<td>70</td>
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</tbody>
</table>

*Phagocytes were cultured for three days in the presence of 10⁻⁸ mol/L 1,25(OH)2D3.

Results are the mean ± SD of three to four separate experiments, each performed in duplicate.

DISCUSSION

TRACP can be isolated in high concentration from bone. Although the cell of origin of this enzyme has not differ only by a carbohydrate moiety present on 5a, which can be removed by sialidase. Band 5b can also be isolated from osteoclasts, the neoplastic cells of leukemia reticuloendotheliosis, and phagocytes of Gaucher’s disease. Freshly isolated monocytes studied by gel electrophoresis demonstrated only band 4 (tartrate-sensitive) activity, as previously reported (Fig 2). Monocyte-derived macrophages cultured for three to seven days expressed band 5b (Fig 3). Monocytes incubated with 1,25(OH)2D3 also demonstrated band 5b on gel electrophoresis (data not shown).

In the U937 cell, only band 4 was detected, whether the cells were replicating or not. Band 4 activity was greater in the U937 cells incubated with 1,25(OH)2D3. Band 5b was not observed under these conditions (Fig 4). These results supported our cytochemical observations (Fig 1). To further evaluate the AcP activity of U937 cells, a cellular extract was eluted over a diethylaminoethyl (DEAE) cellulose column (Fig 5). Over 90% of the AcP activity eluted as a single peak that was tartrate sensitive and migrated on gel electrophoresis as band 4. Band 5b was not observed in this cell extract even after the addition of ferrous iron and ascorbic acid. These compounds are known to enhance the expression of the TRACP. The substance(s) responsible for the TRACP activity observed in the colorimetric assay of the crude U937 extract and freshly isolated human monocytes has not been as yet identified.
been clearly defined, morphologic evidence suggests that TRAcP is associated with the osteoclast.\textsuperscript{17} TRAcP is routinely used to identify osteoclasts isolated from bone or osteoclastlike cells in culture.\textsuperscript{1,5,10} Several lines of evidence suggest that osteoclasts are derived from mononuclear phagocytes, although it is unclear whether peripheral blood monocytes\textsuperscript{30,35} or stem cells\textsuperscript{32,33} are required for such differentiation. In the present study we evaluated the AcP activity of peripheral blood monocytes, monocyte-derived macrophages, and the U937 cell line, a model for monocytic phagocytes.\textsuperscript{21} Our methods included cytochemistry, colorimetric analysis, and gel electrophoresis. Cytochemical and colorimetric assays are widely used, and can be confirmed by gel electrophoresis\textsuperscript{18} or immunoassay.\textsuperscript{24}

Cytochemical analysis revealed only minimal TRAcP activity in freshly isolated normal human peripheral blood monocytes, a finding consistent with previous reports.\textsuperscript{3,27,35} The strong TRAcP in cultured monocytes is consistent with recent observations by Weinberg and colleagues who demonstrated TRAcP activity in polykaryons formed from peripheral blood monocytes cultured in the presence of interferon-\gamma.\textsuperscript{36}

U937 cells replicate in culture, remain in suspension, and are immature monocytes by several criteria.\textsuperscript{21} These cells expressed only tartrate-sensitive cytochemical AcP activity. Cells incubated in the presence of 1,25(OH)\textsubscript{2}D\textsubscript{3}, a known inducer of differentiation, occasionally expressed a speckled pattern of TRAcP activity. This pattern appeared to be located at the plasma membrane and may represent the absorption of the enzyme from serum in the culture medium.\textsuperscript{8}

A colorimetric assay demonstrated TRAcP activity in both monocytes and U937 cells. This finding differed from the results derived from the cytochemical assay. To resolve this discrepancy, extracts from monocytes and U937 cells were evaluated by gel electrophoresis. Only band 4 of the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image1}
\caption{Gel electrophoresis of monocyte-derived macrophages (three days) in which band 5b activity is seen.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image2}
\caption{Gel electrophoresis of U937 cells: (A) Replicating cells have only band 4 activity. (B) In the presence of 1,25(OH)\textsubscript{2}D\textsubscript{3} band 4 activity is enhanced. Band 5 activity cannot be demonstrated.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image3}
\caption{The U937 cell was subjected to sonication for one minute then centrifuged for 15 minutes at 20,000 g. The insoluble particulate fraction was discarded. The soluble proteins in the supernatant fraction were transferred to a DEAE-cellulose column (0.9 x 30 cm), and eluted by a linear concentration gradient of NaCl-10 mmol/L Tris-sulfate, pH 8. The eluate was analyzed for protein concentration and AcP activity by the colorimetric method. Most of the AcP activity was recovered in one peak corresponding to band 4 as detected by the electrophoresis method.}
\end{figure}
ACID PHOSPHATASE EXPRESSION IN MONOCYTES

AcP isoenzymes was observed in the freshly isolated monocytes. Monocyte-derived macrophages expressed band 5b. The U937 cells showed only band 4, which increased after exposure to 1,25(OH)2D3. Anderson and Toverud previously reported that iron and ascorbic acid enhanced detection of TRAcP activity. No band 5b was seen in the U937 cells, even in the presence of ferrous iron and ascorbic acid. Ninety percent of the total AcP activity in the U937 cells was isolated chromatographically and identified as band 4.

These results have several important implications for the study of AcP. It seems clear that the various assays available for the detection of acid phosphatase do not uniformly agree. In our hands the cytochemical assay correlated best with the results of gel electrophoresis. Although the latter technique is relatively cumbersome, the results are more specific than for the colorimetric assays frequently used. Phagocytic cells appear to possess substance(s) that interfere with use of the colorimetric identification of TRAcP.

Isoenzyme 5b was not observed in the U937 cell line even though it is an established monocytic cell model. Band 5b, an isoenzyme characteristic of osteoclasts, was expressed in monocyte-derived macrophages. These findings demonstrate a biochemical link between the monocyte-derived macrophages and osteoclasts.

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REFERENCES

Acid phosphatase activity in mononuclear phagocytes and the U937 cell line: monocyte-derived macrophages express tartrate-resistant acid phosphatase

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