RAPID COMMUNICATION

P2Z Adenosine Triphosphate Receptor Activity in Cultured Human Monocyte-Derived Macrophages

By Suzanne E. Hickman, Joseph El Khoury, Steven Greenberg, Ira Schieren, and Samuel C. Silverstein

The present study shows that human mononuclear phagocytes express a P2Z-like purinergic membrane receptor activity. Extracellular adenosine triphosphate (ATP) induces the formation of nonselective membrane pores in human mononuclear phagocytes that allow the entry of otherwise membrane impermeant fluorescent dyes (YO-PRO-1 or Lucifer yellow) into the cytoplasm of these cells. The percentage of mononuclear phagocytes that was permeabilized by ATP increased as monocytes matured into macrophages. Their response to ATP was inhibited by Mg²⁺ and oxidized ATP. Benzoylbenzoic-ATP (BzBzATP) was approximately 60% as effective as ATP and adenosine-5'-O-(thiophosphate) (ATP-γS) was less than 20% as effective as ATP in permeabilizing human macrophages to YO-PRO-1 or Lucifer Yellow. Thus, the human P2Z-like receptor differs from its murine counterpart because BzBzATP, ATP, and ATP-γS are equally efficacious in permeabilizing murine macrophage-like J774 cells to these dyes. UTP, GTP, and CTP were ineffective in permeabilizing human or murine macrophages to YO-PRO-1. Taken together, these data indicate that human monocyte-derived macrophages express a P2Z-like activity that is pharmacologically distinct from that expressed by their murine counterparts and that expression of these receptors is developmentally regulated in human mononuclear phagocytes.

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EXTRACELLULAR adenosine triphosphate (ATP) mediates a variety of responses in many cell types. These responses include altered contraction in smooth muscle cells,1 neurotransmission,2,3 release of endothelium-derived relaxing factor (EDRF),4,5 cell proliferation in fibroblasts,5 apoptosis in thymocytes,6 degranulation in mast cells,8 and chloride secretion in human airway epithelial cells.7 A distinct effect of the free acid form of ATP (ATP⁺) is permeabilization of the plasma membranes of several types of cells to molecules less than 900 daltons.10 Cellular responses to ATP are mediated by plasma membrane nucleotide receptors, termed purinergic receptors. Pharmacologic studies indicate that there are at least three classes of ATP receptors. P2U/P2Y type receptors are G-protein-coupled receptors that trigger release of intracellular Ca++. through activation of phospholipase C.11,12 P2X receptors directly activate ATP-activated cation channels.13,14 P2Z receptors mediate the opening of large "pores" in plasma membranes, making cells permeable to ions and metabolites up to 900 daltons.10,11 P2Z receptors are present on rat mast cells,8 murine peritoneal macrophages,10 and several murine-transformed cell lines, including J774 and BAC1.2F5 macrophage-like cell lines.10,15 Uptake of membrane impermeant dyes such as Lucifer yellow or ethidium bromide in response to ATP has been used to assess P2Z receptor activity in mouse peritoneal macrophages and in J774 cells.10,16,17 However, it is not known whether human mononuclear phagocytes express P2Z receptor activity. This study presents evidence for the expression of P2Z-like receptor activity in human monocyes maintained in culture, which increases during their differentiation into macrophages.

MATERIALS AND METHODS

Materials. ATP, ADP, adenosine, adenosine-5'-O-(3-thiophosphate) (ATP-γ-S), adenylyl-imidodiphosphosphate (AMP-PNP), adenylyl (β,γ-methylene)-diphosphosphate (AMP-PCP), UTP, GTP, and CTP were obtained from Boehringer Mannheim Corp (Indianapolis, IN). Benzoylbenzoic adenosine 5'-triphosphate (BzBzATP) and iodate oxidized ATP (Ox-ATP) were obtained from Sigma Chemical Co (St Louis, MO). Lucifer yellow and YO-PRO-1 dyes were purchased from Molecular Probes (Eugene, OR).

Cells. Human monocytes were isolated from leukeocyte concentrates (New York Blood Center, New York, NY) by centrifugation over Ficoll hypaque (Sigma) as described.18 The mononuclear cell layer was collected, washed three times in RPMI 1640, and resuspended in the same medium supplemented with 20% pooled human serum (Gemini, Calabasas, CA). The cells were allowed to adhere to tissue culture flasks (Falcon Labware, Oxnard, CA) for 1 hour in the same medium at 37°C. Nonadherent cells were washed away. For some experiments, the adherent cells were detached by a brief incubation with phosphate-buffered saline (PBS) containing 10 mmol/L EDTA at 4°C followed by tapping the side of the dish as described.18 To obtain monocyte-derived macrophages, monocytes separated as above were cultured in RPMI 1640 supplemented with 30% pooled human serum in Teflon beakers, as described.19 In other experiments, the adherent cells were maintained in tissue culture flasks in RPMI media supplemented with 30% human serum and detached with 10 mmol/L EDTA in PBS the day they were used. This method yielded greater than 90% monocytes as evidenced by nonspecific esterase staining and by the ability of the recovered cells to engulf IgG-coated sheep red blood cells (RBCs).17 J774 cells were maintained in spinner flasks in Dulbecco's medium supplemented with 10% bovine serum, penicillin (100 U/mL), and streptomycin sulfate (0.1 mg/mL).
Response to ATP. Human monocytes (freshly isolated or day 1 of culture) or monocyte-derived macrophages (MACs) cultured in Teflon beakers or detached after maintenance in tissue culture flasks were rinsed in RPMI without serum, resuspended in RPMI, plated onto micro-spot slides (Shandon, Pittsburgh, PA) coated with human serum albumin (HSA; 1.0 mg/mL), and incubated for 1 to 2 hours at 37°C. Where indicated, assays were also performed in suspension. Cells were removed from Teflon beakers or detached from monolayers, rinsed in RPMI, and resuspended in 0.1 mL of assay buffer. J774 cells were used as positive controls and were treated in the same way as human cells.

Uptake of Lucifer yellow or YO-PRO-1. Cells plated on HSA-coated slides were rinsed twice in HEPES-buffered saline (HBS; 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L KH₂PO₄, 5 mmol/L glucose, 1 mmol/L NaHCO₃, and 20 mmol/L HEPES, pH 7.6) containing 0.2 mmol/L CaCl₂ and 0.2 mmol/L MgCl₂. The cells were subsequently incubated HBS containing 5 mmol/L ATP or the indicated nucleotides, 5 mmol/L probenecid, and 2 mmol/L Lucifer yellow (LY) for 10 minutes at 37°C. Control cells were incubated in the same solution without ATP. Because LY (457 molecular weight [MW]) is a substrate for the organic anion transporter present in J774 cells, probenecid was included in all buffers used in the assay to prevent efflux of LY. For uptake studies with YO-PRO-1, the cells were incubated in HBS in the presence or absence of 5 mmol/L ATP or the indicated nucleotides and 10 μmol/L YO-PRO-1. Cells were washed three times in HBS containing 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂ (and 5 mmol/L probenecid for LY studies) or HBS containing 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂ for assays with YO-PRO-1. Viability was assessed by incubation for 2 minutes in the appropriate rinse buffer containing 1 μg/mL propidium iodide (PI). Uptake of LY or YO-PRO-1 was assessed by fluorescence microscopy. Cells containing PI alone or PI/YO-PRO-1 were classified as nonviable and were excluded from further consideration. Data are expressed as % YO-PRO-1 positive = no. of cells containing YO-PRO-1/total no. of viable cells. Cell viability exceeded 95% in all assays. For each experimental condition, a minimum of 200 cells and a maximum of 1,000 total cells were counted.

For assays on cells in suspension, cells were rinsed as described and resuspended in 0.1 mL assay buffer. Buffer containing 2× concentration of dye and 10 mmol/L ATP or the indicated nucleotides was added to the cells. Subsequent incubations and rinses were performed as described above. Cells were plated on slides and allowed to settle for 20 minutes at 37°C before microscopic examination.

Flow cytometry. Aliquots of cells were removed from Teflon beakers and assayed in suspension in medium containing YO-PRO-1 and, where indicated, ATP, as described above. Flow cytometry was performed using an EPIC 753 flow cytometer (Coulter Electronics, Hialeah, FL) at an excitation wavelength of 488 nm and band pass filters to collect emissions between 515 and 550 nm. Nonviable cells positive for PI were gated out and represented less than 3% of the total population. Lymphocytes and cell debris were also excluded from the analysis with light scatter gates. Data were collected and analyzed using the CICERO Data Acquisition System (Cytomation, Inc, Ft Collins, CO).

RESULTS AND DISCUSSION

Development of response to ATP in cultured human monocytes. To optimally assay P2Z receptor activity, we used the fluorescent dye YO-PRO-1, a membrane-impermeant cation (629 daltons) whose fluorescence increases 30X upon binding nucleic acids. Unlike LY, it is not a substrate for organic anion transporters that clear dye from the cytoplasm, thereby decreasing the fluorescent signal. Therefore, YO-PRO-1 is an ideal candidate for the sensitive detection of P2Z receptor activity. Monocytes were cultured for 1 to 16 days and were assessed for their ability to respond to ATP by uptake of YO-PRO-1 at different time points. The data presented represent results obtained with monocytes and monocyte-derived macrophages from two different donors, D and E (Fig 1). Nine percent to 11% of monocytes cultured for 1 day became permeable to YO-PRO-1 during treatment with ATP. By day 7 and 14 in culture, 41% and 50%, respectively, of the MACs became permeable to YO-PRO-1 during ATP treatment. These results were typical of the positive responses observed for monocytes and MACs from five different donors. Cells from donor E exhibited half-maximal response by day 2, whereas donor D cells did not reach this point until day 5. The three other donors tested in the time course experiments exhibited half-maximum P2Z response between days 4 and 5. In a separate set of experiments, freshly harvested adherent monocytes from four donors were assessed 1 hour after plating for uptake of YO-PRO-1 in response to ATP. P2Z receptor activity was detected in 13.3 ± 4.2% (mean ± SEM) of these cells.

ATP mediated an increase in YO-PRO-1 uptake that resulted in a diffuse fluorescent staining of the cytoplasm and intense staining of the nucleus (Fig 2), indicating that the dye entered the cytoplasm directly rather than through endocytosis.

MACs obtained from donor C incubated in YO-PRO-1 without ATP and with ATP were analyzed by flow cytometry (Fig 3). The MAC population was selected for analysis with light scatter gates. Cells in the MAC population represented 35% of viable cells. The mean fluorescence of cells incubated with ATP and YO-PRO-1 was 6.8-fold higher than cells incubated with YO-PRO-1 alone. There was no significant increase in fluorescence of cells treated with YO-PRO-1 alone compared with untreated cells.

Response of human culture derived macrophages to ATP analogues and other nucleotides. Rodent P2Z receptors re-
Human MACs cultured for 12 days respond to ATP by uptake of Yo-PRO-1. Phase contrast (b and d) and fluorescence (a and c) micrographs of human MACs treated with 5 mmol/L ATP and Yo-PRO-1. (a) shows uptake of Yo-PRO-1 by MACs treated with ATP (5 mmol/L). (c) represents cells from the same experiment incubated with Yo-Pro but without ATP. We compared the capacity of different ATP analogues to render 5774 cells and human MACs permeable to Yo-PRO-1 (Fig 4). ATP, BzBzATP, and ATPγS were equally effective in promoting entry of Yo-PRO-1 into 5774 cells (90% to 95%), whereas AMP-PNP was only 35% as effective as ATP (Fig 4). Adenosine, ADP, AMP-PCP, UTP, CTP, and GTP did not mediate uptake of Yo-PRO-1 in 5774 cells (data not shown). Responses to the analogue BzBzATP varied among donors. BzBzATP was as effective as ATP in increasing the permeability of MACs from donor C to Yo-PRO-1. However, BzBzATP was only 60% as effective as ATP in increasing permeability of MACs from donors D and E to these dyes (Fig 4). A striking difference between murine and human cells was seen in response to ATPγS. A maximum of 15% of MACs from donor E, 6% from donor D, and 3% from donor C became permeable to Yo-PRO-1 in response to ATPγS. In contrast, 90% of J774 cells became permeable.

Human MACs were analyzed between day 12 and 15 of culture. Forty percent to 50% of the MACs from all donors were permeabilized by ATP. Similar to results obtained with J774 cells, MACs did not take up Yo-PRO-1 in response to adenosine, ADP, AMP-PCP, UTP, CTP, or GTP (data not shown). Responses to the analogue BzBzATP varied among donors. BzBzATP was as effective as ATP in increasing the permeability of MACs from donor C to Yo-PRO-1. However, BzBzATP was only 60% as effective as ATP in increasing permeability of MACs from donors D and E to these dyes (Fig 4). A striking difference between murine and human cells was seen in response to ATPγS. A maximum of 15% of MACs from donor E, 6% from donor D, and 3% from donor C became permeable to Yo-PRO-1 in response to ATPγS. In contrast, 90% of J774 cells became permeable.

Fig 3. Flow cytometric analysis of cultured human MACs treated with ATP. MACs, 14 days in culture, were incubated in suspension with buffer containing Yo-PRO-1 with or without ATP, washed, and analyzed by flow cytometry. The figure shows overlaid histograms representing the fluorescent intensity profiles obtained for control (thin line) and ATP treated (thick line) MACs.

Fig 4. Effect of ATP analogs on MAC membrane permeability to Yo-PRO-1. Responses of MACs from three donors (donor C assayed on day 10; donors D and E tested on day 14) are compared with the responses of J774 cells. Cells were treated with 5 mmol/L ATP or 5 mmol/L ATP analogs as described, plated onto slides, and counted by fluorescent microscopy. Data shown are for a representative experiment using cells from three donors assayed in duplicate (variation among duplicates <10%). (■) Murine J774; (□) donor C; (△) donor D; (□) donor E.
Magnesium inhibits MAC response to ATP. Cells from donors A, C, D, and E were analyzed on days 10 through 16 of culture. Cells were incubated in buffer containing YO-PRO-1 and 5 mmol/L ATP, YO-PRO-1 alone, or YO-PRO-1/5 mmol/L ATP and 10 mmol/L MgCl₂. Assays were performed twice for each donor in duplicate (variation among duplicates was <10%). Data shown are for a representative experiment and are presented as a percentage of cells containing YO-PRO-1.

Inhibition of the ATP response by magnesium and oxidized ATP. P2Z receptors respond to the tetra-anionic form of ATP and ATP⁺, but not to MgATP⁺. The response of MACs to ATP was completely inhibited by the addition of 10 mmol/L MgCl₂ to the medium (Fig 5).

Irreversible inhibition of P2Z receptor activity in J774 is seen after preincubation of these cells with oxidized ATP. Cells from donors A and B (day 19 of culture) and J774 cells were plated onto HSA-coated slides and incubated with 1 mmol/L oxidized ATP for 2 hours. Cells were incubated subsequently with YO-PRO-1 with or without 5 mmol/L ATP. Similar to J774 cells, oxidized ATP markedly inhibited the subsequent response of human MACs to ATP (Fig 6). These findings suggest that the receptor on MACS that responds to ATP is a P2Z receptor.

Uptake of LY in response to ATP. Uptake of LY in response to ATP has been used to demonstrate P2Z receptor activity in J774 cells. We also performed experiments with LY to confirm that results with YO-PRO-1 are comparable to those obtained with LY. Forty percent of the MACs from donors A and B responded to ATP by uptake of LY. Uptake of LY by MACS in response to BzBzATP varied among the two donors, a phenomenon we also observed with YO-PRO-1. As expected, J774 cells became permeable to LY in response to ATP, ATP⁺S, and BzBzATP. However, less than 20% of human MACs became permeable to LY in response to ATP⁺S (Fig 7). MACS treated with ATP in the presence of LY exhibited diffuse cytoplasmic staining (Fig 8). This is similar to results obtained with J774 cells.

Both murine (J774 cells) and human (THP-1 cells) macrophage-like cell lines express P2Z receptors. Exposure of J774 cells to extracellular ATP causes release of intracellular K⁺, influx of extracellular Na⁺, and permeabilizes their plasma membranes to membrane impermeant dyes. If the ATP treatment is brief (<15 minutes), these cells regain membrane integrity and restore intracellularionic balance. However, when they are incubated for prolonged periods (>60 minutes) in ATP-containing medium, J774 cells are lysed. Blanchard et al report that treatment of human blood monocytes and THP-1 cells with interferon γ markedly increases their sensitivity to lysis by extracellular ATP and suggest that ATP’s lytic effect is mediated by P2Z receptors. Thus, their studies suggest that interferon γ induces P2Z receptor expression by human macrophages.

In summary, we have found that a small percentage of
human blood monocytes express P2Z receptor-like activity. This P2Z-like activity is similar pharmacologically to that reported for P2Z receptors of murine macrophages, differing only in the human cells’ relative unresponsiveness to ATPγS (Fig 4 and Blanchard et al15). An increasing percentage of human mononuclear phagocytes express this activity as they mature into macrophages in culture (Fig 1). Increased expression of P2Z receptor activity during differentiation of monocytes into macrophages suggests that this receptor, like the A2 adenosine receptor,4 is developmentally regulated in mononuclear phagocytes.

REFERENCES

P2Z adenosine triphosphate receptor activity in cultured human monocyte-derived macrophages

SE Hickman, J el Khoury, S Greenberg, I Schieren and SC Silverstein