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Platelet polyphosphate induces fibroblast chemotaxis and myofibroblast differentiation

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Abstract

Background: Platelets secrete many pro-wound healing molecules such as growth factors and cytokines. We found that releasates from activated human platelets induced the differentiation of cultured murine and human fibroblasts into a myofibroblast phenotype. Surprisingly, most of this differentiation-inducing activity was heat-stable, suggesting it was not due to the protein component of the releasates. Inorganic polyphosphate is a major constituent of platelet dense granules and promotes blood coagulation and inflammation.

Objectives: We aim to investigate the contribution of polyphosphate on myofibroblast differentiating activity of platelet releasates.

Methods: Using NIH-3T3 cells and primary human fibroblasts, we examined the impact of human platelet releasates and chemically synthesized polyphosphate on fibroblast differentiation and migration.

Results: We found that the myofibroblast-inducing activity of platelet releasates was severely attenuated after incubation with a polyphosphate-degrading enzyme, and that fibroblasts responded to platelet-sized polyphosphate by increased levels of α -smooth muscle actin, stress fibers, and collagen. Furthermore, fibroblasts were chemotactic toward polyphosphate.

Conclusions: These findings indicate that platelet-derived polyphosphate acts as a cell signaling molecule by inducing murine and human fibroblasts to differentiate into myofibroblasts, a cell type known to drive both wound healing and fibrosing diseases. Polyphosphate therefore not only promotes early wound responses through enhancing fibrin clot formation, but also may play roles in the later stages of wound healing — and, potentially, progression of fibrotic diseases — by recruiting fibroblasts and inducing their differentiation into myofibroblasts.

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Authorship Contributions

P.M.S. designed and performed the research, collected data, analyzed and interpreted the data, and wrote the paper. S.A.S. designed the protocol and generated platelet-sized polyphosphate and revised the paper. J.H.M. coordinated and conceived the study, acquired funding, interpreted the data, and revised the paper.

Conflict-of-interest disclosure: J.H.M., S.A.S. and P.M.S. are inventors on patents and patent applications for medical uses of polyphosphate and polyphosphate inhibitors.

Keywords

Blood platelets; Platelet activation; Fibroblasts; Myofibroblasts; Chemotaxis

Introduction

Polyphosphate is a simple inorganic molecule, consisting of linear polymers of phosphate groups that range in length from a few phosphates to over a thousand phosphates long.¹ Polyphosphate has been identified in all kingdoms of life, making it a remarkably conserved molecule.² The majority of polyphosphate studies have been performed in prokaryotes, where polyphosphate has roles in stress endurance, quorum sensing, virulence, phosphate storage, biofilm formation, and heavy metal resistance.^{3–9} In unicellular eukaryotes, polyphosphate has roles in germination, development, migration, nutrient homeostasis, proliferation, and differentiation.^{10–15}

Although the roles and modes of action of polyphosphate in mammalian cells are less understood, extracellular polyphosphate is an emerging regulatory molecule with recently identified functions including blood clotting, complement, biomineralization, inflammation, differentiation, and cell migration.^{16–25} Polyphosphate is contained in the dense granules of platelets, and upon platelet activation it is released into the microenvironment where it modulates coagulation and fibrinolysis by enhancing fibrin clot structure, acting as cofactor for the activation of factor XI by thrombin, accelerating factor V activation by factor XIa, and acting as cofactor for inactivation of alpha tissue factor pathway inhibitor (TFPI α) by FXIa.^{26–31} Platelet-derived polyphosphate acts as a pro-inflammatory and procoagulant molecule for endothelial cells by increasing adhesion molecule expression and von Willebrand factor secretion.^{17,18,32}

Polyphosphate released from activated platelets induces differentiation of monocytes into fibrocytes, a circulating cell type that can promote scar tissue.²⁴ These findings raise the possibility that polyphosphate secreted by activated platelets may act as a cell signaling molecule in wound healing for other cell types. Tissue resident fibroblasts are known to be recruited to wound or injury sites and to differentiate into myofibroblasts, which generate contractile force and secrete tissue matrix proteins to aid in wound closure and scar formation.³³ Myofibroblasts are also the primary effector cells driving fibrotic disease, in which the wound healing process becomes dysregulated in otherwise healthy organs or tissues, which in turn can lead to cellular dysfunction, organ failure, and death.^{34,35} There is currently no cure for fibrosis, which contributes to up to 45% of all deaths in the United States.³⁶ We now report that platelet polyphosphate is chemotactic for mouse and human fibroblasts, and that it potently induces their differentiation into myofibroblasts.

Methods

Materials

α -SMA antibody (14-9760-82), goat anti-mouse IgG2a secondary antibody (A-21133), Dulbecco's Modified Eagle Medium (DMEM), SYBR Green I, polycarbonate cell culture

inserts, ProLong Gold Antifade Mountant, and CyQUANT Cell Proliferation Assay were from Thermo Fisher Scientific (Waltham, MA). Bovine calf serum, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Tween 20, and Triton X-100 were from Fisher Scientific (Hampton, NH). Collagen 1 (84336) and vimentin (5741) antibodies were from Cell Signaling Technology (Danvers, MA). The PAR-1 selective activating peptide, TRAP (S1820), prostaglandin E1, theophylline, sodium pyrophosphate, and sodium tripolyphosphate were from Sigma-Aldrich (St. Louis, MO). Phalloidin-iFluor 647 (ab176759) was from Abcam (Cambridge, UK). Goat anti-rabbit IgG was from Jackson ImmunoResearch Labs (West Grove, PA). Poly-L-Lysine was from ScienCell Research Laboratories (Carlsbad, CA). L-glutamine, penicillin/streptomycin, and insulin-transferrin-selenium were from Corning (Corning, NY). The P2Y₁ receptor competitive antagonist MRS 2279 was from Tocris (Bristol, UK).

Narrowly sized-fractioned polyphosphate was produced from chemically synthesized polyphosphate by preparative PAGE as previously described.³⁷ Approximately platelet-sized polyphosphate, with a modal polymer length of 75 phosphates and a range of 20–280 phosphates, was prepared using differential acetone precipitation of P70 (a more heterogeneous, chemically synthesized polyphosphate preparation that was a kind gift from Dr. Thomas Staffel, BK Giulini GmbH). Briefly, to a stirred solution of 500 mmol/L P70 in 100 mmol/L NaCl, acetone was gradually added to a final concentration of 12.2% v/v. The precipitated polyphosphate was collected by centrifugation for 20 minutes at 13,000 × *g*, dried under nitrogen gas and dissolved in a solution of 20 mmol/L HEPES-NaOH buffer (pH 7.4). Polyphosphate concentrations were quantified as inorganic phosphate after acid hydrolysis as previously described.³⁸ In this paper, polyphosphate concentrations are given in terms of the concentration of phosphate monomer.

Cell culture

The murine embryonic fibroblastic cell line, NIH-3T3 (ATCC CRL-1658), and primary human dermal fibroblasts, GM1381 (Coriell Institute), were routinely cultured in DMEM supplemented with 10 mmol/L HEPES, penicillin, streptomycin, 4 mmol/L L-glutamine, and 10% bovine calf serum, in a 5% CO₂ environment at 37°C. Cells were maintained at less than 90% confluence and were cultured for no more than 2 weeks at a time. Experiments performed in serum-free media were cultured in DMEM supplemented as detailed above, without serum but with the addition of insulin-transferrin-selenium.

Platelet releasates

Recently expired platelet units were kindly provided by the University of Michigan Blood Bank. Platelet releasates were prepared as described.²⁹ Briefly, platelets were washed in Tyrode's buffer and suspended in the same buffer at 2.0×10^9 platelets/mL, then incubated with 5 μmol/L TRAP to activate the platelets. After 20 min, platelets were removed by centrifugation, and the protein content was estimated using a NanoDrop 1000 spectrophotometer (assuming an A₂₈₀ of 1 equals 1 mg/mL protein). Protein concentrations in such releasates averaged 415 ± 86 μg/mL (mean ± standard deviation). Releasates were diluted to 300 μg/mL protein in Tyrode's buffer containing 10 mmol/L EDTA (to inhibit phosphatases), then filter-sterilized and stored at –80°C until use. In some cases, releasates

were heated to 95°C for 30 minutes to denature proteins, then clarified by centrifugation for 10 minutes at $13,000 \times g$. (Polyphosphate is stable during this heat treatment.²⁹) In other cases, polyphosphate was degraded by incubating samples with recombinant yeast exopolyphosphatase (ScPPX1), as previously described.²⁹ Effects of platelet releasates on cell differentiation were tested by incubating adherent cells for 48 hours in medium with 1.5% serum plus twentyfold-diluted releasate (yielding a final releasate protein concentration of 15 µg/mL).

Immunofluorescence

For immunofluorescence studies, cells were seeded on glass coverslips at a density of 3.75×10^3 cells/cm² in DMEM containing 2.5% serum and allowed to adhere overnight. The next day, cells were treated in fresh medium (with 2.5% serum) containing the desired stimulant for varying times, after which cells were washed once with tris-buffered saline (TBS; 50 mmol/L Tris-Cl pH 7.5, 150 mmol/L NaCl) and fixed for 10 minutes at room temperature using 4% paraformaldehyde in TBS. Cells were washed three times with TBS, permeabilized in TBS containing 0.2% Triton X-100 for 5 min, then washed again three times with TBS. Cells were then blocked in TBS plus 0.05% Tween-20 and 5% bovine serum albumin for 1 hour at room temperature, after which they were incubated with primary antibody for 1 hour at room temperature in TBS plus 0.05% Tween-20 and 1% bovine serum albumin, and then washed three times with TBS. Cells were then incubated with secondary antibody plus SYBR green I (to stain DNA, 1:250,000 dilution) in TBS plus 0.05% Tween-20 and 1% bovine serum albumin for 1 hour at room temperature in the dark. Cells were washed three times in TBS, briefly rinsed with water, and mounted onto glass microscope slides using Antifade Mountant. Samples were imaged the next day or stored at 4°C in the dark. Primary antibodies were used at the following dilutions: α-SMA at 1:1000, COL1A at 1:500, and vimentin at 1:100. Secondary antibodies were typically diluted 1:500, while 647-conjugated phalloidin was diluted 1:1000. Images were obtained on a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). A minimum of twenty-five individual cells from a minimum of five representative images were analyzed for each experiment. Integrated density (defined as product of cell area and mean fluorescence intensity) was determined using ImageJ and the average integrated density for each experiment was determined.³⁹ Cell body area was quantified using ImageJ.

Migration assay

5×10^4 cells in DMEM were seeded in the top chamber of a NUNC polycarbonate 24-well transwell (Thermo Fisher Scientific, Waltham, MA) migration assay in which the top chamber insert contained an eight-micron porous membrane growth surface. Media with or without polyphosphate was added into the bottom chamber of the well to generate a polyphosphate gradient. Plates were incubated in a 5% CO₂ environment at 37°C for 20 hours, after which cells in the bottom chamber were collected, subjected to one freeze/thaw cycle and resuspended in RIPA lysis buffer. DNA was quantified in lysates using the CyQUANT proliferation assay using a SpectraMax i3x fluorescent microplate reader (Molecular Devices, San Jose, CA).

Results

Ability of platelet releasates to induce myofibroblast differentiation in NIH 3T3 cells is largely dependent on its polyphosphate content

To characterize platelet-secreted factors that promote wound healing, we examined the ability of platelet releasates to induce differentiation of cultured fibroblastic cells into myofibroblasts, as depicted by increased α -SMA levels. Figure 1A reports a significant increase in α -SMA levels in NIH-3T3 cells 48 hours after incubating the cells with platelet releasates in 1.5% serum, relative to cells cultured in the same medium without releasates. Releasates that had been heated to 95°C for 30 minutes exhibited only a slightly diminished ability to induce an increase α -SMA levels in NIH-3T3 cells, suggesting that the activity was not due to a protein. The inorganic polymer, polyphosphate, is an emerging signaling molecule that is not a protein or peptide, and is released from dense granules of activated platelets.²⁶ Furthermore, polyphosphate is relatively heat-stable under conditions that denature most proteins,¹⁷ so it seemed a good candidate for the differentiation-induce activity in platelet releasate. When platelet releasates were incubated with recombinant yeast exopolyphosphatase (ScPPX1) to degrade polyphosphate,⁴⁰ they lost most of their ability to increase α -SMA levels in NIH-3T3 cells. Finally, the combination of heat-treating the releasates followed by digestion with ScPPX1 completely destroyed their ability to induce an increase in α -SMA levels in NIH-3T3 cells (p value of 0.23). These data support the idea that the polyphosphate content of platelet releasates is essential for most of their ability to induce myofibroblast differentiation.

Platelet releasates also induce myofibroblast differentiation of primary human skin fibroblasts

NIH-3T3 cells are an established cell line derived from mouse embryonic fibroblasts. To examine whether polyphosphate could induce myofibroblast differentiation in primary human cells, we cultured normal human dermal fibroblast cells (GM1381)⁴¹ under the same conditions we used for NIH-3T3 cells. As with NIH-3T3 cells, incubating GM1381 cells with releasates from human platelets induced significant accumulation of α -SMA, while this activity was substantially reduced when the releasates were digested with ScPPX1 (Figure 1B). As with NIH-3T3 cells, heat-treating the releasates resulted in only a modest decrease in accumulation of α -SMA by GM1381 cells. GM1381 cells incubated with platelet releasates that had been both heat-treated and digested with ScPPX1 showed no significant increase in α -SMA over control cells. Taken together, these findings show that primary human dermal fibroblasts respond to platelet-derived polyP in a manner that is very similar to those of NIH-3T3 cells regarding induction of markers of myofibroblast differentiation.

Platelet-sized polyphosphate induces hallmarks of myofibroblast differentiation

To assess directly if polyphosphate can act as a signaling molecule for fibroblasts, we incubated NIH 3T3 cells with chemically synthesized polyphosphate (of approximately the size range released by activated platelets) in low-serum medium for 48 hours. As can be seen in Figure 2A, 5 μ mol/L platelet-sized polyphosphate induced a robust increase in α -SMA levels which localized along stress fibers, both of which are hallmarks of differentiation into myofibroblasts.^{33,35} We further found that concentrations of platelet-size polyphosphate

ranging from 0.1 to 25 $\mu\text{mol/L}$ resulted in significantly increased α -SMA levels and stress fiber formation (Figure 2B and 2C). These polyphosphate concentrations are well within those expected in the presence of activated human platelets.²⁷ In addition to increased α -SMA levels and stress fiber formation, fibroblasts increase in cell body size upon myofibroblast differentiation.⁴² Cells treated for 48 hr with 1 $\mu\text{mol/L}$ platelet-sized polyphosphate increased in cell body area (Figure 2D).

Polyphosphate of a variety of polymer lengths induces myofibroblast differentiation

The biological effects of polyphosphate can depend strongly on polymer length.³⁷ To evaluate the polymer length-dependence of polyphosphate's ability to induce myofibroblast differentiation, we incubated NIH-3T3 cells for 48 hours with a series of narrowly sized-fractioned polyphosphate preparations (all tested at 1 $\mu\text{mol/L}$) and evaluated α -SMA levels and stress fiber localization. Polyphosphate preparations ranging from 14 to 1100 phosphates long showed significant ability to induce α -SMA accumulation (Figure 2E), while polyphosphates ranging from 29 to 1100 phosphates in length supported increased stress fiber formation (Figure 2F). These polymer lengths encompass those secreted by activated platelets, mast cells and basophils, which are approximately 60 to 100 phosphates long, as well as those found in microbes, which can range up to more than 1000 phosphates long.

Platelet-sized polyphosphate induces myofibroblast differentiation of primary human fibroblasts

We next examined whether polyphosphate could induce myofibroblast differentiation in primary human fibroblasts. GM1381 cells were incubated for 48 hours with 1 or 5 $\mu\text{mol/L}$ platelet-sized polyphosphate. Polyphosphate induced significant increases in the levels of α -SMA, in a manner very similar to that observed with NIH-3T3 cells (Figure 2G).

Polyphosphate increases polymerized actin levels

To determine if platelet-sized polyphosphate induced an increase in F-actin levels, we stained polyphosphate-treated NIH-3T3 cells with phalloidin. Polyphosphate increased F-actin levels within 24 hours, and by 48 hours showed clear fibril localization (Figure 3A–B). This further suggests that polyphosphate promotes the formation of actin stress fibers in fibroblastic cells.

Polyphosphate increases collagen synthesis and vimentin levels

We next assessed if platelet-sized polyphosphate induced increases in other markers of myofibroblast differentiation. Myofibroblasts produce high levels of extracellular matrix proteins to propagate scar-tissue formation and promote re-epithelialization.³⁵ Polyphosphate nanoparticles have previously been shown to increase collagen levels in the osteoblastic cell line MC3T3-E1, as well as in vivo when topically applied to skin lesions of mice.⁴³ To determine if polyphosphate induces increased collagen synthesis in fibroblasts, NIH-3T3 cells were stained for collagen 1 alpha after incubation for 48 hours with platelet-sized polyphosphate. Polyphosphate induced an increase in collagen production (Figure 3C–D). The intermediate filament vimentin is a mesenchymal marker that is important in wound

healing, and increases upon myofibroblast differentiation.^{44,45} Polyphosphate caused an increase in vimentin staining (Figure 3E–F). These data suggest that polyphosphate increases the synthesis of collagen and vimentin, both of which are markers of myofibroblasts.

Polyphosphate is a chemoattractant for NIH-3T3 cells

Tissue-resident fibroblasts are recruited to wound sites preceding myofibroblast differentiation.^{33,35} Polyphosphate has been implicated in endothelial cell migration during bone and cartilage formation,⁴⁶ modulates leukocyte migration,⁴⁷ and acts as a chemoattractant for human neutrophils.²⁴ We therefore examined whether polyphosphate was a chemoattractant for NIH-3T3 cells by culturing NIH-3T3 cells in transwell migration plates with a gradient of polyphosphate. Polyphosphate at 1 or 5 $\mu\text{mol/L}$ enhanced NIH-3T3 migration toward the bottom chamber (Figure 4A). To test whether polyphosphate was a chemoattractant rather than simply enhancing motility, polyphosphate was incubated in the top, bottom, or both chambers of transwell migration plates. Cells incubated with polyphosphate in the top chamber remained in the upper chamber in higher numbers compared to untreated cells (Figure 4B). Polyphosphate incubated in both the top and bottom chambers did not induce directional cell migration, while polyphosphate in just the bottom chamber resulted in directional cell migration (Figure 4B). The purinergic receptor P2Y_1 mediates polyphosphate signaling in endothelial cells and astrocytes.^{32,48} Blocking P2Y_1 signaling with the high affinity competitive antagonist MRS 2279 effectively inhibited polyphosphate induced migration (Figure 4C). Thus, in addition to inducing fibroblast differentiation, polyphosphate may also play a role in fibroblast recruitment, mediated by the P2Y_1 receptor. In contrast, we found that MRS 2279 did not significantly diminish the ability of polyphosphate to induce α -SMA accumulation by NIH-3T3 cells (Figure 4D). This suggests that the differentiation-inducing ability of polyphosphate may act via different receptors than its chemotaxis-inducing ability.

Discussion

Here, we show that inorganic polyphosphate promotes myofibroblast differentiation in both the embryonic fibroblast-like cell line, NIH-3T3, and primary human dermal fibroblasts. Platelet-sized polyphosphate induced increased α -SMA expression, which localized along F-actin stress fibers, the two hallmarks of myofibroblast differentiation. Furthermore, polyphosphate promoted increased F-actin levels, collagen synthesis, and vimentin levels. These data suggest that polyphosphate activates fibroblasts to differentiate into a tissue-remodeling phenotype that promotes contractile force generation to aid in wound closure, and increases tissue matrix protein synthesis to generate scar tissue, both essential to wound resolution.

Polyphosphates over a wide range of sizes promoted myofibroblast differentiation, with 29 phosphate groups being the minimum length necessary, and polymer lengths of up to 1100 phosphates showing activity. Eukaryotic cells such as *Dictyostelium discoideum*, mast cells, basophils, and platelets secrete relatively small chains of polyphosphate containing under 100 phosphate groups.^{13,25,26} Prokaryotic organisms, on the other hand, contain

polyphosphates with more heterogeneous size distributions, often well over a thousand phosphate units in length.³⁷ It is therefore possible that microbial polyphosphate could also promote chemotaxis of fibroblasts and differentiation into myofibroblasts. While severe bacterial infection may be detrimental, low levels of bacterial colonization have actually been shown to be beneficial to wound healing.⁴⁹

Polyphosphate is an emerging pro-migratory molecule, enhancing migration of the social amoeba *Dictyostelium discoideum*,¹⁵ human endothelial cells,⁴⁶ and human leukocytes.^{24,47} Our finding that platelet-sized polyphosphate promoted chemotaxis of NIH-3T3 cells suggests that in addition to inducing differentiation into a pro-wound healing and pro-fibrotic phenotype, polyphosphate may act to recruit fibroblasts to sites of tissue injury. The purinergic receptor P2Y₁ mediates polyphosphate signaling in endothelial cells and astrocytes.^{32,48} Migration of NIH-3T3 cells induced by polyphosphate was also mediated by P2Y₁, adding further evidence that P2Y₁ may be a conserved receptor for polyphosphate in mammalian cells. In contrast, inhibiting P2Y₁ receptors did not appreciably diminish 3T3 cell differentiation into a myofibroblast phenotype, suggesting that this aspect of polyphosphate signaling uses different receptors.

Polyphosphate nanoparticles enhance wound healing in vivo, increasing collagen and α -smooth muscle actin (α -SMA) levels in the osteoblast-like cell line, MC3T3.⁴³ Here we show that polyphosphate synthesized and released by human platelets recapitulated the effects of chemically synthesized polyphosphate on myofibroblast differentiation. This was demonstrated in experiments in which releasates from activated human platelets induced fibroblast differentiation, but this activity was largely eliminated following treatment of releasates with the polyphosphate-degrading enzyme, exopolyphosphatase, suggesting that polyphosphate is the major source of activity in platelet releasates. Polyphosphate released from activated platelets and other cell types at sites of tissue damage may therefore act as a pro-wound healing, cell signaling molecule for fibroblasts. It also suggests that polyphosphate may be an appealing therapeutic for treatment of chronic wounds.

In this study, platelet polyphosphate promoted myofibroblast differentiation of both murine fibroblastic cells and human primary dermal fibroblasts. Myofibroblasts drive fibrosis progression in tissues and organs, raising the intriguing possibility that polyphosphate, which also promotes the formation of the pro-fibrotic fibrocyte cell,²⁴ may be a novel target for the suppression of fibrotic diseases, which account for 45% of all deaths in the US.^{24,36} Patients with idiopathic pulmonary fibrosis show increased platelet reactivity and activation, which is due to a currently unknown plasma factor.^{50,51} Primary myelofibrosis, in which the normal spongy marrow of the bone is replaced by dense scar tissue, is thought to be driven in large part by megakaryocytes, which are responsible for platelet production.^{52,53} Megakaryocytes contain pro-fibrotic molecules such as transforming growth factor-beta and platelet derived growth factor.⁵³ The role of platelet-derived and potentially megakaryocyte-derived polyphosphate in these fibrotic disorders has not been investigated, but might provide insight into the progression of these fibrotic pathologies in the lung and bone marrow.

Mycobacterium tuberculosis and *Trypanosoma cruzi* are microorganisms that accumulate polyphosphate.^{54,55} In *Myocobacterium tuberculosis*, polyphosphate plays roles in infection persistence, survival, and virulence, while in *Trypanosoma cruzi*, polyphosphate is important for environmental stress adaptation.^{54,56–58} Tuberculosis infection can lead to the development of pulmonary fibrosis, while *Trypanosoma cruzi* infection can lead to the development of cardiac fibrosis.^{59–61} Microbial polyphosphate may therefore be a source of this fibrosis-inducing activity, which warrants further investigation.

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Essentials

1. Polyphosphate is released from activated platelets
2. Platelet releasates induce myofibroblast differentiation
3. Platelet-sized polyphosphate promotes migration and myofibroblast differentiation
4. Platelet polyphosphate acts as a pro-wound healing and chemotactic molecule for fibroblasts

Figure 1. Platelet releasates induce myofibroblast differentiation, as evidenced by increased α -SMA levels.

(A) NIH-3T3 cells or (B) GM1381 cells were incubated in 1.5% serum in the presence or absence of releasates from activated human platelets (diluted to 15 $\mu\text{g/mL}$ protein). As indicated, some releasates were previously heat-treated to denature proteins, or digested with ScPPX1 to destroy polyphosphate (red indicates treatment with ScPPX1). After 48 hours, antibody staining for α -SMA was quantified by confocal microscopy as a marker of myofibroblast differentiation. All values are mean $\pm$ SEM ($n = 5$). Asterisks highlight statistical significance of the indicated comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (paired t-tests).

Figure 2. Polyphosphate induces myofibroblast differentiation, as evidenced by increased α -SMA levels and stress fiber formation.

(A-C) Platelet-size polyphosphate (polyP) increases α -SMA levels in NIH-3T3 cells. (A) Representative images in which NIH-3T3 cells were cultured in 1.5% serum in the absence (control) or presence of 5 μ mol/L platelet-sized polyphosphate. After 48 hours, cells were stained for α -SMA (red) or DNA (green) and imaged by confocal microscopy (bar = 10 μ m). (B,C) Dose-response of NIH-3T3 cells to platelet-size polyphosphate. In 5 experiments, cells were treated for 48 hours in 1.5% serum plus the indicated concentrations of platelet-size polyphosphate, after which the following were quantified: (B) integrated intensity of α -SMA staining per cell, or (C) percent cells positive for α -SMA staining localized along fibrin tendrils (stress fibers). (D) Platelet-size polyphosphate increases cell body area. NIH-3T3 cells were cultured in 1.5% serum in the absence or presence of 1 μ mol/L polyphosphate for 48 hours and imaged by confocal microscopy. Cell body area of individual cells was quantified. (E-F) Polymer length-dependence of polyphosphate's ability to induce myofibroblast differentiation in NIH 3T3 cells. Cells were incubated in 1.5% serum for 48 hours in the presence or absence of 1 μ mol/L polyphosphate of the indicated polymer lengths (in phosphate units): (E) integrated intensity of α -SMA staining per cell, or (F) percent cells positive for α -SMA staining localized along fibrin tendrils (stress fibers).

(G) Dose-response of GM1381 cells to platelet-size polyphosphate. Cells were incubated in 1.5% serum with varying concentrations of platelet-size polyphosphate for 48 hours, after which the integrated intensity of α -SMA staining was quantified. Values in panels B-C and E-G are mean $\pm$ SEM ($n = 5$). Asterisks highlight statistical significance relative to the no-polyphosphate controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (paired t-tests). Values in panel D are mean $\pm$ SEM of individual cells across five experiments. **** $p < 0.0001$ (unpaired t-test).

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Figure 3. Polyphosphate increases other myofibroblast markers.

(A-B) Phalloidin levels. NIH-3T3 cells were incubated in 1.5% serum for 24 or 48 hours in the presence of the indicated concentrations of platelet-sized polyphosphate, after which phalloidin staining levels from confocal images were assessed. Images at 48 hours are shown in *panel B* (representative of 5 experiments; bar = 10 μm). (C-D) Collagen type 1 alpha (COL-1A) levels. NIH-3T3 cells were incubated in 1.5% serum for 48 hours in the presence or absence of 1 $\mu\text{mol/L}$ platelet-sized polyphosphate, after which COL-1A staining levels from confocal images were assessed. *Panel D* shows representative images from 5 experiments. (E-F) Vimentin levels. NIH-3T3 cells were incubated in 1.5% serum for 48 hours in the presence or absence of 1 $\mu\text{mol/L}$ platelet-sized polyphosphate, after which vimentin staining levels from confocal images were assessed. *Panel F* shows representative images from 5 experiments. Values in panels A, C and E are mean $\pm$ SEM ($n = 5$). Asterisks highlight statistical significance compared to the no-polyphosphate controls: * $p < 0.05$, ** $p < 0.01$ (paired t-tests).

Figure 4. Polyphosphate is a chemoattractant.

(A) NIH-3T3 cells were incubated in 1% serum for 20 hours in the upper chamber of migration chambers, with the indicated concentrations of platelet-sized polyphosphate in the lower chamber. (B) NIH-3T3 cells were incubated in 1% serum for 20 hours with 5 $\mu\text{mol/L}$ platelet-sized polyphosphate in the indicated chamber(s), or without polyphosphate (control). (C) NIH-3T3 cells were incubated in 1% serum for 20 hours in the upper chamber of migration chambers, with the indicated treatments of 3 $\mu\text{mol/L}$ MRS2279 or 5 $\mu\text{mol/L}$ polyphosphate in the lower chamber. Control chambers received no treatment. After collecting and lysing the cells in the lower chamber, CyQUANT signals were expressed as relative fluorescence units (RFU) normalized to the no-polyphosphate control (set to 100 RFU). (D) NIH-3T3 cells were cultured in 1.5% serum in the absence (control) or presence of 5 $\mu\text{mol/L}$ platelet-sized polyphosphate or 3 $\mu\text{mol/L}$ MRS2279. After 48 hours, the integrated intensity of α -SMA staining was quantified. All values are mean $\pm$ SEM ($n = 5$). Asterisks highlight statistical significance compared to the no-polyphosphate controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (paired t-tests).