Signaling pathways mediating adhesion and spreading through extracellular ADP and the P2Y₁₂ receptor

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Introductory note by the Associate Editor

In this *Purinergic Signalling* issue, Babette Fuss of the Virginia Commonwealth University at Richmond discusses two interesting papers dealing with P2Y₁₂ receptor regulation of cell adhesion in two different cell types: microglia (the *Glia* article) and osteoclasts (the *J Clin Invest* article). In Highlight no. 1, new information on the effects mediated by P2Y₁₂ on microglial function are discussed, with special focus on the involvement of the ERK– β -arrestin cascade and on the interactions between ADP-dependent and ADP-independent signaling cascades modulating cell adhesion. In Highlight no. 2, the role of P2Y₁₂ in the formation of adhesive osteoclast structures and pathologic bone remodeling is discussed in view of data showing alleviation of bone resorption in models of inflammatory and tumor-associated bone loss by P2Y₁₂ receptor knockout or pharmacological inhibition. Due to the availability of already marketed P2Y₁₂ antagonists, the latter findings raise new important clinical perspectives for bone diseases.

Highlight N. 1: β -Arrestin 2-dependent activation of ERK1/2 is required for ADP-induced paxillin phosphorylation at Ser⁸³ and microglia chemotaxis

Lee S-H, Hollingsworth R, Kwon H-Y, Lee N, Chung CY. *Glia* 60:1366–1377.

Article summary

S.-H. Lee and colleagues described in this manuscript a novel ADP–P2Y₁₂ receptor signaling cascade that is involved in regulating the turnover of focal adhesions, multi-molecular complexes known to control cell shape and adhesion in response to environmental signals [1], and thereby the chemotaxis of microglia cells. More specifically, the authors investigated signaling events in microglia cells that lead to ERK1/2 activation upon ADP–P2Y₁₂ receptor stimulation, and they analyzed the regulatory roles of ADP–P2Y₁₂ receptor-stimulated ERK1/2 activation in the formation/disassembly of paxillin-containing focal adhesions.

The first set of studies presented in this manuscript is based on the previous observation that ERK1/2 activity is elevated in activated microglia [2]. Using kymographic, chemotaxis, immunofluorescent staining, and live cell imaging assays, the authors demonstrated that ERK1/2 activity is required for ADP-induced membrane ruffle formation and chemotaxis in BV2 microglia cells. Using Western blot analysis and a P2Y₁₂ receptor antagonist, the authors further showed that ADP-induced ERK1/2 activity in BV2 microglia cells is mediated through signaling via the P2Y₁₂ receptor.

In the second part of the manuscript, the authors focused on the role of ADP-stimulated ERK1/2 activation on paxillin phosphorylation and focal adhesion assembly/disassembly. Using Western blot analysis, the authors showed that ADP-induced activation of ERK1/2 leads to phosphorylation of paxillin at Ser⁸³ but not Tyr³¹. Inhibition of the phosphorylation event at Ser⁸³ was further shown to lead to a decrease in the number of paxillin-containing focal adhesions associated with an increase in their size and a decrease in their disassembly rate. Little effect was found for the assembly rate of paxillin-containing focal adhesions. Interestingly, mutation of paxillin's Tyr³¹ site and thus inhibition of phosphorylation

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at this site was found to lead to a decrease in the assembly rate of paxillin-containing focal adhesions with no effect on their disassembly rate. These results were interpreted by the authors to indicate that serine and tyrosine phosphorylation sites have a specific role, respectively, in focal adhesion formation and turnover upon ADP stimulation.

The third part of the manuscript concentrated on identifying additional components of the ADP–P2Y₁₂ receptor–ERK1/2–Paxillin Ser⁸³ signaling cascade. The authors provided convincing evidence that ADP-induced ERK1/2 and Paxillin Ser⁸³ phosphorylation does not require PI3K, PKA, or Src family tyrosine kinase signaling. Interestingly, however, phosphorylation of paxillin at Tyr³¹ was found to be decreased upon inhibition of Src family tyrosine kinase signaling. As a key upstream signaling molecule leading to phosphorylation of ERK1/2 and Paxillin Ser⁸³, the authors were able to identify β -arrestin 2. Consistent with the idea that both assembly/stabilization of focal adhesions as well as disassembly of focal adhesions are necessary for mediating membrane ruffling and chemotaxis, overexpression of both kinase inactive β -arrestin2 and Src mutants was found to decrease lamellipodia extension and chemotaxis of BV2 microglia cells.

Highlight N. 2: The ADP receptor P2RY₁₂ regulates osteoclast function and pathologic bone remodeling

Su X, Floyd DH, Hughes A, Xiang J, Schneider JG, Uluckan O, Heller E, Deng H, Zou W, Craft CS, Wu K, Hirbe AC, Grabowska D, Eagleton MC, Townsley S, Collins L, Piwnica-Worms D, Steinberg TH, Novack DV, Conley PB, Hurchla MA, Rogers M, Weilbacher KN. J Clin Invest 22:3579–3592.

Article summary

X. Su and colleagues investigated in this manuscript the role of ADP–P2Y₁₂ receptor signaling in osteoclasts, multinucleated myeloid lineage cells that are the principal source of bone resorptive activity. More specifically, the authors identified the P2Y₁₂ receptor as the primary ADP receptor in osteoclast, they provided support for the idea that in osteoclasts and similar to platelets ADP–P2Y₁₂ receptor stimulates integrin activation and they demonstrated that inhibition of ADP–P2Y₁₂ receptor signaling can partially protect mice from bone loss under pathologic conditions.

In the first part of the manuscript, the authors characterized bone loss and bone resorption in *P2ry12*^{−/−} mice. When assessing bone mineral density and trabecular bone volume, the authors found no significant changes in younger animals (2 months of age) but significantly increased values in older animals (8 months

of age). Using an osteoclast culture system, the authors were able to demonstrate that *P2ry12*^{−/−} cells exhibit intact osteoclast differentiation but decreased formation of bone resorption pits compared to wild-type (WT) cells. Importantly, ADP-stimulated osteoclast differentiation and bone resorption pit formation as seen in WT cells was found impaired in *P2ry12*^{−/−} cells.

In the second part of the manuscript, the authors made a first attempt in characterizing an ADP–P2Y₁₂ receptor signaling cascade in osteoclasts. In platelets, ADP stimulation through the P2Y₁₂ receptor has been described to lead to integrin activation and platelet adhesion via a PI3K–RAP1 pathway [3]. In analogy, the authors demonstrated that ADP–P2Y₁₂ receptor signaling in osteoclasts is involved in the ADP-stimulated formation of actin rings, adhesive osteoclast structures, and in ADP-mediated PI3K–RAP1 activation.

In the third part of the manuscript, the authors explored the role of P2Y₁₂ receptor signaling in bone loss under pathologic conditions. Using serum transfer arthritis as a model of inflammatory bone loss, the authors could show that while WT and *P2ry12*^{−/−} mice exhibit similar clinical inflammatory responses, WT mice show a decreased osteoclast surface per bone surface as well as a decreased bone marrow density compared to *P2ry12*^{−/−} mice. Using syngeneic inoculation of osteolytic B16-F10 melanoma cells as a model of tumor-associated bone loss, the authors could demonstrate that WT mice exhibit an increased bone loss compared to *P2ry12*^{−/−} mice. Similarly, pharmacological inhibition of P2Y₁₂ receptor signaling using clopidogrel was found to yield an increase in bone marrow density and trabecular bone volume compared with vehicle-treated tumor-bearing mice in a tumor model of intracardiac injection of syngeneic osteolytic 4T1-GFL-FL murine mammary carcinoma cells. No difference in tumor burden was noted. Lastly, the authors used a model of estrogen deficiency-induced osteoporosis, in which they could demonstrate that lack of P2Y₁₂ receptor expression (*P2ry12*^{−/−} mice) or P2Y₁₂ receptor inhibition (clopidogrel treatment) provides partial protection from bone loss.

Commentary

ADP signaling via the P2Y₁₂ receptor has been implicated in the regulation of cell adhesion and motility in a variety of cell types, in particular in microglia cells and platelets [4, 5]. Interestingly and as evidenced by the above-presented highlights, ADP–P2Y₁₂-initiated signaling may play various roles in the regulation of adhesion, the functional outcome of which may be dependent on the cell type.

In microglia cells, ADP–P2Y₁₂ signaling has been implicated in mediating process extension and chemotaxis [4, 6]. Such cellular motility is regulated by the dynamic assembly and disassembly of multi-molecular complexes

referred to as focal adhesions. In particular, it has been shown that paxillin-containing adhesions near the leading edge are highly dynamic and rapidly form and turn over [7]. In this regard, the data presented by S.-H. Lee and colleagues suggest that an ADP–P2Y₁₂–ERK– β -arrestin 2–paxillin Ser⁸³ signaling cascade promotes preferentially focal adhesion disassembly and turnover and thereby potentially a cellular state that has been previously referred to as an intermediate state of adhesion [8]. Thus, this ADP–P2Y₁₂–ERK– β -arrestin 2–paxillin Ser⁸³ signaling cascade is somewhat reminiscent of signaling mediated by matricellular proteins. In this context, it is noteworthy that the matricellular protein thrombospondin was found to stimulate focal adhesion disassembly via a PI3K–ERK activation cascade [9]. At the same time, however, it appears that ADP–P2Y₁₂ signaling also promotes focal adhesion assembly, in particular via a Src-mediated signaling cascade. How the two branches of the ADP–P2Y₁₂ signaling cascade are being coordinated and integrated, however, remains to be elucidated.

Somewhat different from microglia cells, ADP–P2Y₁₂ receptor signaling in platelets has been well characterized to primarily promote adhesion. As described by X. Su and colleagues, a similar adhesion-promoting effect was observed in osteoclasts. In both cell types, ADP–P2Y₁₂ receptor signaling likely promotes adhesive cellular states via activation of integrins and through PI3K–RAP1 signaling. However, to our knowledge, the effect of this signaling cascade on focal adhesion assembly and turnover has not yet been investigated.

The two manuscripts highlight the perplexing diversity of signaling cascades initiated by ADP–P2Y₁₂ receptor signaling and affecting the adhesive interactions of cells with their extracellular environment. Further complicating is the fact that P2Y₁₂ receptor signaling activated by a non-ADP ligand has also been reported to affect the adhesive state of cells [10]. More specifically, it has been demonstrated that the expression of the P2Y₁₂ receptor is necessary for the matricellular extracellular matrix protein autotaxin (ATX) to mediate an intermediate state of adhesion in differentiating oligodendrocytes, the myelinating cells of the central nervous system. This effect of ATX was found to be mediated by its modulator of oligodendrocyte remodeling and focal adhesion organization domain and to be associated with a decrease in the number of paxillin-containing focal adhesions. More comparative studies, however, will be necessary to dissect the contributions of

each of the described signaling cascades to the adhesive states of cellular lineages as diverse as microglia, platelets, osteoclasts, and oligodendrocytes.

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About the author

Babette Fuss is a professor at Virginia Commonwealth University in Richmond, Virginia, USA. Her primary research interest relates to the biology of the myelinating cells of the central nervous system, namely oligodendrocytes. In particular, her laboratory is interested in elucidating how (and by which intracellular signaling pathways) extracellular cues, such as autotaxin or ADP, regulate the differentiation of cells of the oligodendrocyte lineage and the process of central nervous system myelination during development as well as under pathological conditions.