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Review

Purinergic signaling: a novel mechanism in immune surveillance

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Purinergic receptors and the associated signaling cascades are known to play critical roles in cardiovascular, nervous, respiratory, gastrointestinal and urinogenital systems. Recent studies have also shed light on the importance of nucleotides and purinergic receptors in the regulation of the immune response. With a better understanding of the distribution and the receptor subtypes, the purinoceptors have the potential to become important therapeutic targets in inflammation, chemotaxis and immune-related diseases.

Keywords: nucleotides; purinergic receptor; immune surveillance; inflammation; chemotaxis

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Introduction

Extracellular nucleotides and their metabolites are important signaling molecules that enact various biological effects through specific receptors. The receptors for these molecules can be divided into two major families: the P1 receptor for adenosine and the P2 receptor for ATP, ADP, UTP, and UDP[1]. Based on differences in structure and transduction mechanism, P2 receptors can be sub-classified as ligand-gated ion channel receptors and G protein-coupled receptors (GPCRs), referred to as P2X and P2Y receptors, respectively. To date, seven P2X and nine P2Y subtype receptors have been cloned, and these receptors are widely distributed in various mammalian organs. All immune and inflammatory cells express P2Y and P2X receptors, and their expression is modulated by inflammatory cytokines during development. Among the P2 receptors, P2Y2, P2Y6, P2Y11, P2Y12, P2X4, and P2X7 are closely associated with immune responses.

In particular, the physiological role of P2X7 on the release of IL-1β has been clearly demonstrated[2]. During infection, tissue injury, inflammation, tumor immunity and apoptosis, nucleotides are released or secreted from cells, contributing not only to host defense but also to phagocytic clearance. Thus, both modulation of the release of these nucleotides and influence on the receptors represent important therapeutic strategies for the treatment of immune-related diseases.

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P2 receptors and chemotaxis

It has long been known that the activation of phagocytic cells is accompanied by ATP release. However, the specific receptor and signaling are still unknown. Neutrophils, the leukocytes that play a central role in host defense by destroying microorganisms, also secrete ATP when they become activated or start clearing dying cells. Further, it was found that ATP was released predominantly from the protruding regions at the leading edge of the migrating neutrophils. Hydrolysis of ATP by apyrase or CD39, which hydrolyze ATP to AMP, strongly affects chemotaxis, suggesting that the hydrolysis of ATP is crucial in the initiation of migration[3]. Early reports speculated that activation of P2X7 receptors was one of the most efficient stimuli for IL-1β release[4]. Chen reported that P2Y2 and A3 receptors were concentrated at the leading edge of the polarized cells and concluded that P2Y2 receptors controlled cell orientation. Additionally, this group suggested that following the hydrolysis of ATP and the formation of adenosine, the A3 adenosine receptors further facilitated the speed of chemotaxis. This hypothesis was confirmed by studies in gene knockout mice[5]. Recently, Elliott et al. targeted a "find me" signal on extracellular nucleotides, and these signals potently induced monocyte activation both in mammalian cell culture and in mice. They used an air-pouch model, which allowed direct tracking of cell movement in vivo, and showed that apoptotic cells selectively attracted monocytes and macrophages. Further experiments identified P2Y2 as the specific receptor involved in the process of clearance. Enzymatic breakdown of ATP and UTP, pre-incubation with a P2Y2 inhibitor and use of gene knockout mice all impaired

thymic apoptosis-induced chemotaxis[6]. For this reason, ATP seems not only to activate macrophages but also to regulate some scavenging functions. For instance, the chemotaxis and degranulation of mast cells are also mediated by P2X7R-induced calcium release[7].

Microglia are the principal immune cells in the brain. Previous studies found that microglial processes rapidly converged on the site of injury, isolating normal from injured tissue. The motility of these cells can be inhibited by ATP-hydrolyzing enzymes or P2Y receptor inhibitors[8]. Although the specific subtype of P2Y receptors are yet to be identified, the report from Haynes et al. showed that microglia from P2Y12$^{-/-}$ mice were unable to polarize, migrate or extend processes toward nucleotides. Moreover, P2Y12 receptor expression was high basally but dramatically reduced after activation, indicating that P2Y12 receptors may initiate the early stage of the microglial response[9]. Furthermore, the platelet P2Y12 receptor also mediates morphological changes, suggesting that clopidogrel (a P2Y12 inhibitor) may have an extra-neuroprotective role. In addition to ATP, UTP and other nucleotides may also play important regulatory roles, as indicated by a more recent report from Koizumi[10]. Following injury, the P2Y6 receptor was up-regulated with concurrent down-regulation of P2Y12, and this event triggered phagocytosis. Koizumi *et al* also found that after chemical injury of the hippocampal region *in vivo*, the levels of UTP increased. It was speculated that UTP and its degradation product, UDP, mediated the unique functions of microglia: ATP regulates motility through the P2Y12 receptor using a "find me" signal, whereas UTP triggers phagocytosis through its specific P2Y receptor by an "eat me" signal[11]. In other words, ATP releases a kind of diffuse signal, orientating the phagocyte, while UTP and UDP work more locally to accurately guide the phagocytic process. This hypothesis was fully supported by a recent report[12]. The activation of phagocytes must be stringently regulated because the body must be protected from an unwanted response by these cells. Purinergic signaling serves as one of the crucial parts in this complicated crosstalk (see Figure 1). Developing new agents that target the purinergic signaling system may prevent unwanted inflammatory responses.

P2 receptors and inflammation

Normally, inflammation is triggered by invading microorganisms via stimulation by various cytokines. Atarashi et al. reported that bacteria could activate a unique subset of colon lamina propria cells through an increase in luminal ATP concentration, leading to the differentiation of T_H17 cells, which are crucial in the pathogenesis of colitis[13]. However, it is also known that cell stress or tissue damage can initiate inflammation even in the absence of pathogens. Compelling evidence demonstrated that immune cells responding to injury release molecules that are normally located inside the cell. Among these "damage-associated molecular patterns," ATP has all the right features: its concentration is high within the cytoplasm and low in the extracellular space, and it is quickly released after cell damage, rapidly inactivated by ecto-ATPases and further regulated by the adenosine receptor[14].

Accumulation of ATP in the extracellular space generates a dual activity. Moderate ATP release causes mainly anti-inflammatory effects. Here, ATP activates P2Y11 receptors, causing maturation of dendritic cells (DCs) and the induction of Th2 cells[15], or it facilitates T-cell activation through P2X7-mediated calcium influx[16]. However, the release of ATP at high concentrations brings other P2 receptors into play, leading to a massive release of pro-inflammatory mediators and initiating the process of apoptosis. For example, high ATP accumulation in the airways in an asthmatic animal model could enhance Th2 sensitization, resulting in over-activated lung myeloid DCs[17]. Special attention should be paid to the P2X7 subtype, as it is closely related to IL-1β and IL-18. IL-1β is a potent pro-apoptotic agent that can be driven by P2X7 activation rather than by a passive process, as previously thought.

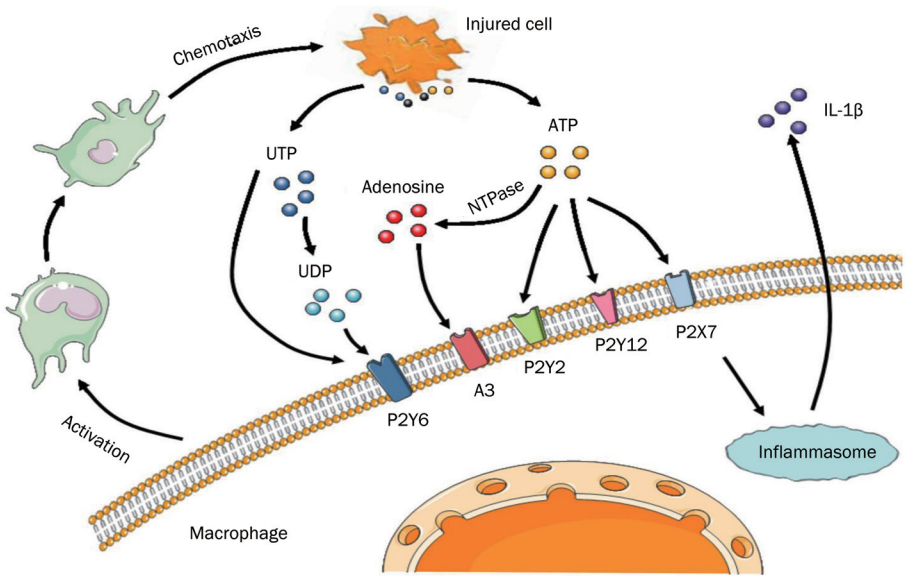

Figure 1. Purinergic signaling regulates phagocyte migration.

It is possible that P2X7 activation simultaneously opens the potassium channel and facilitates IL-1β secretion[18]. For example, monocytes from patients with rheumatoid arthritis were more sensitive to release of IL-1β following ATP activation of the P2X7 receptor[19]. Mice deficient in P2X7 exhibited markedly reduced inflammation, pain and IL-1β-mediated IL-6 production[20]. On the other hand, it is also possible that there may be a subset of pro-inflammatory cells, such as P2X7-expressing DCs and natural killer cells (NK), that are exquisitely sensitive to ATP-induced apoptosis. Hence, high levels of ATP could drive these cells out of the inflammatory milieu, causing immuno-suppressive activity[21].

The inflammasome, a caspase-activating platform, consists of a central scaffold protein that coordinates a number of additional proteins[22]. These proteins allow interactions with inflammatory caspases. Following inflammatory stimuli, caspase-1 converts pro-IL-1β to IL-1β. Recent studies found that the P2X7 receptor activates the K^+ current and panx-1, a member of the pannexin family, through a protein-protein interaction, suggesting that P2X7 is a key activator of the inflammasome[23]. Finally, nucleotides, adenosine and their metabolizing enzymes, CD39 and CD73, form a sophisticated surveillance network in immunomodulation. Studies on CD39, a recently discovered marker of $Foxp3^+$ Treg cells, may lead to a new understanding of the role of purinergic signaling in the immune system[24].

P2 receptors and cancer

Adenine nucleotides were first described as having anticancer activity in 1983. Recent evidence suggests that extracellular ATP inhibits human tumor growth, including cells from prostate, breast, colon and liver. In addition, ATP induces resistance of normal tissues to chemo- and radiation therapy and causes weight loss, anorexia and cachexia in older patients by expanding blood plasma ATP levels[25]. However, the mechanism by which ATP participates in these antitumor activities has yet to be resolved.

Multiple forms of P2 receptors have been studied in various types of cancer[26]. For example, mRNA for P2Y1, P2Y2, P2Y4, and P2Y6 receptors have been detected in melanomas. It is also known that the P2Y1 receptor stimulates a decrease, whereas the P2Y2 receptor subtype mediates an increase in cell number. The changes in cell number are likely to be regulated through PLC-coupled increases in intracellular Ca^{2+} levels. However, in some other type of cancer, such as esophageal and colorectal cancers, activation of P2Y2 receptors causes a decrease in cellular proliferation[27]. These seemingly contradictory data suggest that different second messenger systems may possess opposing effects in addition to the effects of ATP. Functional P2X7 receptors have been studied extensively in human cervical cancer cell lines. Treatment with a P2X7 antagonist led to cell apoptosis, possibly through the activities of caspase-3 and caspase-9 or by the IL-1β pathway[28].

Several signaling pathways have been suggested as being coupled to the P2 receptor. The most common signaling pathway described begins with an increase in PLC activity, leading to Ins(1,4,5)P3 mobilization and Ca^{2+} release from the endoplasmic reticulum[29]. In addition, the P2Y receptor coupled to adenylyl cyclase affects cAMP levels. It is not clear at present whether any specific P2Y receptors activate or inhibit the production of cAMP in cancer cells. Clearly, this presents a novel challenge to develop effective agents selectively targeting cancer cells either through specific signaling pathways or by working in conjunction with other chemotherapy drugs.

P2 receptors in cells of the CNS

The central nervous system (CNS), an avascular tissue previously thought to be "immunoprivileged," is clearly involved in immune reactions. Recent reports revealed that ATP is not only a neurotransmitter but is also involved in glial proliferation, motility, survival, differentiation and myelination and in facilitating interactions between neurons. Presently, it is well known that purinergic signaling profoundly affects neuroinflammation[30].

In some cases, the release of ATP during brain injury is neuroprotective but in other circumstances, ATP contributes to the initiation of harmful immune reactions. Under normal physiological conditions, peri-neuronal ATP concentrations are maintained at a low level. However, brain injury can induce increased concentrations of ATP, triggering uncontrolled immune reactions. Various P2 receptors are expressed by astrocytes in both central and peripheral nerves. On one hand, reactive astrocytes can impede neuronal regeneration and synthesize toxic molecules. On the other hand, astroglial-mediated neuroinflammation can also be beneficial[2]. Among the mediators driving reactive astrogliosis, ATP has a prominent role. P2 receptors induce gliosis via extracellular-regulated kinase (ERK) and protein kinase B/Akt pathways[31]. Astrocytes can sense the severity of damage in the CNS by the concentration of ATP and can modulate tumor necrosis factor-α (TNF-α)-mediated inflammatory responses via P2 receptors[32]. The specific activation of P2X7 in astrocytes facilitates the release of glutamate, GABA and ATP, which might influence the excitability of neurons under pathological conditions[33].

Microglia, the immune cells of the CNS, are also activated by nucleotides to release inflammatory cytokines, such as IL-1β and TNFα, and are involved in chemotaxis, as mentioned earlier. In addition, P2X7 receptors are up-regulated around amyloid-β plaques in a transgenic model of Alzheimer's disease via superoxide production[34]. ATP can also selectively suppress the synthesis of inflammatory proteins through calcium influx via P2X7 receptors. If pain is regarded as a sort of inflammation, blockade of P2X4 receptors can reverse tactile allodynia caused by peripheral nerve injury, suggesting the involvement of P2X4 receptors[35]. Thus, blocking P2X4Rs in microglia may be a new therapeutic strategy for pain induced by nerve injury.

Studies in various CNS injury models suggest that activation of P2X7 receptors leads to neuronal death. Survival of retinal ganglion cultures declined with increasing exposure to ATP[36]. It is believed that this process is at least partially

mediated by intracellular Ca^{2+} accumulation[37]. *In vivo* studies revealed that exposure to high levels of ATP led to irreversible increases in cytosolic calcium and cell death, and this effect is believed to be mediated through P2X7 receptors. P2X7 receptor antagonists could inhibit this excitotoxicity-based neuronal degeneration, reducing both the extent and functional sequelae of acute spinal cord injury[38]. Although numerous studies suggest that blockade of P2X7 receptors may reduce excessive neuronal inflammation, work to date using $P2X7^{-/-}$ animals in an ischemic model did not confirm this effect. Clearly, further experiments are required to develop a consensus on the role of P2 receptors in CNS inflammation and to elucidate the crosslink between purinergic and adenosinergic signaling. For example, depression of synaptic transmission in the hippocampus during hypoxia was alleviated by A1 receptor agonists[39].

Conclusion

The immune system represents an important and unique defense mechanism in our body. Dysfunction of the immune response may lead to numerous diseases. In this sophisticated defense network, nucleotides play a recognized role in physiological and pathophysiological processes, such as chemotaxis, proliferation, cytokine secretion, cell death and intercellular communication. The pharmacological modulation of nucleotide-mediated signaling in cells represents a desirable new therapeutic approach for the treatment of inflammatory diseases and cancer. In recent years, several P2X receptor agonists were used to treat inflammatory diseases. The use of nonselective P2X receptor antagonists was proposed for the treatment of rheumatoid arthritis and pain induced by nerve injury. ATP was successfully applied to limit T cell–mediated inflammation in mouse models of type 1 diabetes and inflammatory bowel disease. Thus, purinergic receptor blockade might represent a promising therapeutic strategy for the treatment of a number of diseases. Although P2 holds great potential in the treatment of many immune diseases, it is essential for future investigations to understand how ATP affects the immune system mechanistically and to identify ADP's precise role in coordinating adenosine signaling. It is conceivable that new techniques such as fluorescence resonance energy transfer (FRET)[40] and the real-time assessment of ATP release[41] will help to elucidate the underlying mechanism of purinergic signaling and allow us to develop more specific and effective immuno-therapies.

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