P2 receptors in cancer progression and metastatic spreading

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Abstract

The tumor microenvironment is rich in nucleosides and nucleotides. Very recent observations have highlighted the crucial role of adenosine in determining the highly immunosuppressive properties of the tumor interstitium. Mounting evidence suggests that also extracellular ATP heavily affects host-tumor interactions, albeit effects of this nucleotide on the host-tumor interaction are still incompletely understood. ATP-selective plasma membrane receptors, P2 receptors, are expressed on both host and tumor cells. P2 receptor activation may drive an anti-tumor response or rather promote tumor progression, depending on the receptor subtype, the inflammatory infiltrate and the tumor cell type. It is anticipated that an in depth knowledge of the pharmacology, biochemistry and functional activity of the P2 receptors will allow a better understanding of the essentials of host-tumor interaction and the development of innovative anti-cancer therapy.

Introduction

The delicate interactions between host and tumor occur in a protected milieu known as tumor microenvironment (TME). The cellular and biochemical composition of the TME is strikingly different from that of healthy tissues, but akin to that of inflammatory sites, and in fact the TME is an inflammatory microenvironment [1]. It has been known for several years that the inflammatory infiltrate present in the TME strongly affects tumor progression. Depending on the stage of the tumor and the immune cell phenotype, the inflammatory infiltrate can either suppress or promote cancer progression. An equilibrium (dormancy) can also be achieved whereby the tumor is not eradicated but is kept under check by the host immune response [2]. A dormant tumor can wake up at any time, escape control by the immune system, invade neighboring tissues and metastasize. All these processes are crucially dependent on the immunological properties of the TME.

The TME of established and advanced tumors is strongly immunosuppressive, due to the recruitment of specific cell types and to the presence of specific soluble factors. Cell types mainly responsible for immunosuppression are type 2 macrophages (M2), myeloid-derived suppressor cells (MDSCs), and T regulatory lymphocytes (Treg) [3]. The TME is also enriched in IL-10, TGF-β, indoleamine 2,3-dioxygenase, arginase, kynurenine, adenosine and other factors that suppress anti-tumor immunity. Among these factors adenosine has recently spurred interest for its strong immunosuppressive activity and for exciting pre-clinical data showing that immunosuppression in the TME can be reverted by interfering with adenosine generation and with adenosine receptors [4] [5]. About at the same time it was formally proved that the TME is enriched in extracellular ATP, the biochemical adenosine precursor [6]. These two topical findings brought adenosine and ATP (i.e. extracellular purines) at the leading edge of tumor immunology.

Metabolism and plasma membrane receptors of extracellular purines

The bulk of adenosine is generated within tissue interstitium at the expenses of extracellular ATP. Availability of a novel genetically-encoded molecular probe (i.e. the plasma membrane-expressed luciferase named pmeLUC) has provided direct *in vivo* demonstration that, in striking contrast to healthy tissues, the TME contains very high (hundred micromolar) ATP concentrations pellegatti [6]. High ATP levels are the biochemical prerequisite for adenosine accumulation in the TME. Within the tissue interstitium ATP serves as an adenosine precursor as well as a ligand for specific receptor (P2 receptors, P2Rs) expressed on both host and tumor cells [7]. P2R subtypes are differentially expressed by host and tumor cells, and are characterized by widely different affinity for ATP. These features endow the P2R system with a remarkable plasticity and allow modulation of selected pathophysiologic responses by targeting specific P2Rs. In addition, pharmacological targeting of cellular ATP release pathways and plasma membrane nucleotidases allows efficient modulation of adenosine accumulation in the TME.

P2Rs are classified in metabotropic P2Y and ionotropic P2X receptors (P2YRs and P2XRs, respectively) [8]. According to their coupling to specific G-proteins, P2YRs can be further sub-divided into two additional subfamilies: P2Y1R, P2Y2R, P2Y4R and P2Y6R (which couple to Gq to activate phospholipase Cβ, and P2Y12R, P2Y13R and P2Y14R (which couple to Gi to inhibit adenylyl cyclase). The P2Y11R couples to both Gq and Gs, thus its activation causes an intracellular Ca^{2+} rise as well as an increase in cAMP levels. Different nucleotides are agonist at P2YRs, P2Y11R being the only genuine ATP-selective receptor. The other P2YRs are preferentially activated by ADP (P2Y12R, P2Y13R), UTP (P2Y4R), UDP(P2Y6R), UDP-glucose or UDP-galactose (P2Y14R), or with equal potency by ATP and ADP (P2Y1R) or ATP and UTP (P2Y2R) [7]. Half maximal nucleotide stimulatory concentrations for P2YRs are in the high nano molar-low micromolar range [8].

P2XRs are homo-/hetero-trimeric cation-selective plasma membrane channels made by the assembly of seven different subunits (P2X1-7) [9;9;9]. The only known physiologic ligand at P2XRs is ATP, but there is evidence that at least one P2XR subtype, the P2X7R, can also be activated by non –nucleotide agonists such as the the pro-inflammatory mediator LL-37 cathelicidin released by neutrophils [10] or the 1-42 amyloidogenic peptide [11]. P2X1,2,3,4,6 subunits can form homotrimeric or heterotrimeric channels, while the P2X5 subunit is functional only in association with other subunits, i.e. P2X1/P2X5, P2X2/P2X5 or P2X4/P2X5 heterotrimers [12;12] [13] [14]. Quite interestingly, the P2X2/P2X5 heterotrimeric receptor exhibits functional properties, e.g. large pore formation, typical of P2X7R [12]. The P2X7 subunit, at variance with the other subunits, forms only homotrimeric channels, although it has been reported that under certain conditions can also form homoexamers [15] [16]. All P2XRs activate transmembrane ion fluxes, thus the main signal transducing mechanism associated to these receptors is the modulation of the intracellular ion concentration. Little is known of additional signal transduction pathways and intracellular proteins interacting with P2XRs, with the exception of the P2X7Rs subtype that has been shown to interact directly with at least 11 intracellular proteins, among which heat shock proteins, β–actin and phosphatidylinositol 4-kinase [17].

P2YRs and P2XRs participate in a complex extracellular purinergic homeostatic system consisting of ligands (ATP and adenosine), receptors (P2Rs and P1Rs) and degrading enzymes (CD39, CD73 and adenosine deaminase, ADA). The CD39 and CD73 ecto-nucleotidases that degrade with different affinities ATP and ADP to AMP and AMP to adenosine, are major determinants of the concentration of extracellular nucleotides in the TME. CD39 is a member of the ecto nucleoside triphosphate diphosphohydrolase (E-NTPDase) family [18]. CD73 is the only member of the 5’ ectonucleotidase enzyme family expressed on the outer surface of the plasma membrane and the most important extracellular enzyme responsible for adenosine generation. The final step in this enzyme chain is adenosine degradation to inosine by ADA. CD39, CD73 and ADA are extremely

important players in the overall process of host-tumor interaction because they set the levels of ATP and adenosine in the TME, and therefore its intrinsic immunosuppressive properties. In support of this crucial role, increased expression of CD39 and CD73 has been reported in several cancers [19], [20], [21].

Purinergic signalling in tumor progression

P2YRs and P2XRs are ubiquitously distributed in many different cell types, both excitable and non-excitable and, although it is not by any means a generalized finding, some P2Rs subtypes are reportedly expressed to higher level in cancer versus healthy tissues (e.g. P2X7R, P2X3R, P2X5R, P2Y1R, P2Y2R, P2Y6R) [22] [23] [24] [25] [26]. However present data are insufficient to conclude that this is a generalized feature, and it is fair to say that as of now there is no clear-cut differential expression pattern of P2Rs in normal and neoplastic cells. Nevertheless, virtually all tumor cell lines respond to stimulation with ATP, ADP or UTP [27]. P2Y1R and P2Y2R activation stimulates proliferation of healthy and neoplastic cells, therefore these receptors are potential mediators of the growth-promoting activity of extracellular ATP in the TME. In prostate cancer P2YRs have also been shown to support invasiveness and metastatic spreading [28] [29] [30]. In the human breast cancer cell line MDA-MB-231 P2Y2 stimulation causes HIF-1α activation, lysyl oxidase release and THP-1 macrophage recruitment, events that are conducive to metastatic niche formation [31]. The P2Y2R has been solidly implicated in metastases formation by a recent study by Schumacher et al [32]. These authors have shown that tumor emboli in the blood as they come in close contact with the endothelial lining trigger ATP and ADP release from nearby platelets. Nucleotides diffuse onto the endothelial cell surface to activate endothelial P2Y2Rs which in turn open endothelial cell barrier and facilitate tumor cell extravasation [32]. To further support the key role of P2Y2Rs, metastatic dissemination is reduced by pharmacologic P2YR blockade and in mice genetically deleted of P2Y2R or harboring platelets defective in ATP secretion. However, care should be exercised to generalize these effects as opposite responses due to P2Y1R or P2Y2R activation, i.e.

inhibition of proliferation or outright apoptosis, have been described in nasopharingeal carcinoma or in human colon carcinoma, respectively [24], [33]. The P2Y12R subtype is well known for being a major player in platelet activation and aggregation, and a target for anti-coagulant therapy [34], but recent findings are also highlighting its role in cancer. P2Y12R blockade has been shown to reduce growth and metastatization of B16 melanoma and gliomas by acting on circulating platelets and directly on the tumor cells [35] [36]. In gliomas the effect of P2Y12R blockade seems to be mediated by an increase in intracellular cAMP levels and by the activation of an autophagic pathway eventually leading to cell death [36]. P2YRs are likely to have a much wider role in cancer, as witnessed by the recent finding that P2Y1R, P2Y2R and P2Y6R are included in the number of genes responsible for driving resistance to ALK-inhibitors in the lung [23].

Most tumors express high levels of the P2X7R [37], [27]. This receptor is well known for its cytotoxic effects, but there is now strong indications that low intensity stimulation might also support growth [37]. Cytotoxic responses are triggered by P2X7R especially following stimulation with high (pharmacological) ATP doses which are likely to build up in the extracellular milieu only under very particular conditions, as for example those present in the TME. Under most other conditions of basal ATP release, tonic autocrine or paracrine stimulation of P2X7R has a trophic effect. Thus, the P2X7R behaves as a bifunctional receptor that, depending on the level of activation and the given cell type, can either precipitate cell death or drive proliferation [38]; [39] [40] [41] [42]. Under conditions present in the TME (i.e. high ATP levels) it is anticipated that cytotoxic effects due to P2X7R activation should prevail, but surprisingly this is not the case. It appears that cancer cells are resistant to the high (hundred micromoles/liter) ATP concentrations present in the TME. The reason for such refractoriness to ATP stimulation is unknown, however there is evidence that in at least some cancer cell types, P2X7R is uncoupled from major intracellular death pathways [43]. Other cell types present in the TME (e.g. myelod-derived suppressor cells, MDSCs) are also resistant to very high extracellular ATP concentrations despite

expression of fully functional P2X7Rs [44]. While the mechanism underlying cancer cell or MDSC refractoriness to P2X7R-mediated cytotoxicity is largely unknown, details of the trophic P2X7R activity are being unveiled. Low intensity P2X7R stimulation causes a small elevation in mitochondrial Ca^{2+}, an increase in mitochondrial potential, stabilization of the mitochondrial network, and an overall increase in efficiency of oxidative phosphorylation [45]. At the same time P2X7R also stimulates aerobic glycolysis, thus leading to a large increase in lactic acid output. Most of the key glycolytic enzymes (i.e Glut-1, glyceraldehyde 3-phosphate dehydrogenase, phosphofructokinase, pyruvate kinase M2 and pyruvate dehydrogenase kinase 1) are up-modulated and intracellular glycogen stores are expanded in P2X7R-expressing versus P2X7R-less cells [46]. The net result of the enhancement of both oxidative phosphorylation and glycolysis is a large increase in the total cellular ATP content [45]. The strong stimulation of glycolysis on one hand help replenish the ATP pool, and on the other makes available abundant biosynthetic intermediates for the synthesis of nucleic acids, phospholipids and non-essential aminoacids (anaplerotic reaction), and generates reducing equivalents in the form of NADPH [47]. The positive effect of P2X7R expression on both energy metabolism and anaplerosis is witnessed by the striking proliferative advantage of P2X7R-expressing cells [26;43;45] [48] [49] [50] [51] [52] [53] [54] [55].

Besides its effect on energy metabolism, the P2X7R activates several key intracellular growth-promoting pathways, including NFATc1, ERK, Akt and HIF-1α [46;48;56] [57] [58], and in turn P2X7R is itself modulated by the Akt/PI3K pathway [55]. Most of these actions are triggered by subtle changes in endoplasmic reticulum and mitochondrial Ca^{2+} homeostasis [45] [57]. Another striking feature of the P2X7R is the ability to trigger massive release of VEGF from immune cells as well as from tumor cells in vitro and in vivo [59] [26]. Many tumors are known to release large amounts of microvesicles/microparticles (MVs/MPs) into the extracellular space, and into the blood. MVs/MPs carry a cargo of several bioactive factors (miRNAs, DNA, metalloproteinases,

tissue factor) that modify blood homeostasis and may also be horizontally transferred to host cells [60]. ATP acting at P2X7R turns out to be one of the most potent stimuli for microparticle release from healthy as well as cancer cells [61] [62] [63] [64]. Whether and how MV/MP release might contribute to invasiveness and metastatic dissemination is an entirely unfathomed field of study. In this regard it is noteworthy that P2X7R triggers release of active cysteine cathepsins and metalloproteinases from cancer cells [65]. Although there are no data in tumors, experiments in mononuclear phagocytes suggest that P2X7R-stimulated MVs/MPs release is a main pathway. Cathepsins and metalloproteinases are overexpressed by cancer cells, from which are released to digest the extracellular matrix and promote infiltration in soft agar. To support these findings, P2X7R stimulation promotes migration of PC9 lung carcinoma and T47D breast cancer cells [66] [67], while on the contrary its silencing inhibits prostate cancer cell migration and invasiveness *in vitro* and *in vivo*. P2X7R silencing might also affect metastatic spreading by down-modulating expression of genes involved in epithelial/mesenchimal transition such as Snail, E-cadherin, Claudin-1, IL-8, and MMP-3 [68]. Several loss- or gain-of-function single nucleotide polymorphisms (SNPs) are known in the P2X7R. No association with cancer has been documented for any particular SNPs, with the exception of the A1513C SNP (rs3751143) in familiar chronic lymphocytic leukemia [69]. Very recently, a specific genetic profiles of P2X7R and VGFR2 polymorphisms associated to a favorable or unfavorable prognosis in prostate cancer has been identified [70]. Although single P2X7R (rs3751143, rs208294) or VGFR2 (rs2071559, rs11133360) polymorphisms were not associated with a significant change in overall survival, their combination identified patient cohorts with a better or worse prognosis. Overall, the P2X7R exhibits striking oncogene-like features as it promotes aerobic glycolysis, activates the PI3K-Akt pathway, induces HIF-1a, stimulates glycogen synthase kinase (GSK)-3β, trigger release of VEGF, tissue factor and matrix metalloproteinases, promotes growth, invasiveness and metastatic spreading [71] [65] [72].

Full comprehension of the role of the P2X7R in host-tumor interaction is made more complicated by the high level of expression of this receptor on immune cells and its strong pro-inflammatory activity [14]. Experiments in P2X7R-KO mice or with P2X7R blockers show that lack of P2X7R impairs allogeneic responses [73] [74]. In addition, tumors grow faster and metastasize more readily in P2X7R-KO versus wt mice [75] [76]. These findings suggest that the role played by host P2X7R versus tumor P2X7R is quite different and likely differently modulated by the biochemical composition of the TME. A similar antithetic role for host versus tumor CD73 has been recently highlighted in prostate cancer [77]. However, in vivo data show that the anti-tumor effect due to blockade of P2X7R expressed by cancer cells prevails on potential inhibition of the anti-tumor immune response due to blockade of P2X7R expressed on the host cells, thus suggesting that P2X7R blockade is a viable anti-cancer therapy.

Among other P2XRs, P2X3R and P2X5R have recently attracted interest in cancer. In tumor cells from hepatocellular carcinoma patients P2X3R overexpression has been shown to stimulate the JNK pathway, promote proliferation and be associated with an unfavorable prognosis [78]. These observations are intriguing as the P2X3R is rapidly desensitized in the presence of physiologic ATP concentrations suggesting that cell activation should be short lasting. On the other hand, it is possible that in tumor cells desensitization of this receptor is defective, or the TME contains factors (positive allosteric modulators?) that prevent desensitization. The P2X5R has been implicated in epithelial cell homeostasis [27], is present in human basal cell and squamous cell carcinomas and is overexpressed during differentiation, suggesting that it might have a negative effect on cancer cell growth [79]. Human P2X5 subunits are unable to form a functional ion channel because the most common P2X5 splice variant misses exon 10, or both 3 and 10. A defective human P2X5 splice variant has been recently shown to be expressed on human leukemia cells where it acts as a minor histocompatibility antigen. This novel marker of leukemia cells might be a suitable target for immunotherapy [80].

The conversion of ATP to adenosine by CD39 and CD73 is a crucial feature of purinergic signalling in the TME [4]. The discovery that adenosinergic receptors mediate immunosuppression has prompted numerous attempts to target this signalling pathways in the attempt to restore an anti-tumor immune response in the TME. In a mouse model of triple negative breast cancer (TNBC) combined treatment with doxorubicin together with anti-CD73 mAbs or A2A receptor antagonists produced a significant increase in survival [81]. Similar results were also obtained by combining targeting of the adenosinergic pathway with anti-PD-1 or anti-CTLA4 therapy [82]. In preclinical models, simultaneous blockade of immune checkpoints and A2A receptors enhanced cytotoxic T lymphocyte and NK cell activity and reduced metastatic dissemination in mouse models of melanoma and breast cancer [83]. Purinergic signalling in the TME is also dramatically affected by hypoxia. Hypoxia driven HIF-1α activation increases expression of CD39 and CD73 and adenosine production via transcription factors Spi and HIF-1a, respectively [84] [85]. Contrary to hypoxia, hyperoxia might counteract immunosuppression and restrains tumor growth [86].

Conclusion

Purinergic signalling is one of the most widely diffused extracellular messenger systems. It is a fact that virtually all cell types communicate via nucleotide release and generate an fundamental additional messenger (adenosine) by controlled hydrolysis of extracellular ATP. We are now learning that nucleotides and nucleosides are exquisitely abundant in the TME where they play a crucial role in host-tumor interaction. Nucleotide and nucleotides, chiefly ATP and adenosine, modulate the host immuneresponse as well tumor growth, invasion and metastatic spreading. Pre-clinical data show that blockade of adenosinergic signalling may restore an efficient anti-tumor immune response, and that combinatorial therapy based on the administration of A2AR blockers and immune checkpoint inhibitors is highly effective in reducing tumor burden. These exciting findings bring purinegic signalling in the limelight of the fight against cancer.

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