Signaling pathways involving adenosine A_{2A} and A_{2B} receptors in wound healing and fibrosis

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Abstract Collagen and matrix deposition by fibroblasts is an essential part of wound healing but also contributes to pathologic remodeling of organs leading to substantial morbidity and mortality. Adenosine, a small molecule generated extracellularly from adenine nucleotides as a result of direct stimulation, hypoxia, or injury, acts via a family of classical seven-pass G protein-coupled protein receptors, A_{2A} and A_{2B}, leading to generation of cAMP and activation of downstream targets such as PKA and Epac. These effectors, in turn, lead to fibroblast activation and collagen synthesis. The regulatory actions of these receptors likely involve multiple interconnected pathways, and one of the more interesting aspects of this regulation is opposing effects at different levels of cAMP generated. Additionally, adenosine signaling contributes to fibrosis in organ-specific ways and may have opposite effects in different organs. The development of drugs that selectively target these receptors and their signaling pathways will disrupt the pathogenesis of fibrosis and slow or arrest the progression of the important diseases they underlie.

Keywords Adenosine · Collagen · Fibrosis · Scar · Wound

Introduction

Tissue injury is followed by a series of events that allow the organism to contain potential infection and restore the structural integrity of the organ involved. The process of wound

healing is initially characterized by inflammation and later involves neovascularization and matrix synthesis (granulation tissue formation), re-epithelialization, and, over time, formation of a scar and ultimately contraction and remodeling. In many organs, the wound healing process is diverted or overly active leading to excess matrix production, fibrosis. Fibrosis underlies a wide range of clinically important diseases including idiopathic pulmonary fibrosis, penile fibrosis, cirrhosis, hypertrophic scarring, keloid formation, radiation fibrosis, and scleroderma, among others. Scleroderma and other diffuse fibrosing disorders, although uncommon, cause major morbidity for affected people including disfigurement and diminished range of motion as well as cardiac, gastrointestinal, and renal dysfunction leading to premature mortality. Treatment of common cancers, such as breast and head and neck cancers, with ionizing radiation is often complicated by radiation dermatitis in the short term and dermal fibrosis in the long term. Disfiguring scars are a common cause of physical and psychological disability after trauma and result in more than 171,000 scar revision surgeries each year, representing a major healthcare cost. Adenosine and its receptors are involved in the initiation and progression of fibrosis, which will be reviewed.

Adenosine formation and physiologic actions

That such a diverse range of diseases characterized by fibrotic change suggests a common set of cellular and molecular factors in their pathogenesis. Indeed, there is a complex interplay among many effector cells including fibroblasts, myofibroblasts, and bone marrow-derived cells that signal via a variety of small molecules, chemokines, and cytokines. For example, transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF) is important for

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normal wound healing but also contribute to pathologic fibrosis in virtually every organ studied [1]. Another small molecule involved is adenosine, a purine nucleoside, which is generated extracellularly from adenine nucleotides, such as ATP or ADP, released by cells as a result of direct stimulation, hypoxia, injury, or metabolic stress [2]. Endothelial cells and neutrophils release ATP via connexin hemichannels [3, 4]. Pannexin hemichannels also mediate ATP egress under both physiologic conditions and during cellular apoptosis [5]. Extracellular ATP has a short half-life before it is converted to adenosine via spontaneous hydrolysis or by the serial dephosphorylation of ATP to AMP by nucleoside triphosphate dephosphorylase (CD39) followed by dephosphorylation to adenosine by 5'-ectonucleotidase (CD73) [6]. Adenosine acts via a family of classical seven-pass G coupled protein receptors (A_1 , A_{2A} , A_{2B} , and A_3 receptors). These receptors are found throughout the body including in blood vessel endothelium, T lymphocytes, neutrophils, platelets, and fibroblasts, among others [7, 8]. A_{2A} and A_{2B} receptors, the subtypes most relevant to fibrosis, both signal via a G_s protein and activate adenylyl cyclase leading to an increase in intracellular cAMP [9, 10]. The rise of cAMP, in turn, activates PKA and other downstream targets that influence gene expression [11]. Termination of adenosine signaling involves equilibrative nucleoside transporters which transports adenosine from the extracellular to the intracellular space down its concentration gradient [12]. Once within the cell, adenosine is converted to inosine via adenosine deaminase or AMP by adenosine kinase [13]. The signaling pathways involving adenosine and its receptors in wound healing and fibrosis have begun to be elucidated which holds promise for development of targeted therapeutics.

The role of adenosine and its receptors in granulation tissue formation

Granulation tissue is new connective tissue formed after wounding and is composed of matrix elements, new blood vessels, and supporting cells. Adenosine, acting at its receptors, promotes granulation tissue formation both directly and indirectly [14–16]. It has been shown that A_1 R and A_{2A} R hasten the resolution of acute inflammation by a variety of mechanisms [17–19]. All four adenosine receptor subtypes have also been implicated in revascularization although the A_{2A} R and A_{2B} R play the dominant roles in fibrosis. Thus, it has been reported that mice with genetically disrupted A_{2A} R form fewer vessels in healing wounds and that treatment with an A_{2A} R agonist promotes vessel formation by endothelial cells in wild-type mice [16]. Stimulation of A_{2A} R and A_{2B} R directly stimulates endothelial cell proliferation and A_{2A} R stimulation leads to tube formation in vitro. Moreover, A_{2B} R occupancy upregulates VEGF, bFGF, and IL-8 in human

endothelial cells via G_q and possibly $G_{12/13}$ and activation of phospholipase C. A_{2A} R also upregulates VEGF in macrophages in a PKA-independent mechanism [14, 15]. Additionally, A_{2A} receptor stimulation suppresses thrombospondin-1 expression in microvascular endothelial cells, and thrombospondin-1 suppresses angiogenesis in vitro in microvascular endothelial cells [20] although others have reported that A_{2A} and A_{2B} receptor stimulation promotes angiogenesis through increased macrophage expression of thrombospondin-1 [21].

Expression of adenosine receptor subtypes by a particular cell is dependent on its milieu and is influenced by characteristics such as hypoxia and differential exposure to cytokines. For example, hypoxia induces expression of A_{2B} R in human endothelial cells and smooth muscle cells, which is coupled with an upregulation of VEGF and represents an “angiogenic” phenotype [22]. Interferon-gamma, a Th1 cytokine, also increases expression of A_{2B} R and decreases expression of A_{2A} R in human microvascular endothelial cells [23]. Interestingly, A_{2A} R knockout mice have more disorganized granulation tissue after wounding compared to wild-type mice although the re-epithelialization rate is not impacted [16, 24]. Finally, there is evidence that the inhibitor of angiogenesis endostatin modulates dermal collagen deposition and that inhibition of adenosine receptor-mediated angiogenesis alters the composition of scars thus demonstrating that the two key elements of granulation tissue formation are at least partially interdependent [25].

Adenosine receptors regulate collagen production and fibroblast activation via cAMP and its downstream signaling molecules PKA and Epac

Collagen is the essential building block of the extracellular matrix. A_{2A} R stimulates fibroblasts to produce collagen I (Col1) and collagen III (Col3) and to downregulate matrix metalloproteinase (MMP) 9, 2, and 14, which are involved in collagen breakdown [26]. In a bleomycin-induced fibrosis mouse model, A_{2A} R stimulation directly promoted diffuse collagen production and this effect was abrogated in A_{2A} R knockout mice or when wild type mice were treated with receptor antagonist [26]. Mice lacking adenosine deaminase are also protected from dermal fibrosis [27]. A_{2A} R receptor stimulation increases intracellular cAMP and the A_{2A} R has been shown not to couple with the G_q /PLC/PKC pathway [10]. Rather, direct PKC activation represses both Col1 and Col3 in human dermal fibroblasts [28]. Taken together, these results suggest that Col1 and Col3 increased by A_{2A} R activation occur directly via cAMP.

A_{2A} R activation increases hepatic stellate cell (HSC) proliferation and reduces HSC apoptosis via PKA-dependent

inhibition of the Rac1/p38 MAPK pathway. Activation of this pathway leads to downregulation of core cell fate pathways involving p53 and Rb [29]. This cross-talk between the cAMP/PKA and MAPK pathways has been well characterized, wherein PKA-dependent phosphorylation disrupts the interaction between p21^{ras} and c-RAF, which results in cAMP-mediated suppression of the MAPK pathway [30]. A_{2A}R occupancy can also lead to direct activation of MAPK by phosphorylation as well as inhibition of its upstream kinase, MEK-1, which suppresses adenosine-mediated collagen production in dermal fibroblasts [26]. These pathways are the target of both deleterious and beneficial pharmacologic modulation. For example, stimulation of HSCs with acetaldehyde, a potent hepatotoxin that is a byproduct of ethanol metabolism, significantly increases expression of cAMP, PKA, and p-CREB compared to control, and the magnitude of the increases is greater in A_{2A}R agonist-treated cells and is inhibited in cells treated with a A_{2A}R-specific antagonist. Therefore, acetaldehyde-induced HSC activation and the resulting fibrosis from chronic exposure likely occur via A_{2A}-mediated cAMP/PKA signaling [31]. Caffeine, on the other hand, a nonselective adenosine receptor antagonist, has been shown to be protective against hepatic fibrosis in an animal model by inhibition of this pathway [32]. The inhibitory effects of caffeine on procollagen I are thought to be mediated by a separate PKA-Src-ERK1/2 pathway downstream of A_{2A}R, whereas the A_{2A}R-P38-MAPK pathway is involved in mediating its effects on the synthesis of procollagen III [33].

The ratio of Col1 to Col3 is 4:1 in normal skin; however, in immature scars, Col3 is increased and the ratio is closer to 2:1 [34]. As the scar matures, Col3 is replaced by Col1, which increases its tensile strength [34]. The mechanism underlying the different ratio of Col1:Col3 is mediated by A_{2A}R via differential activation of PKA and Epac2 [35]. With nanomolar concentrations of A_{2A}R agonist, PKA promotes expression of Col1 and represses expression of Col3. At higher concentrations of agonist, with correspondingly higher concentrations of cAMP, PKA inhibition of Col3 is offset by Epac2 induction (Fig. 1) [34]. Epac1, another cAMP effector, promotes fibroblast migration by activating its target Rap1. Activated Rap1 can bind to RAPL, a Rap1 binding molecule, and promote cell polarization by activating integrins [36]. PKA may also directly activate and regulate Epac1 and Rap1 [36] [37]. Interestingly, TGF- β inhibits Epac1 expression via a Smad3-dependent process [36]. Overexpression of Epac1, on the other hand, inhibits TGF- β -induced collagen synthesis, which indicates that a reduction in Epac expression is required for a profibrotic response. Taken together, these findings demonstrate an example of cross talk between a receptor tyrosine kinase and a GPCR [36].

Paradoxically, Epac and PKA have also been shown to decrease collagen production in human fibroblasts at supra-physiologic levels of stimulation [36]. Additionally, PKA and

Epac differentially regulate fibroblast migration and morphology: Epac promotes migration via Rap1, whereas PKA inhibits migration and decreases fibrogenicity. Experimental evidence indicates that lower cAMP concentrations tend to promote migration via Epac [36]. Thus, it is likely that cAMP serves as a switch where different concentrations can produce opposite physiologic effects. Modest intracellular increases in cAMP promote fibroblast activation and Col1 deposition whereas supra-physiologic levels inhibit fibroblasts and reduce Col 1 [35].

Other signaling pathways by which adenosine receptors regulate collagen expression

A_{2A}R stimulation increases collagen production by human fibroblasts and hepatic stellate cells by several other distinct pathways involving cAMP. At low levels of receptor stimulation, Col1 expression is promoted in a pathway involving AKT phosphorylation and activation. However, at higher levels of receptor stimulation, Col3 expression is induced through a p38 MAPK-mechanism (Fig. 1) [35]. This likely reflects the fact that Col3 expression is negatively regulated by ERK1 and ERK2 and thus requires higher levels of receptor activation to overcome this inhibition. MMP-1 expression in human dermal fibroblasts is also diminished by cAMP via ERK inhibition by PKA [35]. Low levels of A_{2A}R engagement are insufficient to overcome ERK inhibition; however, at higher levels of A_{2A}R activation, Col3 is increased via ERK inhibition by PKA (Fig. 1). Contrary to what is seen with Col1, Col3 is increased at maximal cAMP concentrations. These findings help to further explain the reduction in the Col1:Col3 ratio in hypertrophic and immature tissues where adenosine levels are increased. Additionally, PKA inhibition of ERK on G_s-protein-coupled receptor activation perpetuates skin fibrosis progression and represents a potential therapeutic target. However, cAMP still suppresses MMP-1 expression even when ERK activity is unaffected by it so there must be other pathways involved. Interestingly, AKT has also been reported to downregulate MMP1 in human dermal fibroblasts [38].

A_{2A}R also promotes Col1 synthesis by a connective tissue growth factor (CTGF)-dependent mechanism, and this effect is not abrogated with AKT knockdown indicating that it is an independent pathway [39]. Constitutive expression of CTGF in fibroblasts is sufficient to cause accelerated diffuse tissue fibrosis in vivo [40]. The Ets factor, Fli1, may be of central importance in this pathway. It has been shown to be an important regulator of collagen production in vivo by repressing collagen genes such as Col1A1, Col1A2, and Col3A1. Fli1 has also been shown to be significantly reduced in clinically involved skin of scleroderma patients [39]. A_{2A}R occupancy in dermal fibroblasts promotes reduced nuclear Fli1 mRNA

Fig. 1 Cross-talk of adenosine receptor signaling pathways leading to collagen deposition and fibrosis. Collagen III is separately regulated by p38, inhibition of ERK via PKA, and Akt pathways. Collagen I is shown being positively regulated by Epac2. Additionally, $A_{2A}R$ inhibits Fli1, which promotes CTGF transcription and fibrosis

and protein expression with correspondingly increased CTGF protein expression and secretion [39]. TGF- β pathway activation has also been shown to acetylate Fli1 in dermal fibroblasts, which renders Fli1 less stable thereby promoting extracellular matrix production. Fli1 thus serves as another potential link between these two important pro-fibrotic pathways [39].

Recently, the cannabinoid receptor CB1 has been reported to form a heteromer with $A_{2A}R$ and following $A_{2A}R$ blockade; nonselective cannabinoid receptor stimulation results in a substantial anti-fibrotic effect. Additionally, the CB1-specific antagonist VD60 has been shown to reduce the expression of $\alpha_2(I)$ pro-collagen mRNA and exert an antiproliferative effect on hepatic stellate cells. Taken together, these results suggest cross-talk between the CB1 and A_{2A} receptors [41]. The inhibition of reactive oxygen species production and phosphorylation of Akt, ERK, and Smad3 may also explain the underlying mechanisms behind the antiproliferative effect of VD60 [42]. This evidence of a synergistic anti-fibrotic effect between the adenosine and cannabinoid systems in scleroderma dermal fibroblasts may represent a novel therapeutic target.

Unique aspects of adenosine receptor signaling in organ fibrosis

Extensive, reversible cardiac fibrosis and decreased Akt expression have been shown in a mouse model of inducible A_1

overexpression, suggesting a pro-fibrotic role for the receptor. This effect is mediated by phosphatidylinositol-3 (PI3) kinase and Akt kinase signaling which have been shown to play an important role in both physiologic and pathologic cardiac hypertrophies and in the regulation of cell survival [43]. Antagonism of A_1R with the highly selective inhibitor SLV320 attenuates myocardial fibrosis and albuminuria without influencing blood pressure in a uremic cardiomyopathy model [44]. Interestingly, this effect is likely mediated by both counteracting the pro-fibrotic role of A_1 and increasing the activation of A_{2B} receptors, which are anti-inflammatory and have a protective role in cardiac fibrosis via multiple mechanisms. Increased expression of $A_{2B}R$ leads to a decrease in levels of collagen and protein synthesis, while underexpression of $A_{2B}R$ yields increased protein and collagen synthesis [45]. In rat cardiac fibroblasts, adenosine activates the A_{2B} -G_s-adenylyl cyclase pathway, and the resultant cAMP reduces collagen synthesis via a PKA-independent, Epac-dependent pathway that involves PI3K [46]. Additionally, cAMP has been shown to have an anti-proliferative effect on cardiac fibroblasts possibly by inhibiting MAP kinase activity [47]. Activation of $A_{2B}R$ also promotes nitric oxide release from endothelium partially through a cAMP-dependent process, which has an inhibitory effect on fibrosis [48]. $A_{2B}R$ activation has also been shown to increase intracellular levels of calcium and activate calcium-dependent signaling pathways [22, 49]. Therefore, the anti-fibrotic effects of the $A_{2B}R$ may involve several different signal transduction

pathways acting simultaneously. Finally, $A_{2B}R$ activation may also influence collagen synthesis through indirect effects like suppression of cytokine synthesis [50]. Thus, A_{2B} receptors are protective against cardiac fibrosis, which is the opposite of their effects in other organs, and represents a potential therapeutic target in fibrosing cardiac diseases.

The A_{2B} receptor promotes renal fibrosis in the setting of hypoxia by induction of endothelin-1 expression [51]. Endothelin-1, in turn, promotes mesangial cell proliferation and collagen production in the kidney [52]. Notably, adenosine, signaling via $A_{2B}R$, also modulates the release of IL-6 and ET-1 in interstitial lung disease leading to collagen deposition, vascular remodeling, and, over time, pulmonary hypertension; this represents a convergent signaling pathway in fibrosis of the two organs [53]. The transcription factor hypoxia inducible factor (HIF) is also overexpressed in renal hypoxia and is in part responsible for the increased expression of $A_{2B}R$ in this setting [54]. Another transcription factor, cAMP receptor binding protein (CREB), also increases $A_{2B}R$ expression [55, 56]. As discussed above, the $A_{2B}R$ is a G_s -coupled protein receptor, which activates adenylyl cyclase and increases cAMP production; thus, this serves as a positive feedback mechanism wherein $A_{2B}R$ increases its own expression and promotes renal fibrosis in the setting of hypoxia. Importantly, bone marrow-derived cells involved in inflammation seen in early hypoxia co-express $A_{2A}R$ and $A_{2B}R$; however, $A_{2A}R$ has a much greater affinity for adenosine; thus, its effects likely predominate under physiologic conditions [57]. Under pathologic conditions, such as hyperglycemia and buildup of angiotensin II, local levels of adenosine increase and promote fibrosis by activation of $A_{2B}R$ on mesangial cells and fibroblasts. Selectively, inhibition of $A_{2B}R$ represents a novel therapeutic target in chronic kidney disease characterized by interstitial fibrosis and glomerulosclerosis.

Adenosine promotes elaboration of Col1 and Col3 by hepatic stellate cells (HSCs) in the liver via two different mitogen-activated protein kinase dependent (MAPK) pathways: extracellular signal-related kinase 1/2 (ERK1/2) and p38MAPK, as described above. It has also been shown to promote production of TGF-beta [58]. Recently, it has been demonstrated that adenosine, signaling via $A_{2B}R$, stimulates TGF-beta secretion from corpus cavernosum fibroblasts in an autocrine fashion, thereby promoting fibroblast proliferation and increased production of collagen [59]. This represents a common final pathway in hepatic and penile fibrosis. Interestingly, adenosine signaling also has an anti-fibrotic role in the liver. For example, occupancy of the $A_{2A}R$ blocks cytoplasmic increases of Ca^{++} in HSCs, which inhibits their chemotaxis by platelet-derived growth factor (PDGF). This serves as a potent stop signal for fibrosis [58]. This creates a system with a start signal (inflammatory chemokine or cytokine) and an independent stop signal (adenosine), allowing HSCs to stop at the site where they are most needed and maintain them there until hepatocyte apoptosis ceases and

adenosine levels fall. The adenosine stop signal is reversible so that after local injury has resolved, HSCs can respond to new chemokines in other locations. Additionally, hepatic ischemic preconditioning, which protects against fibrosis, is thought to involve adenosine by a mechanism wherein PKA phosphorylates $A_{2A}R$, which activates PI3K by coupling it with G α protein through Src [60]. This leads to downstream activation of PKB/Akt-mediated phosphorylation, which in turn inactivates the apoptosis promoting-factor BAD and glycogen synthetase kinase 3 [60]. Taken together, these pathways promote tolerance to hypoxia and lessen the stimulus for fibrosis. Adenosine, at least in the context of hypoxia, may initially be protective against the development of fibrosis but later contributes to its pathogenesis. Once the process is initiated, however, it may be part of a “breaking” mechanism to prevent further recruitment of HSCs and help promote its resolution.

Conclusion

Both adenosine A_{2A} and A_{2B} receptors signal for regulation of matrix production via classic G_s -mediated activation of cAMP and its downstream signaling molecules. The regulatory actions of the receptors likely involve multiple interconnected pathways, and one of the more interesting aspects of this regulation is the opposing effect of the different levels of cAMP generated. Whether this relates to incorporation of alternative signaling pathways at higher levels of cAMP or the activation of discrete signaling pathways at different sites in the cell remains a subject for further investigation. Additionally, adenosine signaling also contributes to fibrosis in organ-specific ways and, as with the case of $A_{2B}R$, may have opposite effects in different organs.

The deposition of collagen by fibroblasts is an essential part of wound healing but also contributes to pathologic remodeling of organs, which can lead to substantial morbidity and mortality. Many small molecules contribute to the pathogenesis of fibrosis including the purine adenosine which signals primarily through A_{2A} and A_{2B} receptors. These receptors help to reduce inflammation and promote wound healing, but excessive activation leads to architectural disruption and loss of organ integrity in a variety of fibrosing disorders. The development of drugs that selectively target these receptors will disrupt the pathogenesis of fibrosis and slow or arrest the progression of these important diseases.

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