Purinergic signaling in scarring

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ABSTRACT   Adenosine (ADO) and nucleotides such as ATP, ADP, and uridine 5′-triphosphate (UTP), among others, may serve as extracellular signaling molecules. These mediators activate specific cell-surface receptors—namely, purinergic 1 and 2 (P1 and P2)—to modulate crucial pathophysiological responses. Regulation of this process is maintained by nucleoside and nucleotide transporters, as well as the ectonucleotidases ectonucleoside triphosphate diphosphohydrolase [ENTPD; cluster of differentiation (CD)39] and ecto-5′-nucleotidase (5′-NT; CD73), among others. Cells involved in tissue repair, healing, and scarring respond to both ADO and ATP. Our recent investigations have shown that modulation of purinergic signaling regulates matrix deposition during tissue repair and fibrosis in several organs. Cells release adenine nucleotides into the extracellular space, where these mediators are converted by CD39 and CD73 into ADO, which is anti-inflammatory in the short term but may also promote dermal, heart, liver, and lung fibrosis with repetitive signaling under defined circumstances. Extracellular ATP stimulates cardiac fibroblast proliferation, lung inflammation, and fibrosis. $P2Y_2$ (UTP/ATP) and $P2Y_6$ [ADP/UTP/uridine 5′-diphosphate (UDP)] have been shown to have profibrotic effects, as well. Modulation of purinergic signaling represents a novel approach to preventing or diminishing fibrosis. We provide an overview of the current understanding of purinergic signaling in scarring and discuss its potential to prevent or decrease fibrosis.—Ferrari, D., Gambari, R., Idzko, M., Müller, T., Albanesi, C., Pastore, S., La Manna, G., Robson, S. C., Cronstein, B. Purinergic signaling in scarring. *FASEB J.* 30, 3–12 (2016). www.fasebj.org

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Tissue repair and wound healing are complex, dynamic, multistep processes tightly interwoven with the immune defense response. The replacement of devitalized or missing cellular structures and tissues can last for years, and the optimal outcome is the complete re-establishment of pre-existing tissue size and functional homeostasis (1). The reparative process starts at the first (bleeding) phase that occurs as a consequence of injury or trauma. The following (inflammatory) phase is essential to tissue repair. Inflammation occurs early, with a rapid increase in magnitude before a gradual decrease until resolution. During the next (proliferative) phase, generation of the cellular and noncellular repair material takes place.

Proper scar formation is not achieved until late in the overall repair process, and the so-called remodelling phase is responsible for the final organization and functional quality of the scar. The process of tissue repair can go awry, however, with two main consequences (2). If the reparative process is inefficient, the outcome will be insufficient healing, resulting in a hypotrophic or atrophic scar; in contrast, when tissue repair ends with an abundance of fibroblasts and matrix deposition, the improper outcome is a hypertrophic scar (if confined to the wound site) or generalized organ fibrosis, extending beyond the area of the original insult and often heavily impairing organ function. Two additional important aspects of noncanonical matrix deposition are reduced tissue resistance of mechanical stress, often accompanied by pain.

Fibrosis underlies many pathologic states initiated by traumatic insults. Keloids, for example, are markedly hypertrophic scars of the skin, but fibrosis also causes tendon adhesions, reduced or arrested nervous signal transmission after nerve injury, and partial or total organ loss of function, such as kidney or gut fibrosis. Localized or systemic fibrosis is the hallmark of different immune diseases, culminating in systemic sclerosis or scleroderma, with clinically significant vascular occlusion, major organ (lung, gut, and cardiac) dysfunction and diffuse skin fibrosis. Aberrant matrix deposition causing the intestinal fibrosis that results in formation of small intestine and colon stricture is

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the primary reason for surgical treatment in Crohn's disease (3). Replacement of damaged liver tissue with fibrotic tissue is the characteristic pathologic feature of liver cirrhosis caused by hepatitis B and C, alcohol abuse, and nonalcoholic fatty liver disease (4). Fibrosis necessitating surgery causes strictures and consequent impaired passage in the esophagus and urethra or formation of capsules surrounding breast implants (5).

Because extracellular matrix (ECM) synthesis and turnover are mainly caused by tissue fibroblasts, investigation of the molecular basis of fibrosis has been concentrated mostly on mediators that modulate fibroblast functions (2). Growth factors, such as angiotensin (ANG) II and TGFβ and its receptor connective tissue growth factor (CTGF), which are known to stimulate fibroblast proliferation, migration, and profibrogenic activity, are at the basis of the development of tissue fibrosis (6–8). Recent data show that the fibrotic process is under control of previously unrecognized cellular molecules. The Nod-like receptor family pyrin domain containing (NLRP)3 inflammasome, as well as Notch signaling, have been linked to fibrosis (9, 10). Compelling evidence points to aberrant expression of peroxisome proliferator–activated receptor-γ during fibrosis (11), and recent data have indicated the participation of a newly identified cell population potentially involved in lung fibrosis. Human adult lung tissue contains a population of perivascular mesenchymal stem cells (ABCG2pos), the precursors of myofibroblasts, which cause the detrimental matrix remodeling that is at the basis of pulmonary fibrosis (12). A recent contribution to a better understanding of fibrogenesis came from the comparison of the microRNA (miR) expression pattern in matched normal and hypertrophic scar samples. In particular, miR-200b appears to be down-regulated by more than 2-fold in hypertrophic scar tissues and human hypertrophic scar fibroblasts (13), by affecting the synthesis of collagen I and III, fibronectin expression, and TGF-β1/α-smooth muscle actin signaling and ultimately modulating proliferation and apoptosis of human hypertrophic scar fibroblasts (13). Another microRNA, miR-21, was shown to control fibroblast cell growth in hypertrophic scars by regulating hTERT expression *via* the phosphatase and tensin homolog (PTEN)/PI3K/protein kinase B (AKT) signaling pathway. However, despite these recent advances, fibrosis is still a critical pathologic condition, and new pharmacological compounds and strategies aimed to prevent and reverse the process are greatly needed (1, 2).

THE PURINERGIC NETWORK

Adenosine (ADO) and nucleotides, such as ATP, ADP, uridine 5′-triphosphate (UTP) and uridine 5′-diphosphate (UDP), are present both inside and outside living cells. In

the extracellular fluid, they mediate cell-to-cell communication (14). Receptors for extracellular nucleosides and nucleotides are currently classified as two subgroups: purinergic 1 (P1) receptors activated by extracellular ADO and P2 receptors activated by ATP and other nucleotides (15). The first group includes 4 receptor types: ADO receptor a (Adora)-1 (A_1), Adora 2 (A_{2A}), Adora 2B (A_{2B}), and Adora 3 (A_3), and the second comprises 7 P2X and 8 P2Y human subtypes. Increased levels of the purinergic receptor agonists ADP, AMP, and ADO can result from the activity of 2 membrane enzymes, the ectonucleoside triphosphate diphosphohydrolase (ENTPD; CD39), which converts ATP/ADP to AMP, and ecto-5′-nucleotidase (CD73), which converts AMP to ADO (16, 17) (**Fig. 1**). An increase in the extracellular concentration of ATP can be achieved by transport of the nucleotide through the plasma membrane by ATP-binding cassette transporters, connexin hemichannels, and pannexin channels (18–20); vesicular exocytosis; and extracellular synthesis *via* plasma membrane F(1)/F(0)-ATP synthase. ATP is included in the so-called damage-associated molecular patterns (DAMPs), because it is a normal intracellular constituent that is released into the extracellular milieu as a consequence of tissue damage, cell stress, or cell death. Physiologic release and degradation of ATP and related nucleotides must be tightly regulated to avoid perpetuation of the immune response, even in the absence of microbes (16, 17). A moderate, controlled release of ADO, ATP, or UTP exerts beneficial effects by activating reparative homeostatic responses, whereas the uncontrolled, prolonged release of these mediators induces excessive activation of immune and nonimmune cells, leading to increased, prolonged secretion of the inflammatory mediators [*i.e.*, prostaglandins, leukotrienes, and reactive oxygen intermediates (ROIs)] and proinflammatory cytokines that mediate tissue damage, induce massive recruitment of immune cells, and establish the chronic inflammatory conditions often accompanied by fibrosis.

Sequential CD39 and CD73 activity generates ADO in the extracellular milieu. Involvement of these enzymes in modulating tissue function during both physiologic and pathologic conditions has been appreciated in several tissues (18, 19). Recent evidence points to a role for ectonucleotidases in the fibrotic process, by reducing nucleotide concentration, thus preventing or reducing P2 receptor activation, and by generation of ADO, which in turn stimulates different P1 ADO receptor subtypes. The concentration of ADO in the extracellular milieu ranges from 100 to 500 nM and increases to levels in the low micromolar range in response to inflammation, hypoxia, and ischemia (20, 21). The P1 receptors interact with ADO in different manners. The high-affinity receptors A_1, A_{2A}, and A_3 are consistently activated by low concentrations (>10–50 nM) of extracellular ADO. In contrast, the low-affinity receptor A_{2B} is activated by much higher ADO concentrations, as in cell injury or cell death (>1 μM).

The nucleoside promotes tissue remodelling and repair, but depending on cell type and stimulated receptor subtype, it can favor nonregulated extracellular matrix elaboration, abnormal deposition, and misalignment of collagen (*i.e.*, fibrotic responses in liver, lung, and skin); in contrast, it decreases heart fibrosis (8, 22) (**Table 1**). Herein, we report on recent advancements in elucidating the contribution of
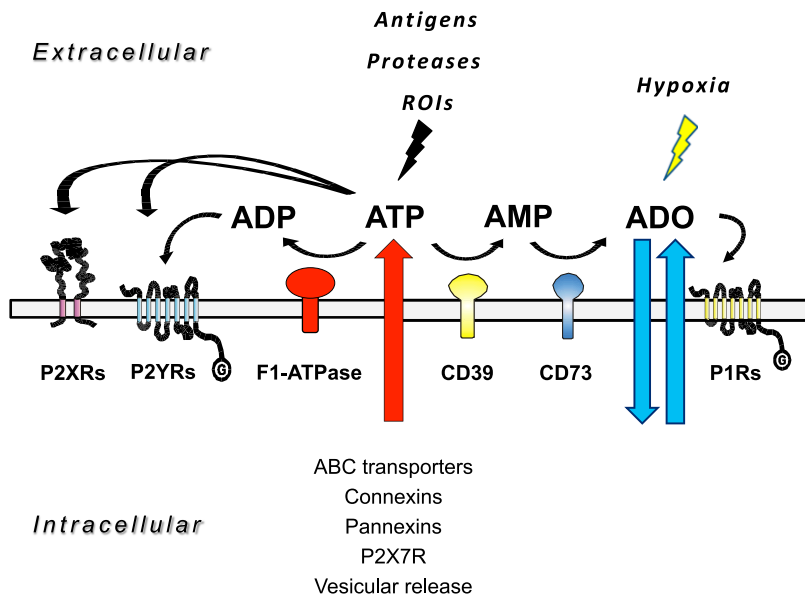
Figure 1. Purinergic signaling network. ADO and ATP are released in the extracellular compartment by different means. Other than by lesions of cell membranes with injury, ADO is released by means of equilibrium transporters, whereas ATP escapes *via* vesicles, connexins, pannexins, ABC transporters, and P2X$_7$ receptors. Both ADO and ATP function as signaling molecules *via* activation of the P1 receptor (A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$) and the P2 receptor (P2X and P2Y) subtypes, respectively. In the extracellular space, ADO is inactivated by ADA to inosine, ultimately to form hypoxanthine, by deribosylation. P1 receptor-mediated signaling is also terminated by cellular uptake of ADO by the equilibrative nucleoside transporters ENT1 and -2. In contrast, ATP is metabolized by enzymatic phosphohydrolysis in a 2-step process *via* CD39 conversion of ATP (or ADP) to AMP and CD73 phosphohydrolysis of AMP to ADO.

molecular components of the purinergic signaling network to the genesis of fibrosis.

PURINERGIC MODULATION OF THE IMMUNE SYSTEM IN WOUND HEALING AND FIBROSIS

Fibrosis occurs in tissues that have frequent exposure to chemical and biologic insults that provoke chronic stimulation of immune and nonimmune cells of the injured organs. Immune cells participate in a coordinated series of defensive and tissue repair events with the main objective of isolating and destroying nonself molecules and bodies. Innate immune functions, including chemotaxis, phagocytosis,

and collagen degradation and remodeling, ultimately leading to neoangiogenesis and organ repair or re-epithelization, are crucial for reaching this goal.

An important ability of immune cells is to detect and discriminate between self and nonself antigens, but also to become activated when normal intracellular constituents are liberated extracellularly as a consequence of cell stress or damage (21). Among the latter, nucleosides and nucleotides are important activators of immune responses, once they reach proper extracellular concentration. Therefore, expression of P1 and P2 receptors by immune cells enables them to detect the release of diverse ADO and ATP concentrations, thus continuously monitoring tissue stress or injury arising from disparate causes (18, 23). Neutrophil extravasation and

TABLE 1. *Effects of single purinergic receptors or ectonucleotidases on tissue and organ fibrosis*

Receptor	Agonist	Stimulus	Tissue	Profibrotic	Antifibrotic	Species	Reference
A$_{2A}$	ADO		Heart	+			73
		UUO	Kidney		+	M	88
		CCl$_4$, TAA	Liver	+		M	100
		Ethanol	Liver	+		M	99, 101
		CG, PDF	Peritoneum	+		M	109
			Skin	+		H, M	62, 71, 73, 111
A$_{2B}$	ADO		Heart		+	M	72, 111, 114
			Kidney	+		M	90, 91
			Liver	+		H	102, 103
			Lung	+		H	58
A$_3$	ADO	UUO	Kidney	+			92
P2X$_7$	ATP	CCl$_4$	Liver	+		M	98
		Bleomycin	Lung	+		M	51
		BzATP	Kidney	+		R, M	86, 87
P2Y$_2$	ATP		Lung	+		H	55
P2Y$_4$	UDP		Lung	+		H	56
CD39		Bleomycin	Skin	+		M	71
CD73		CCl$_4$, TAA	Liver	+		M	104
			Lung	+		H	58
		Bleomycin	Skin	+		M	8, 71

CG, chlorhexidine gluconate; H, human; M, mouse; PDF, peritoneal dialysis fluid; R, rat; TAA, thioacetamide.

infiltration of wounded tissues is an early crucial response that protects wounds from invading microbes; however, excessive neutrophil infiltration and the consequent prolonged release of proinflammatory cytokines, proteolytic enzymes, and ROIs leads to abnormal tissue repair and chronic nonhealing wounds. Therefore, the role of neutrophils in wound healing is still debatable and, according to recent data obtained in mice, the cells tend to inhibit tissue repair (24).

Bacterial constituents stimulate ATP release and IL-8 production from neutrophils and lead to further chemo-attraction of these cells (24). Neutrophils are also attracted by the ATP and ADP released during platelet clotting (25). Cooperation between P1/P2 receptors and ectonucleotidases (particularly $P2Y_2$, A_3 subtypes, and CD39) is involved in sensing and producing receptor ligands, thus modulating the speed of neutrophil migration (26–29). Macrophages are also a prominent cell population in wounds, and besides the important defensive function, they remove apoptotic cells and prompt cell proliferation and matrix elaboration after an injury. Because of their phenotypic plasticity, macrophages have been compared with lymphocytes. They acquire the M1 proinflammatory phenotype to counteract microbe invasion, whereas they become M2 reparative and immune suppressive, to complete the wound-healing process and therefore represent an important target in promoting wound healing and reducing fibrosis (30). Many responses induced by ADO have been shown in macrophages, among them secretion of pro- and anti-inflammatory cytokines and mediators and even modulation of the antigen-presenting function (31). The A_{2A} receptor synergizes with Toll-like receptor-2, -4, -7, and -9 to switch macrophage activity from defensive to proangiogenic and reparative, thus contributing to tissue repair (32, 33). A fibroblast-secreted protein, osteopontin (OPN), has been convincingly shown to act as a potent profibrotic factor. Macrophages and mast cells induce expression of fibroblast OPN by secretion of platelet-derived growth factor (34). The $P2X_7$ subtype is a central proinflammatory macrophage ATP receptor, the triggering of which induces secretion of IL-1β, IL-18, prostaglandin E_2 (PGE_2), phosphatidylserine, and ROIs by fibroblasts (35). $P2X_7$ activation has been linked to inflammation and defense against microbes and, recently, to fibrosis. Accordingly, bleomycin-treated $P2X_7$-knockout (KO) mice show reduced inflammation and lung fibrosis (36). Activation of this receptor in mice is linked to silica-induced lung inflammatory and fibrotic response. Mice lacking the receptor show lower infiltration of inflammatory cells and collagen deposition in lung parenchyma (37).

Cells of the adaptive immune response also appear in the wound tissue; however, their role in healing is likely less important. In T-cell population–depletion studies performed in mice, T-helper lymphocytes had no effect on wound healing, whereas activity of T-suppressor and cytotoxic lymphocytes was inhibitory, as depletion of these cells increased collagen synthesis and improved wound resistance (38). Lymphocyte involvement in fibrosis is under investigation. In systemic sclerosis, patients subjected to a therapy based on anifrolumab, a monoclonal antibody targeting IFN-α and -β receptors, undergo suppression of T-lymphocyte activation and a decrease in collagen accumulation (39). Another example of fibrosis in which T lymphocytes are likely to play an important role is encapsulating peritoneal

sclerosis [*i.e.,* an excessive fibrotic response that occurs in the peritoneum after long-term peritoneal dialysis]. In this case, inflammatory T helper 1 cells may play a role (40).

It has been shown recently that stimulation of the A_3 receptor in a human mast cell line down-regulates expression of this subtype and heavily affects the gene expression profile of these cells. Both A_3 stimulation and knockdown lead to upregulated expression of genes involved in tissue remodeling (*i.e.,* IL-6, IL-8, VEGF, amphiregulin, and OPN) (41). An interesting point that has yet to be addressed is the increased presence of mast cells in some fibrotic tissues and whether their contribution is profibrotic *via* release of proteases and digestion of wound proteins, with the production of profibrotic peptides. This hypothesis seems to be confirmed by the high chymase gene expression and activity in mast cells that are present in keloids, compared with normal skin, and by the fact that chymase promotes proliferation of fibroblasts and collagen synthesis by activating TGF-β1 in keloids (42). Thus, the type and degree of inflammation has a critical impact on scar formation and fibrosis. Therefore, a deeper understanding of inflammation would be likely to lead to an enhanced capacity to counteract fibrosis.

LUNG SCARRING AND FIBROSIS, CHRONIC OBSTRUCTIVE PULMONARY DISEASE, AND PURINERGIC SIGNALING

Pulmonary fibrosis is a chronic disease of the lung characterized by tissue fibrosis with consequent loss of elasticity, shortness of breath, and the presence of inflammatory infiltrate, which can develop after an increased immune response to inhaled organic molecules, such as those from bacteria, fungi, mites, parasites, or occupational chemicals (43). In contrast, idiopathic pulmonary fibrosis (IPF) is a lung disease of unknown etiology, with an even greater degree of fibrosis, resulting in a very poor prognosis. Though some progress has been made, there are few therapeutic options available at the moment (44–46). Although the persistence of inciting pathologic agents and the consequent chronic inflammatory state have been suggested to be prerequisites to different forms of pulmonary fibrosis, the situation is likely to be more complex for IPF, because of the poor efficacy of standard anti-inflammatory therapies, including corticosteroids (47). In chronic obstructive pulmonary disease (COPD) consisting of chronic inflammation, oxidative stress, and bronchial obstruction, increased ECM deposition underlying the bronchial epithelium produces fibrosis and thickening of the airway wall (48).

Cells of the human respiratory system express receptors for extracellular nucleosides and nucleotides (49). Lung hypoxia causes ATP release, thus enhancing adventitial fibroblast proliferation and transformation to myofibroblasts (50). Increased ATP in bronchoalveolar lavage fluid has been found, both in patients with IPF and in animals with experimental lung fibrosis (51). Similar observations have been made in individuals or animal models of COPD (52, 53). In experimental lung fibrosis induced by the antitumor drug bleomycin, ATP, through the connexin-1/$P2X_7$ receptor axis, induces inflammatory cell recruitment and lung fibrosis by stimulating IL-1β secretion and tissue inhibitor of metalloproteinase (TIMP)-1 production (51). Moreover, release of ATP is paralleled by that of other

intracellular molecules. In bleomycin-induced lung fibrosis, uric acid released by damaged dying cells represents an additional DAMP that activates NLRP3 with subsequent IL-1β release, inflammation, and fibrosis (54). That ATP is a crucial trigger of epithelial stress-induced inflammation and fibrosis has been convincingly shown by various means. The ATP-degrading enzyme apyrase greatly reduces bleomycin-induced inflammation, as does the absence of the $P2X_7$ subtype. $P2X_7$-KO mice show much less lung inflammation and fibrosis markers (lung collagen content, and the matrix-remodeling proteins TIMP-1 and matrix metalloproteinase-9) than do wild-type mice (51).

P2Y receptors are involved in the repair of human airway epithelia. The $P2Y_2$ subtype induces TNFα-converting enzyme activation, which then releases the membrane-bound ligands of the epidermal growth factor receptor, thus inducing cell migration and proliferation (49, 55). However, P2Y receptor signaling is most likely also involved in lung fibrosis, as in vitro stimulation of human lung fibroblasts by nucleotides increases the expression of $P2Y_4$, TGF-β, collagen A_1, and fibronectin (55). ADO receptors are expressed in the lung, where they regulate inflammation and airway remodeling by favoring differentiation of lung fibroblasts into myofibroblasts that are typically found in fibrotic tissues (56). Subjects with COPD and IPF show an altered ADO metabolism (57). CD73 and A_{2B} receptor expression is increased in surgical lung biopsies of patients with severe COPD or IPF. Moreover, the profibrotic mediators IL-6, IL-8, and OPN are increased in these samples (58, 59). The profibrotic activity of ADO in the lung has been elegantly shown in mice deficient in adenosine deaminase (ADA), the enzyme that transforms ADO to inosine, which is inactive at ADO receptors, thus eliminating the P1 receptor agonist from the extracellular milieu. Hence, the absence of ADA induces ADO-dependent pulmonary fibrosis in these animals (60). Furthermore, a recent study demonstrated that A_{2B} receptor expression on myeloid cells is necessary for the development of pulmonary fibrosis and pulmonary hypertension in response to bleomycin (61). Consistent with these findings, ADO has been shown to mediate many of the anti-inflammatory effects of low-dose, weekly methotrexate treatment, which is used to treat rheumatoid arthritis and psoriasis (62, 63). A feared side effect of this therapy is pulmonary fibrosis, and it is possible that ADO release in the lung plays a role in the pathogenesis of this drug toxicity as well. Therefore, in the respiratory system, dysregulation of purinergic signaling with activation of ATP and ADO receptors appears to contribute substantially to the development of fibrosis.

SKIN REPAIR AND SCARRING ARE MODULATED BY P2 AND P1 RECEPTORS

Keloids are a classic example of the skin fibrosis that occurs during wound healing. They are different from hypertrophic scars, as they do not contain myofibroblasts (64). Fibroblasts that are present in keloids produce greater concentrations of collagen, probably because of increased expression of TGF-β and TGF-β receptor. Skin cells express purinergic receptors (65), and the gene expression profile of ATP-treated keratinocytes reveals marked changes (66). If it is obvious that, upon acute epidermal injury (e.g., lacerations, trauma, burns, or abrasions or after tape stripping), ATP is released from damaged keratinocytes (67), it is less intuitively obvious that ATP is normally released by keratinocytes during epidermal homeostasis and in response to mild mechanical and thermal stimuli. It is even less obvious that air stimulation induces release of ATP from human neonatal keratinocytes in vitro (68). Moreover, opening of pannexin hemichannels in keratinocytes as a consequence of nonmetal hapten stimulation causes release of ATP (69).

The positive effects of ADO on wound healing have been described. The nucleoside promotes wound neovascularization by stimulating the A_{2A} receptor (70). However, excessive ADO generation by CD39 and CD73 promotes dermal fibrosis (8, 71), activating the same receptor that has been recognized as a fine-tuned regulator of the collagen1:collagen3 balance (72). A_{2A}-stimulated collagen secretion by human fibroblasts occurs via at least 2 pathways: a cyclic AMP- and AKT-dependent pathway (73) and a Fli1- and CTGF-mediated mechanism (74). Despite previous failures of A_{2A} receptor agonists to promote wound healing in patients with diabetic foot ulcers (unpublished data), a more recent study indicates that the ADO A_{2A} receptor agonist polydeoxyribonucleotide effectively promotes wound healing of diabetic foot ulcers better than a placebo (75).

The demonstration that A_{2A} agonists could promote wound healing, including matrix production in the wound, suggests that A_{2A} receptors play a role in fibrosis (73). The pivotal role of ADO and A_{2A} receptor in skin fibrosis has been elegantly shown in ADA-deficient mice. These animals have increased levels of TGF-β1, CTGF, and IL-13, which are accompanied by increased collagen deposition and fibrosis. Accordingly, the A_{2A} receptor antagonist ZM-241385 prevents the development of dermal fibrosis in ADA-KO mice (76). Reported observations show that purinergic signaling modulates skin physiology and fibrotic response, identifying novel opportunities for the development of purinergic-based treatments to prevent or block dermal fibrosis.

KIDNEY TRANSPLANT, FIBROSIS, AND SCARRING: WHAT IS THE ROLE FOR PURINES?

Kidney fibrosis is present in most forms of chronic renal disease, and progressive accumulation of ECM often leads to renal failure, necessitating dialysis or kidney transplantation (77). Major cellular actors in renal fibrosis are kidney fibroblasts and mesangial cells under the control of the renin–angiotensin–aldosterone system or of TGF-β1/bone morphogenic protein-7. Renal injury often starts with monocyte/macrophage and T-lymphocyte infiltration, leading to chronic inflammation and eventually to glomerulosclerosis, tubulointerstitial fibrosis, and loss of renal parenchyma (77, 78). Polarization of macrophages seems to be central in kidney fibrosis in mice. In particular, macrophages polarize to an M2 subtype after renal injury; M2 macrophages secrete high levels of TGF-β1 levels and promote ECM accumulation (79). Subclinical inflammation is also a consistent risk factor for the development of the interstitial fibrosis associated with chronic humoral rejection of kidney grafts (80). Accordingly, initial fibrotic levels in the transplanted kidney (significantly correlating with donor

age) strongly affects graft outcome (81, 82). Among the factors contributing to renal fibrosis, nucleotides have been shown to play an important role. Purinergic receptors are expressed in the kidney and are involved in modulating physiologic organ functions, such as vascular tone and glomerular pressure (83). Different stimuli induce ADO and ATP release in the kidney, and the absence of CD73 in mice diminishes renal function and induces autoimmune inflammation (84). Responses induced by ATP, UTP, and the pharmacological ATP analog 2′(3′)-O-(4-benzoylbenzoyl)ADO-5′-triphosphate (BzATP) have been shown in rat mesangial cells. Whereas ATP and UTP induce cell proliferation via $P2Y_2$ and $P2Y_4$ receptors, stimulation of the $P2X_7$ receptor by BzATP causes cell death (85). A role for the $P2X_7$ subtype in kidney fibrosis has also been hypothesized (86). Opposing effects of P2Y and P2X receptors on TGF-β and collagen production have also been reported. ATP and BzATP increase TGF-β and collagen secretion, and UTP decreases them (87). ADO-mediated responses in the kidney are also complex and intriguing because, depending on the type of P1 receptor subtype stimulated, they can induce both beneficial and detrimental effects. Although the nucleoside plays a positive role in protecting the organ tissue during ischemia, the chronic profibrotic effect of ADO has also been shown by different groups by means of the unilateral ureteral obstruction (UUO) model in mice (88). Thus, in this model, there is renal tissue hypoxia and ADO release. Stimulation of A_{2A} receptor reduces renal damage, down-regulates expression of fibrosis markers, and decreases collagen deposition (88). Similarly, activation of the A_{2A} subtype down-modulates macrophage-mediated inflammation, which plays a central role in the pathogenesis of crescentic glomerulonephritis in Wistar-Kyoto rats (89). The role of the A_{2B} receptor in kidney inflammation and fibrosis is opposite that of the A_{2A} receptor, as stimulation of the A_{2B} receptor induces IL-6 secretion (90), and its inhibition during hypoxic conditions reduces proliferation and secretion of profibrotic cytokines (91). The role of the A_3 subtype has not been well investigated in kidney fibrosis; so far, its activation has been linked to UUO-induced tubulointerstitial fibrosis in mice (92).

LIVER AND GUT REGENERATION, REMODELING, AND SCARRING ARE MODULATED BY NUCLEOTIDES

Liver fibrosis is caused by the hepatic chronic injury often accompanied by inflammation (93). Among the initial damaging agents are viral infections (hepatitis B or C viruses), alcohol abuse, accumulation of iron in hemochromatosis or copper in Wilson disease, and biliary obstruction (94). Excessive collagen accumulation can lead to portal hypertension and loss of the canonical hepatic architecture (cirrhosis). Advanced cirrhosis causes liver failure, often resulting in liver transplantation.

Long-term liver fibrosis has also been linked to the development of hepatocellular carcinoma (95). Myofibroblasts, portal fibroblasts, and hepatic stellate cells have been identified as major players in abnormal matrix deposition and among the fibrogenic signals, leptin, ANG II, and TGF-1β, are crucial in hepatic fibrosis (96).

Liver cell functions are modulated by nucleotides and nucleosides (97). Involvement of the $P2X_7$ receptor in the pathogenesis of carbon tetrachloride (CCl_4)-induced liver fibrosis has been described in mice (98). Subcutaneous injection of this chemical up-regulates $P2X_7$ expression and increases collagen deposition, whereas inhibition of this subtype by the specific antagonist A438079 reduces TGF-β1 secretion and collagen formation (98).

ADO also plays a role in liver fibrosis, as triggering of the A_{2A} receptor induces expression of collagen by stellate cells (99), and inhibition or deletion of this subtype prevents CCl_4-, thioacetamide-, and ethanol-induced fibrosis in mice (100, 101). ADO-mediated effects are dependent, at least in part, on A_{2B} activation, as the receptor antagonist MRS1754 reduces liver fibrosis, as well (102, 103). CD73 has been linked to hepatic fibrosis for its ability to produce extracellular ADO (104). However, participation of this ectonucleotidase in healing appears complex, given that the knockdown of CD73 message induces an increase in collagen I and augments cell migration in stellate cells (105). Another interesting observation is that CD73 gene expression increases during myofibroblastic differentiation (106).

Of note are recent epidemiologic observations indicating major protective effects of coffee in the setting of progressive liver fibrosis and cirrhosis (107). Laboratory studies suggest that these salutary effects are mediated by stellate cell expression of A_{2A}, where caffeine may serve as an inhibitor. In a similar vein, low-dose weekly methotrexate has been used for the treatment of psoriasis and rheumatoid arthritis for many years, and prior studies have indicated that ADO accounts for many of the anti-inflammatory effects of this drug (63). One well-described toxicity of methotrexate therapy is hepatic fibrosis; it is possible that ADO mediates this effect as well.

An emerging role for purinergic signaling in the evolution of gut fibrosis and stricturing has also been suggested. $P2Y_2$ receptor is highly expressed by rat intestinal myofibroblasts, and ATP stimulation has been shown to stimulate cellular activation and contraction (108).

MODULATION OF PERITONEAL AND CARDIAC FIBROSIS BY ADO

Peritoneal fibrosis is a common complication of chronic peritoneal dialysis or surgery and may lead to diminished efficacy of peritoneal dialysis, development of intestinal adhesions, and obstruction. Recent work indicates that either pharmacological blockade or deletion of ADO A_{2A} receptors can diminish peritoneal fibrosis (109). In contrast, others have reported that topical application of ADO and phosphorylated ADO derivatives could, by acting via ADO receptors, diminish peritoneal adhesions in a rodent model (110). Recent work suggests an explanation for these diametrically opposed findings with respect to the effects of ADO A_{2A} and A_{2B} receptor-mediated promotion and inhibition of collagen production and fibrosis (111). Of interest, A_{2A} receptors stimulate a modest increase of cAMP, which inhibits collagen production, but that the much higher cAMP levels induced by forskolin (and potentially A_{2B} receptors) suppresses collagen production (72). Thus, chronic A_{2A} stimulation leads to fibrosis, but stimulation of A_{2B} receptors, which requires higher levels of endogenous

ADO for stimulation, inhibits collagen production. Moreover, in contrast to other tissues, the preponderance of evidence indicates that the principal P1 receptor subtype in the heart that regulates cardiac fibroblast function is the A_{2B} receptor. In 1986, Dubey *et al.* (112) first reported that ADO inhibits cardiac fibroblast collagen production *via* stimulation of A_{2B} receptors. Later work further indicated that maneuvers that increase intracellular cAMP diminish collagen production by cardiac fibroblasts (113, 114). This observation led to the demonstration of the homeostatic role of ATP hydrolysis, by the action of ENTPD (CD39) in diminishing collagen production by increasing extracellular ADO levels, which act at A_{2B} receptors to raise cAMP and diminish fibroblast production of collagen (115). Similarly, hydrolysis of AMP by CD73 on the surface of immune cells protects against cardiac fibrosis (116). These studies further suggest that regional and tissue differences in the expression of A_{2B} receptors can lead to opposing effects of ADO on fibrosis.

CONCLUSIONS AND FUTURE PERSPECTIVES

Fibrosis underlies different pathologic conditions that, because of disfigurement, may lead to psychologic, social, and economic problems, but can also cause organ failure and increased risk of mortality (117). It is therefore crucial to find new therapeutic solutions, especially for diffuse organ fibrosis and for those fibrotic states, such as IPF, that do not respond to medical therapy.

It has become clear that excessive ligand-mediated activation of specific nucleoside and nucleotide receptor subtypes as a consequence of uncontrolled ligand release gives the tissue microenvironment a surplus of signals, promoting abnormal replication of smooth muscle cells, fibroblasts, and myofibroblasts, with pathologic matrix deposition causing fibrosis. Recent reports demonstrate the significant contribution of extracellular nucleosides and nucleotides in promoting abnormal matrix production (8, 55, 62). However, at present, this field is at an initial stage and many developments have still to be made. Moreover, relevant scientific and clinical questions are awaiting an answer. A central question dogging the field is why the same receptor subtype can have both anti- and profibrotic effects, depending on tissue expression.

Therefore, collaboration between purinologists and clinicians (dermatologists, rheumatologists, gastroenterologists, cardiologists, and pulmonologists) may open unexpected opportunities for deeper investigation of this subject and, more important, for development of treatments of fibrotic diseases. FJ

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