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Commentary

Adenosine receptor agonists for promotion of dermal wound healing

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ARTICLE INFO

Keywords:
Adenosine receptors
Impaired healing
Inflammation
Diabetic foot ulcer
Angiogenesis
Granulation tissue

ABSTRACT

Wound healing is a dynamic and complex process that involves a well-coordinated, highly regulated series of events including inflammation, tissue formation, revascularization and tissue remodeling. However, this orderly sequence is impaired in certain pathophysiological conditions such as diabetes mellitus, venous insufficiency, chronic glucocorticoid use, aging and malnutrition. Together with proper wound care, promotion of the healing process is the primary objective in the management of chronic poorly healing wounds. Recent studies have demonstrated that A_{2A} adenosine receptor agonists promote wound healing in normal and diabetic animals and one such agonist, Sonedenoson, is currently being evaluated as a prospective new therapy of diabetic foot ulcers. We will review the mechanisms by which adenosine receptor activation affects the function of the cells and tissues that participate in wound healing, emphasizing the potential beneficial impact of adenosine receptor agonists in diabetic impaired healing.

1. Introduction

The mechanisms underlying the normal repair process, cell migration and proliferation, and extracellular matrix deposition and remodelling, have been extensively studied [1–3]. Cellular responses to inflammatory mediators, growth factors, cytokines, and to mechanical forces must be appropriate and precise in order to obtain optimum healing of a cutaneous wound. However, even during the normal process of wound healing complications can occur, including infection, thrombosis, and ischemia [4,5]. More importantly, the orderly progression of the healing process is impaired in chronic wounds, including those due to diabetes.

Impaired wound healing is a major concern for diabetic patients because their wounds do not heal properly and are a source of major suffering and cost. Only two-thirds of diabetic foot ulcers eventually heal and up to 28% may result in amputation [6]. The pathogenesis of diabetic foot ulcers is complex and it is well recognized that a number of contributory factors working together ultimately lead to impaired healing. Several intrinsic factors, such as peripheral neuropathy, foot deformity, peripheral vascular disease and peripheral oedema have been identified as the commonest factors responsible of impaired healing after trauma. In addition, extrinsic factors, such as wound infection, callus formation, and excessive pressure to the site, further aggravate the healing process [7].

Recent studies suggest that nerves play a central role in tissue homeostasis and can orchestrate complex reparative as well as destructive processes. First, an intact nociceptor system of primary afferent sensory nerves is important for

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.11.002

the initiation of the inflammatory process and successful tissue repair [8]. Apart from the loss of pain perception, which is a key factor in the development of neuropathic foot ulcers, loss of autonomic function and small fibre neuropathy can result in impaired neurogenic control of local microcirculatory blood flow, impaired fluid homeostasis, diminished energy metabolism, oxygen delivery, and inflammatory responses. These processes could render the feet of diabetic patients with neuropathy more susceptible to tissue damage and infection [9].

Given the complexity of the pathogenesis of diabetic foot ulcers, many different interventions have been proposed to accelerate the healing process, but few have been subjected to formal evaluation. Despite the relatively large number of studies of growth factors like PDGF (becaplermin), EGF, basic FGF and other agents modulating aspects of wound physiology, there is currently little evidence to suggest that any of the reported interventions should be adopted in routine practice. Diabetic foot ulcer management is based on the simple principles of eliminating infection, debridement, cleansing and the use of dressings to maintain a moist wound bed, and lastly, becaplermin is the only promoting agent approved for use of those ulcers resistant to simpler interventions [6].

Based in studies carried out in vitro and in experimental animal models, we are proposing a new strategy for the promotion of impaired wound healing, the use of adenosine receptor agonists. We will summarized the biology of adenosine and review its actions on different tissues and cells implicated in the healing of cutaneous wounds, as well as its effect on experimental wounds in animals.

2. Purine metabolism and biology

Adenosine is a ubiquitous purine nucleoside produced by stepwise dephosphorylation of ATP by the coordinated action of ecto-apyrase (CD39) and ecto-5′-nucleotidase (CD73) (Fig. 1). While extracellular ATP and other nucleotides (ADP, UTP and UDP) have many biological effects through direct activation of cell surface receptors for adenine nucleotides (seven P2X ionotropic and eight P2Y metabotropic receptor subtypes), adenosine modulates cellular and organ function via occupancy of four specific cell surface receptors (A_1, A_{2A}, A_{2B} and A_3), all members of the large family of 7-transmembrane spanning, heterotrimeric G protein-associated receptors [10,11]. The A_1 and A_3 adenosine receptors coupled with Gi proteins are associated with two effector systems, namely, adenylate cyclase and phospholipase C. The binding of adenosine or its agonists to A_1 and A_3 adenosine receptors either induce inhibition of adenylate cyclase leading to a decrease in intracellular cAMP levels or stimulate phospholipase C and the release of intracellular Ca^{2+}. A_{2A} and A_{2B} receptors are associated with Gs proteins and their activation stimulates an increase in intracellular cAMP. In addition, they couple to mitogen-activated protein kinases (MAPK), which may give them a role in cell growth, survival, death and differentiation [12]. The affinity of selected agonists at the different adenosine receptor subtypes is summarized in Table 1.

Extracellular actions of purines in non-neuronal cells, including fast signalling roles in exocrine and endocrine secretion, platelet aggregation, cardiovascular effects and kidney function, have been known for a long time. More recently, slow purinergic signalling has been implicated in embryological development, wound healing, restenosis, atherosclerosis, ischemia, cell turnover of epithelial cells in skin and visceral organs, inflammation, neuroprotection and cancer [13,14].

More interestingly, purines and pyrimidines have major roles in the activities of neurons. This includes nociceptive mechanosensory transduction, as well as acting as a cotransmitter and neuromodulator in most, if not all, nerve types in the peripheral and central nervous systems [13]. This raises the innovative hypothesis that diabetic patients suffering from peripheral neuropathy will have altered purinergic neurotransmission.

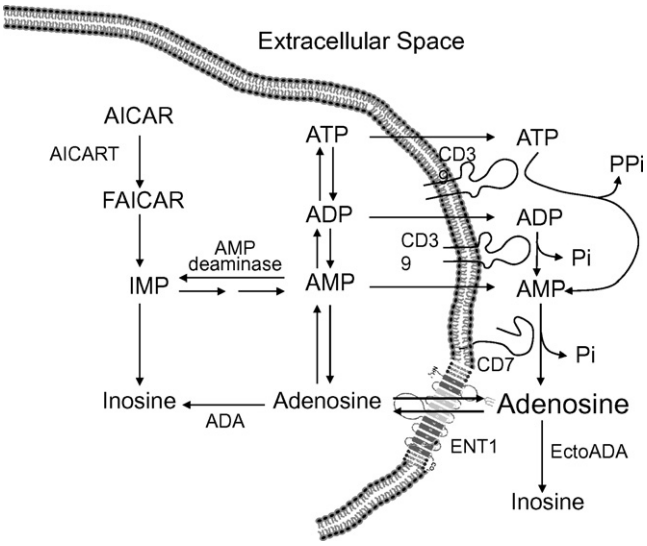

Fig. 1 – Adenosine metabolism. AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AICART, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; FAICAR, formyl 5-aminoimidazole-4-carboxamide ribonucleotide; ADA Adenosine deaminase; EctoADA Ectoadenosineaminase; CD73 Ecto-5′-nucleotidase; CD39 ecto-nucleoside triphosphate diphosphohydrolase (NTPDase); ENT1 equilibrative nucleoside transporter 1.

3. Could adenosine play a role in normal wound healing?

Under basal conditions, the extracellular adenosine concentration is rather constant (30–300 nM), and held in tight check by the equilibrium between adenosine production/release into the extracellular space and adenosine uptake by cells or catabolism to inosine (Fig. 1). In contrast its concentration can increase dramatically to micromolar or even higher ranges when there is an imbalance between energy use and energy supply, such as in oxygen depletion, or under conditions of cellular or tissue necrosis or stress as a result of ATP catabolism [12].

Table 1 – Binding affinity of selected adenosine receptor agonists at the four receptor subtypes.

Ki (nM)	A_1	A_{2A}	A_{2B}	A_3
Adenosine* [77]	310 (EC_{50})	700 (EC_{50})	24,000 (EC_{50})	290 (EC_{50})
2-Cl-adenosine [11]	1.39	180	N.D.	19
NECA [11]	14	20	330	6.2
CPA [11]	2.3	790	21,000	43
CGS21680 [11]	290	27	361,000	67
ATL-146e [14]	77	0.5	N.D.	45
Sonedenoson (MRE0094)** [66,72]	>10,000 (IC_{50})	490 ± 50 (IC_{50})	>10,000 (IC_{50})	N.D.
IB-MECA [77]	3.7	2500	54,000	1.2

Ki, Dissociation constant of unlabeled compounds in radioligand competition experiments at recombinant human A_1, A_{2A}, A_{2B} and A_3 adenosine receptors in Chinese hamster ovary (CHO) cells.
N.D. Not determined or not disclosed.
NECA 5′-N-ethyl-carboxamidoadenosine.
CPA N6-cyclopentyladenosine.
CGS 21680 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine.
ATL146e 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester.
Sonedenoson (MRE0094) 2-[2-(4-chlorophenyl)ethoxy] adenosine.
IB-MECA N6-(3-iodobenzyl)adenosine-5′-N-methyluronamide.
* Adenosine data from a cyclic AMP functional assay in CHO cells stably transfected with recombinant human A_1, A_{2A}, A_{2B} and A_3 adenosine receptors.
** Data provided by King Pharmaceuticals at a variety of receptor systems by radioligand-binding studies.

All cell subtypes involved in wound healing, macrophages, epidermal cells, fibroblasts and microvascular endothelial cells, differentially express functional adenosine receptors, although the receptor expression patterns vary between cellular types and have not been fully established. Moreover, even the same cellular type such as endothelial cells express different adenosine receptor subtypes depending on the vascular bed of origin [15]. Adenosine A_{2A} receptors, in particular, are expressed on most cell types involved in wound healing, including macrophages, fibroblasts and microvascular endothelial cells [15–17]. We have reported that A_{2A} receptor-deficient mice suffer from disordered wound healing with poor matrix formation and diminished blood vessel formation in the granulation tissue of excisional wounds and mechanically injured skin, an observation that indicates a role for adenosine, acting at A_{2A} receptors, in normal wound healing [18]. We have further observed that cytokines released during the inflammatory phase of wound healing, TNF-α and IL-1, up-regulate adenosine A_{2A} receptor expression in human monocytoid cells (THP-1) and microvascular endothelial cells [16,19,20]. Similarly, A_{2B} receptor expression in murine bone marrow-derived macrophages is also up-regulated by IFN-gamma [21]. The differential adenosine receptor expression in cells from diabetic patients has not been established yet. In this regard, streptozotocin-induced diabetes in rats altered adenosine receptors expression in liver, heart and kidney and administration of insulin returned their levels to normality [22].

4. Adenosine in inflammation

Hemostasis and inflammation constitute the first phase of tissue repair. The formation of a blood clot re-establishes tissue hemostasis and provides a provisional matrix for cell migration. Released cellular mediators initiate inflammatory leukocyte recruitment necessary for removing necrotic tissue and preventing infection [1]. In addition, inflammatory cells release a variety of cytokines and chemokines that play an important role in the evolution of granulation tissue through stimulation of fibroblasts and epithelial cells [4]. However, despite its importance, persistent or exaggerated inflammation could be detrimental for the healing process in some pathological settings. In this respect, the concept that diabetes is a low-level chronic inflammatory disease is commonly accepted [23,24].

Numerous studies indicate that adenosine, through the activation of its different receptor subtypes, is a potent regulator of inflammation and innate immunity [25,26]. In fact, genetic deficiency in adenosine deaminase (ADA), the enzyme responsible of the deamination of adenosine to its less potent derivative inosine (Fig. 1), is characterized by a severely compromised immune system [27]. Moreover, several studies have established that adenosine mediates the anti-inflammatory effect of mainstay anti-rheumatic drugs such as methrotexate and sufasalazine, and also salicylates, in different animal models of acute and chronic inflammation [28–30].

Adenosine A_1 receptor activation has been associated with pro-inflammatory properties in most inflammatory cell types [31,32]. Nevertheless, studies in vivo have demonstrated the anti-inflammatory effect of selective A_1 agonists acting in the Central Nervous System by increasing adenosine concentration at the inflamed site [33,34].

Adenosine A_{2A} receptors are generally regarded as the receptor subtype most relevant for the anti-inflammatory effect of adenosine. Their activation inhibits neutrophil and monocyte oxidative burst, degranulation and release of cytokines and chemokines [35,36]. Activation of A_{2B} receptors selectively inhibits collagenase mRNA accumulation in synovial fibroblasts, mediates neutrophil-stimulated intestinal epithelial leakiness and prevents vascular leakage and edema formation [37–39].

The role of adenosine A_3 receptors in inflammation has been more controversial, maybe due to the observed difference in agonist affinity between species, have also been described as anti-inflammatory in human blood leukocytes and in murine models of inflammation [40,41]. We have confirmed the anti-inflammatory effects of adenosine acting at A_3 receptors in experimental animals, since animals deficient in this receptor show an exacerbated response to an inflammatory insult when compared to their wild-type littermates [42].

It has been firmly established that adenosine modulates the production of both inflammatory and anti-inflammatory cytokines including TNFα, IL-10, and IL-12 [16,43]. The anti-inflammatory effect of adenosine could be beneficial since some reports depict a deleterious effect of TNF in wound healing [44].

5. Adenosine in tissue formation

Driven by growth factors synthesized by local and migratory cells, fibroblasts migrate on the provisional fibrin scaffold into the wound where they proliferate and construct a more robust collagen rich extracellular matrix. Wound fibroblasts acquire a distinctive contractile and secretory phenotype, known as myofibroblasts, responsible for wound contraction, a very important event in full thickness wounds. In response to many of the same growth factors, epidermal cells migrate from the edge of the wound over the surface of the injured area and proliferate until there is complete wound closure [1,5].

Many studies have shown that purinoceptors are involved in the regulation of proliferation and differentiation of most target cells. Thus, activation of adenosine A_{2B} receptor and P2Y2 receptors have mitogenic effects in murine keratinocytes [45], contrasting with earlier reports showing that adenosine and its related nucleotides (ATP, ADP, AMP) were antiproliferative for normal human epidermal keratinocytes cultured in the absence or presence of exogenous epidermal growth factor [46]. Similarly, the result of adenosine receptor activation in fibroblast proliferation remains unclear [47,48]. Studies in our laboratory indicated that adenosine A_2 receptor occupancy, both A_{2A} and A_{2B}, contributes to enhanced fibroblast and endothelial cell migration [49].

We have recently reported that activation of adenosine A_{2A} receptors promotes collagen synthesis by human dermal fibroblasts and that blockade or deletion of this receptor in mice protects against bleomycin-induced dermal fibrosis, a murine model of scleroderma [17]. The stimulation of collagen synthesis in human dermal fibroblasts occurs through an A_{2A}R/mitogen-activated protein kinase kinase-1/mitogen-activated protein kinase-mediated pathway [17]. Adenosine deaminase (ADA), the principal catabolic enzyme for adenosine in vivo, and its deficiency leads to the spontaneous development of pulmonary and skin fibrosis in mice, in which increased collagen deposition is accompanied by increased levels of key mediators of fibrosis, including transforming growth factor beta1, connective tissue growth factor, and interleukin-13. Pharmacological treatment of ADA-deficient mice with the A_{2A} receptor antagonist ZM-241385 prevented the development of dermal fibrosis in this model of elevated tissue adenosine, by reducing dermal collagen content and expression of profibrotic cytokines and growth factors. These data confirm a fibrogenic role for adenosine in the skin [50]. We also found an increased number of myofibroblasts associated with elevated skin adenosine concentration, a phenomenon that was prevented by pharmacological blockade of A_{2A} receptors [51]. Although these results are consistent with a fibrogenic role for adenosine A2A receptor activation, a possible role for adenosine A2B receptors cannot be ruled out, since the concentration of the antagonist ZM-241385 used in these studies is high enough to also antagonize rodent A2B receptors [52]. Wound-healing models have not been studied in ADA-deficient mice.

6. Adenosine in neovascularization

Revascularization of the wound bed is essential to supply oxygen, nutrients, and inflammatory cells to the newly growing tissue. Two mechanisms contribute to the development of new vessels in the adult: angiogenesis, the formation of new vessels from pre-existing ones; and vasculogenesis, the initial series of events in vascular growth in which endothelial cell precursors (angioblasts) differentiate in situ and assemble into solid endothelial cords [53]. This multistep process is highly regulated by a variety of soluble angiogenic growth factors, proteolytic enzymes, which allow endothelial cell detachment and extracellular matrix invasion, and a close interaction between adhesive proteins of the extracellular matrix and their integrin receptors [54,55]. Among the growth factors implicated in the angiogenic process, bFGF and vascular endothelial growth factor (VEGF) are known to be potent angiogenic molecules that induce the growth of new blood vessels during wound healing and embryonic development [56,57]. Diabetic patients frequently suffer from macro and microvascular complications characterized by an early dysfunction of vascular endothelium that could further aggravate their impaired healing.

Many studies have shown that the administration of adenosine or adenosine agonists as well as the up-regulation of endogenous adenosine can increase the expression of vascular endothelial growth factor (VEGF) in a variety of different cells studied in vitro [58–62] and after intravenous infusion of adenosine in humans [63]. Adenosine A_{2A} receptors activation stimulates macrophage production of VEGF as well [60], meanwhile adenosine A_{2B} receptors induce VEGF release by retinal endothelial cells [59,61].

The elevated expression of VEGF might account for the angiogenic effects of adenosine; however, it is more likely that adenosine also stimulates angiogenesis via other secondary angiogenic and anti-angiogenic mediators or by way of an intracellular action [64]. Thus, the adenosine analog NECA also increased the expression of the proangiogenic factors insulin-like growth factor-I (IGF-I) and basic fibroblast growth factor (bFGF) in human retinal endothelial cells as well as the expression of the proangiogenic factors interleukin-8 (IL-8) and angiopoietin-2 in human mast cells. A_{2B} receptors mediated the IL-8 and bFGF responses and A_3 receptors may have mediated the angiopoietin-2 response [65]. We have shown that adenosine and A_{2A} agonists can promote in vitro

angiogenesis by inhibiting endothelial secretion of the anti-angiogenic factor thrombospondin-1 [66].

7. Adenosine in remodeling

The reparative process culminates with the remodelling of the newly formed granulation tissue in order to restore complete functionality. During this extended phase, the provisional extracellular matrix rich in type 3 collagen is degraded by serine proteases and metalloproteases and sequentially substituted for the definitive matrix rich in type 1 collagen [1].

Activation of plasminogen plays a role in proteolytic degradation of extracellular matrices in tissue remodelling events and it is required for normal repair of skin wounds in mice [67]. Earlier reports showed that the non-selective adenosine receptor agonist NECA increased plasminogen activator release in rabbit alveolar macrophages due to intracellular elevations of cAMP [68]. Similarly, cAMP elevating agents increased the acute release of tissue plasminogen activator in human umbilical endothelial cells [69,70] and intraarterial infusions of ATP, an adenosine precursor, to healthy volunteers induce tPA release [71].

8. Adenosine agonist promotion of wound healing in animal models

Much of the knowledge of the normal healing process of cutaneous wounds and the mediators involved has evolved from information derived from experimental wounds in animals, especially genetically modified mice. Unfortunately, a valid model of chronic wounds in animals has not yet been developed. From the in vitro experiment data it is difficult to predict which adenosine agonists, if any, will be useful for promoting impaired wound healing in humans.

There are few reports of the use of adenosine agonists for the enhancement of wound healing. We have demonstrated pharmacologically and by the use of mice lacking A_{2A} receptors that topical application of adenosine A_{2A} receptor agonists accelerate healing of dermal wounds in both healthy animals and in streptozotocin-induced diabetic rats with impaired wound healing [18,49,72]. Histological analysis showed faster re-epithelialization and increased matrix deposition, fibroblast density and vascularity in the granulation tissue of the agonist treated wounds as soon as 3 days after injury [18]. In a model of pressure ulcer formation as a result of recurrent ischemia-reperfusion of the skin, an adenosine A_{2A} receptor agonist infused via osmotic mini-pumps reduced leukocyte infiltration and protected from ischemia/reperfusion injury in skin [73]. No signs of fibrosis were described in these experimental animal models.

When studying the dose-response effect of the selective A_{2A} receptor agonist CGS-21680, a narrow therapeutic window was observed. Both low (0.5 μg/wound) and high concentrations (10 μg/wound) did not affect the rate of wound closure; while intermediate doses were equally effective. Loss of efficacy may be caused by the loss of specificity of CGS-21680 at higher concentrations. In contrast Sonedenoson did not lose its accelerating effect at the highest concentration studied. Moreover, it stimulated more rapid wound closure than CGS-21680, suggesting the possibility that a higher selectivity of MRE0094 for A_{2A} receptors may be important [72]. It is worth noting that the pharmacokinetics of these compounds administered topically in a hydrophilic gel have not been determined and could have important repercussions in the final effect, in terms of penetration or systemic absorption and elimination rate. In the same study 0.01% Becaplermin gel (rhPDGF-BB), the only growth factor approved for clinical use, did not accelerate the rate of wound closure as compared to the control, and both CGS-21680 and Sonedenoson treated wounds closed significantly faster [72].

Topical application of the non-selective adenosine receptor agonist 5′-N-ethyl-carboxamidoadenosine (NECA) also normalized the impaired healing induced by subcutaneous injection of dexamethasone in mice. This effect was only partially abrogated by a selective A_{2B} antagonist, but the contribution of other receptors was not determined [45]. Topical application of the adenosine A_1 receptor agonist, N(6)-Cyclopentyladenosine (CPA), also promoted healing of incisional and excisional wounds on the dorsum of both diabetic (db/db) and normal (db/+) mice, although the concentration used was above the selectivity threshold and the participation of other receptors cannot be ruled out. Interestingly, hair growth along the wound margin was enhanced in agonist-treated mice, an effect that has not been described for adenosine A_{2A} agonists [48].

Finally, using an incisional skin-wound model produced on the back of female diabetic mice, intraperitoneal administration of Polydeoxyribonucleotide, a compound mixture of deoxyribonucleotide polymers, improved the impaired wound healing and increased the wound-breaking strength in diabetic mice through adenosine A_2 receptors activation. The promoting effect of this compound was blocked by an antagonist, DMPX (3,7-dimethyl-1-propargilxanthine), which blocks both A_{2A} and A_{2B} receptors [74].

As inferred from the in vitro studies previously described, many factors contribute to the effect of adenosine receptor activation in promoting wound healing. The synergistic interaction between Toll-like receptor and adenosine A_{2A} receptor signaling switches macrophages towards an angiogenic phenotype [60,75], and plays a role in an excisional wound-healing model. Mice lacking MyD88, an adapter protein in the signal transduction pathway of Toll-like receptors, heal at a markedly slower rate than wounds in wild-type mice, showing delayed contraction, decreased and delayed granulation tissue formation, and reduced new blood vessel density. CGS21680, an A_{2A} receptor agonist, promoted wound closure and angiogenesis in wild-type mice, but had no significant effect on healing of MyD88(−/−) mice, suggesting the synergistic interaction between Toll-like receptors and adenosine A_{2A} receptors signaling in wound healing in vivo [76].

9. Conclusion

Nowadays there is a high awareness of the problem that diabetic foot ulcers represent in terms of costs and quality of

life for those suffering them. Studies to better understand the normal healing process and the pathology of impaired healing have been extremely useful to improve wound management and care. Randomized controlled trials have shown that topical application of becaplermin gel is effective in increasing healing rates for diabetic neuropathic foot ulcers with adequate blood supply. However, this efficacy has not translated to positive clinical experience, and the drug is not widely used. Moreover, it is an expensive medication of relative short storage stability and an increased risk of mortality secondary to malignancy was observed in patients. Adenosine receptor agonists represent an attractive and novel alternative to growth factors for promotion of diabetic foot chronic ulcers. They are small synthetic molecules of longer stability and lower cost than growth factors. Given the complexity of the pathogenesis of foot ulcers and the differential adenosine receptor expression in the cells involved in the impaired tissue repair process, it is difficult to predict which agonists, selective or not, could be most useful. To add more complexity, all four receptor adenosine subtypes are G protein-coupled receptors, but also exert cAMP-independent actions. In addition, they couple to mitogen-activated protein kinases by mechanisms that appear to differ substantially, both between receptor subtypes in the same cell type and between the same receptor in different cell types. From our studies, selective adenosine A_{2A} receptor agonists present the advantages of reducing inflammation and increasing vascularization and extracellular matrix deposition. Nevertheless, the fibrogenic potential and increased risk of infection should also be monitorized. Other important factors to be considered are the pharmaceutical formulation and the pharmacokinetics of the agonist used, in order to minimize its systemic effects. As any new strategy in the management of chronic wounds, the use of adenosine agonists has to be evaluated and validated before taking it into practice.

Acknowledgments

MCM is beneficiary of the Ramón y Cajal program from the Spanish Government (Ministerio de Educación y Ciencia) and a grant from the Instituto de Salud Carlos III (FIS 05/1659) of the Ministry of Science and Innovation, Government of Spain. B.N.C. is a consultant for King Pharmaceuticals, Can-Fite Biopharma, Inc., Bristol-Myers, Squibb, and Tap Pharmaceuticals. He is funded by grants from King Pharmaceuticals and the National Institutes of Health (AA13336, AR41911, GM56268, and HL70952) and is the recipient of honoraria for speaking from Merck Pharmaceuticals, Tap Pharmaceuticals, and Amgen.

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