Brief Definitive Report

Wound Healing Is Accelerated by Agonists of Adenosine A\(_2\) (G\(_{\text{as}}\)-linked) Receptors

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Summary

The complete healing of wounds is the final step in a highly regulated response to injury. Although many of the molecular mediators and cellular events of healing are known, their manipulation for the enhancement and acceleration of wound closure has not proven practical as yet. We and others have established that adenosine is a potent regulator of the inflammatory response, which is a component of wound healing. We now report that ligation of the G\(_{\text{as}}\)-linked adenosine receptors on the cells of an artificial wound dramatically alters the kinetics of wound closure. Excisional wound closure in normal, healthy mice was significantly accelerated by topical application of the specific A\(_{2A}\) receptor agonist CGS-21680 (50% closure by day 2 in A\(_{2}\) receptor antagonists. In rats rendered diabetic (streptozotocin-induced diabetes mellitus) wound healing was impaired as compared to nondiabetic rats; CGS-21680 significantly increased the rate of wound healing in both nondiabetic and diabetic rats. Indeed, the rate of wound healing in the CGS-21680–treated diabetic rats was greater than or equal to that observed in untreated normal rats. These results appear to constitute the first evidence that a small molecule, such as an adenosine receptor agonist, accelerates wound healing in both normal animals and in animals with impaired wound healing.

Wound healing is a complex process characterized by three overlapping phases: inflammation, tissue formation, and tissue remodeling. During tissue formation, growth factors synthesized by local and migratory cells stimulate fibroblasts to migrate into the wound where they proliferate and construct an extracellular matrix. In response to many of the same growth factors, keratinocytes migrate from the edge of the wound over the surface of the injured area and proliferate until the wound is completely covered. Both matrix formation and epithelialization, in turn, depend upon angiogenesis, a process that occurs through the migration, proliferation, and organization of vascular endothelial cells. Earlier studies demonstrated that a variety of peptide growth factors may promote reepithelialization, migration of fibroblasts into a wound, and angiogenesis, but no small molecules of potentially greater use have yet been shown to enhance the rate at which wounds close in normal healthy animals.

Because of their potent effects on some of the cells involved in wound healing, we hypothesized that adenosine receptor agonists promote wound healing both in vitro and in vivo. Previous studies have demonstrated that adenosine, acting at A\(_{2}\) receptors, inhibits neutrophil accumulation and function (2) and promotes endothelial cell proliferation, migration, and secretion of growth factors (3–6). In contrast, adenosine receptor occupancy inhibits keratinocyte proliferation (7) and its effects on fibroblast proliferation are inconsistent (3, 8). Four adenosine receptors have been cloned and the deduced sequences reveal that all four are members of the large family of 7 transmembrane–spanning, G protein–coupled receptors. Three of the adenosine receptor subtypes, A\(_{1}\), A\(_{2A}\), and A\(_{2B}\), are highly conserved throughout evolution (80–95% sequence homology), whereas the A\(_{3}\) receptors differ significantly among species (for review see reference 9). Adenosine A\(_{1}\) and A\(_{2}\) receptors were first differentiated by their opposing effects on cAMP accumulation (10, 11), effects mirrored, in some cell types (e.g., neutrophils), by opposing functional effects (for review see reference 2).

Because results of preliminary in vitro studies indicated that adenosine A\(_{2}\) receptor agonists promoted fibroblast and endothelial cell migration into an artificial wound, we tested the effects of a specific adenosine A\(_{2A}\) receptor agonist, CGS-21680 (4-[[N-ethyl-5'-carbamoyladenos-2-yl]aminoethyl] phenylpropionic acid), on wound healing in normal mice and rats and in rats rendered diabetic. We report here that
adenosine, acting at specific A$_{2}$A receptors, promotes healing both in normal, healthy, young animals and in diabetic animals with impaired wound healing.

Materials and Methods

Materials. CGS-21680, DMPX (3,7-dimethyl-1-propargyl-xanthine), and CSC (8-(3-chlorostyryl)caffeine) were obtained from Research Biochemicals, Inc. (N. abick, M.A.). Tissue culture media and reagents were obtained from Gibco BRL (Bethesda, MD).

Cell Lines. Fibroblasts (CCD-25sk) were obtained from the American Type Culture Collection (Rockville, MD) and were originally derived from normal human dermal fibroblasts. These cells were grown to confluence in standard tissue culture medium consisting of MEM/10% fetal bovine serum (vol/vol). HUVEC were obtained by modifications of the method of Jaffe et al. (12). In brief, HUVEC were obtained by collagenase treatment of fresh human umbilical cords and grown to confluence in medium 199 with 20% fetal bovine serum, antibiotics, and endothelial growth supplement at 37°C in a 5% CO$_2$ atmosphere (12, 13). The experiments in the study were performed on HUVEC in their third passage.

Reverse Transcriptase-PCR of A$_{2}$A Receptor mRNA. Total RNA from confluent monolayers of either HUVEC or CCD-25sk cells was isolated by the RNeasy$^{TM}$ B method (Tele-Test, Inc., Friendswood, TX) and first-strand cDNA was synthesized by GeneAmp$^{TM}$ RNA PCR Core Kit (Perkin-Elmer Corp., Branchburg, NJ) according to the directions. The amplification primers for the adenosine receptor messages have been previously described by Nielsen et al. (14). Upstream primers for the A$_{2}$A and the A$_{2}$B receptors were GGTGGAATTCAACAACTGCGGC, GGTGGAATTCAAACACTGCGG-TCAGCAGAATA, and GGTGGAATTCAACACTGCGG-TCAGCAGAATAAGCTGC, respectively, and the downstream primers were GGTTGAGATTTCCGACTCACTTGATCCC, GGTTGAGATTTCCGACTCACTTGATCCC, and GGTGGAATTCAACACTGCGG-TCAGCAGAATAAGCTGC, respectively. The nested amplification primers for A$_{2}$A receptors were CAGCTTTCCAGGC, GGTGGAATTCAACAACTGCGG-TCAGCAGAATA, and GGTGGAATTCAACACTGCGG-TCAGCAGAATAAGCTGC, respectively. The nested amplification primers for A$_{2}$B receptors were GGTTGAGATTTCCGACTCACTTGATCCC, GGTTGAGATTTCCGACTCACTTGATCCC, and GGTGGAATTCAACACTGCGG-TCAGCAGAATAAGCTGC, respectively. The nested amplification primers for A$_{2}$A receptors were AACGTGTGCTTACCTGCTGGTGTC (upstream), GTAGCTTACCTGCTGGTGTC (downstream), and CAGCGACCCACCAAGGAAAAGCTGC (nested). Template first-strand cDNA (150 ng) was added to a reaction mixture which included dNTPs (0.2 mM each), Mg$^{2+}$ (25 mM) and appropriate primers (1 mM), and Taq DNA polymerase (0.025 U/µl) in a final volume of 50 µl. The PCR was carried out in a thermocycler (GeneAmp 2400; Perkin-Elmer) programmed as follows: 94°C (2 min), and then 35 cycles of 94°C (1 min), 58°C (1 min), 72°C (3 min), followed by a 10-min terminal extension (72°C) for the A$_{2}$A receptor. The A$_{2}$A, A$_{2}$B, and A$_{3}$ receptors were amplified using the following program: 94°C (2 min), and then 35 cycles of 94°C (30 s), 58°C (30 s), and 72°C (45 s), followed by a 10-min terminal extension (72°C). PCR products were separated in a 1.8% agarose gel. Sequencing of the PCR products confirmed their identity with previously described primers of the appropriate adenosine receptors.

Excisional Wound Formation in Rats Rendered Diabetic. A adult (330–400 g) female Sprague-Dawley rats were given a single intraperitoneal injection of streptozotocin (60 mg/kg). Animals were then rested for 8 d before excision of 2.0 cm wounds on the dorsum of the rats (15, 16). Wounds were treated daily with topical application of 20 µl of either the adenosine agonist CGS-21680 (250 µg/ml) or vehicle (1.5%, wt/vol carboxymethylcellulose in PBS). The animals were kept in individual cages to minimize the licking of the wounds or applied agents. The rate of wound closure was determined as described above.

Histologic Analysis. Some animals were killed on the stated day by CO$_2$ poisoning, and then wounds were excised and histologic slides were made using standard methods. Slides, stained with hematoxylin and eosin, were graded using a variation of the scoring described by Tsuboi and Rikfin (17). In brief, reepithelialization was measured on a score from 1 to 10 (1 = no closure; 10 = complete closure). Matrix density was scored from 1 to 4 (1 = edematous with little matrix; 2 = a small amount of coarse matrix; 3 = a moderate amount of matrix; and 4 = dense matrix). Fibroblast infiltration was scored from 1 to 4 (1 = many fibroblasts; 2 = a moderate number of fibroblasts; 3 = many cells and 4 = very many cells). Inflammatory cells were graded from 1 to 4 (1 = many cells; 2 = many cells; 3 = a moderate number of cells; 4 = few cells). A maximum composite score of 22 can be obtained. All slides were graded in a blinded fashion.

Results

Endothelial C cells and Fibroblasts Express Messenger RNA for Adenosine A$_{2}$ Receptors. To establish the profile of adenosine receptors expressed by fibroblasts and endothelial cells, we determined whether mRNA for adenosine A$_{2}$A, A$_{2}$B, and A$_{3}$ receptors was present in cultured dermal fibroblasts and HUVEC by use of reverse transcriptase-PCR. As shown in Fig. 1, message for A$_{2}$A, A$_{2}$B, and A$_{3}$ receptors was present in both fibroblasts and HUVEC. In contrast, message for A$_{2}$A receptors was expressed in HUVEC, but not in fibroblasts. Rats with other in vitro studies with these cells indicated that occupancy of adenosine A$_{2}$ receptors, both A$_{2}$A and A$_{2}$B receptors, promoted migration of both fibroblasts and HUVEC into an artificial in vitro wound by a cAMP-dependent mechanism (data not shown).
and the wounds of the diabetic animals healed more slowly than those of the control animals (50% closure by day 9 versus by day 7, respectively; \( P < 0.0001 \); Fig. 4). As with the normal young mice, topical application of CGS-21680 significantly promoted wound healing in the healthy nondiabetic rats (50% closure by day 4; \( P < 0.0001 \), Fig. 4). More importantly, application of CGS-21680 increased the rate at which the diabetic animals closed their wounds (50% closure by day 6, \( P < 0.0001 \), versus control diabetic rats, Fig. 4) but did not affect the serum glucose concentration (432 ± 31 mg/dl versus 407 ± 40 mg/dl in the control and CGS-21680-treated diabetic rats, respectively; \( P = N S \)). Indeed, the rate of wound healing in CGS-21680-treated diabetic animals was as good as or better than that in the untreated controls (Fig. 4).

**Discussion**

The results reported here demonstrate that occupancy of adenosine A2A receptors increases the rate at which wounds

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**Figure 1.** Endothelial cells (HUVEC) and a fibroblast cell line (CCD-25sk) express messenger RNA for adenosine receptor subtypes. RNA was isolated from confluent monolayers of either HUVEC or CCD-25sk cells, as described. CDNA was generated from the isolated mRNA by reverse transcriptase and the message for the adenosine receptors was amplified by antiense primers as described. Shown is one of two experiments yielding similar results.

**Figure 2.** The effect of the adenosine A2A agonist CGS-21680 (250 \( \mu \)g/ml) on wound closure. (A) Wounds were excised on the dorsum of mice and treated with carrier (1.5% methylcellulose), CGS-21680, the adenosine A2 antagonist DMX (2.5 mg/ml), or their combination, as described. Wounds were traced daily and the area was determined after computer digitization of the wounds. (B) Wounds were excised on the dorsum of mice and treated with carrier (1.5% methylcellulose), CGS-21680, the adenosine A2 antagonist CSC (250 \( \mu \)g/ml), or their combination, as described. Each point represents the mean (± SEM) of 10 wounds. Similar results were found in two other experiments.
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To our knowledge, this is the first demonstration that a small nonpeptide agent, such as a purine nucleoside, promotes wound healing. Equally striking is the finding that this phenomenon occurs in normal, healthy animals, since only a few of the known peptide growth factors that accelerate wound healing in sick animals also accelerate wound closure in healthy animals (1).

Preliminary studies in our laboratory indicated that adenosine A2 receptor occupancy, both A2A and A2B, contributes to enhanced fibroblast and endothelial cell migration. Signal transduction at adenosine A2A and A2B receptors proceeds, at least in part, via activation of heterotrimeric G proteins leading to both cAMP-dependent and cAMP-independent signaling events (2, 9, 18). Our studies demonstrate that adenosine receptor occupancy promotes fibroblast and endothelial cell migration by a cAMP- and PKA-dependent pathway. In contrast, Sextl et al. have reported that adenosine A2A receptor occupancy modulates endothelial cell proliferation by a cAMP-independent mechanism (4, 19). These disparate findings suggest that stimulation of migration and proliferation in endothelial cells are mediated by different signal transduction pathways which diverge after occupancy of adenosine A2A receptors. However, there is no evidence that these divergent effects of adenosine receptor occupancy occur in other cell types. Regardless of the signal transduction pathway involved, our data clearly indicate that agents that occupy adenosine A2 receptors, receptors linked to Gs, signal transduction proteins, promote wound healing.

It is unlikely that an adenosine receptor-mediated increase in fibroblast migration and angiogenesis is solely responsible for accelerating wound closure. Previous studies have demonstrated that adenosine and its analogues, acting at A2A receptors, increase secretion of vascular endothelial growth factor in addition to promoting endothelial cell proliferation and migration [3–6]. These observations suggest that one mechanism by which adenosine receptor occupancy increases the rate of wound closure is by promoting secretion of growth factors that act locally. Alternatively, by inhibiting the secretion of a variety of inflammatory cytokines (TNF-α, IL-6, IL-8) adenosine receptor occupancy might diminish the secretion of agents that inhibit wound healing (20–24). Another explanation for the effects of ade-
Adenosine receptor occupancy on wound healing is suggested by the work of Boyle et al. who reported that adenosine A2 receptor occupancy specifically inhibits synthesis and secretion of collagenase by synovial fibroblasts (14). Thus, diminished matrix degradation within the wound might also enhance wound closure. Therefore, it is likely that there are a number of adenosine-mediated effects that contribute to the accelerated rate of wound closure and that are mediated by ligation of adenosine receptors.

We conclude that adenosine A2 receptor agonists promote wound healing in normal and diabetic animals. This is the first example of a member of the 7 transmembrane-spanning, heterotrimeric G protein–associated family of receptors that, when occupied, promote wound healing by itself. Moreover, unlike some growth factors, occupation of adenosine A2a receptors promotes wound healing even in young, healthy animals (1). The observation that this same adenosine receptor agonist promotes wound healing in both normal individuals and individuals with impaired wound healing.

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