Brief Definitive Report

Wound Healing Is Accelerated by Agonists of Adenosine A_2 ($G_{\alpha s}$-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 $G_{\alpha s}$-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 (1).

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

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adenosine, acting at specific A_{2A} 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-propargylxanthine), and CSC (8-(3-chlorostyryl)caffeine) were obtained from Research Biochemicals, Inc. (Natick, MA). 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 Adenosine Receptor mRNA. Total RNA from confluent monolayers of either HUVEC or CCD-25sk cells was isolated by the RNAzol™ B method (Tel-Test, Inc., Friendswood, TX) and first-strand cDNA was synthesized by GeneAmp™ 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 Nilsen et al. (Nilsen, D., A. Talbot, C. Aston, R. Hirschhorn, B.N. Cronstein, and J. Reibman, manuscript submitted for publication) and Boyle et al. (14). Upstream primers for the A_1, A_{2A} and the A_{2B} receptors were GGTGGAATTCTCCATCTCAGCTTTCCAGGC, GGTGGAATTCAACAACTGCGGTCAGCCAAA, and GGTGGAATTCGAACCACGAATGAAAGCTGC, respectively, and the downstream primers were GGTGAAGCTTTCGAACTCGCACTTGATCAC, GGTGAAGCTTCAGCTGCCTTGAAAGGTTCT, and GGTGAAGCTTTGACCATTCCCACTCTTGAC, respectively. The nested amplification primers for A_3 receptors were AACGTGCTGGTCATCTGCGTGGTC (upstream), GTAGTCCATTCTCATGACGGAAAC (downstream), and CTGCAGACCACCACCTTCTATTTC (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_1 receptor. The A_{2A}, A_{2B}, 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 portions of the appropriate adenosine receptors.

Excisional Wound Formation. Two sterile, full-thickness excisional wounds (12 mm in diameter) were formed on the dorsum of anesthetized 6–8-wk-old male and female mice (BALB/c) using a template and scissors. 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) in the presence or absence of either the adenosine receptor antagonist DMPX (2.5 mg/ml) or CSC (250 μg/ml). Mice were kept in individual cages to minimize licking of the wounds or applied agents. To determine the rate of closure, wounds were traced onto clear plastic sheets on a daily basis and the area of the wounds was quantitated by digitization of the wound tracings using a WACOM digitizing pad and Scan Analysis (Specom Research, Ferguson, MO) software. These experiments were approved by the Institutional Animal Care and Use Committee (New York University School of Medicine).

Excisional Wound Formation in Rats Rendered Diabetic. 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. Mean serum glucose, tested on the final day of the experiment using the Easy Test® Strips for glucose with an Accu-Chek® Easy™ Monitor (Boehringer-Mannheim, Indianapolis, IN), was 156 ± 18.5 mg/dl in the nondiabetic rats as compared to 432 ± 25 mg/dl in the diabetic rats. Seven rats died after injection of streptozotocin but before excision of the wounds and seven other diabetic rats died during the course of these experiments (five control rats and two CGS-21680–treated rats).

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 Rifkin (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 infiltrate was scored from 1 to 4 (1 = few fibroblasts; 2 = a moderate number of fibroblasts; 3 = many cells; and 4 = very many cells). Inflammatory cells were graded from 1 to 4 (1 = very 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 Cells and Fibroblasts Express Message for Multiple Adenosine Receptors. To establish the profile of adenosine receptors expressed by fibroblasts and endothelial cells, we determined whether mRNA for adenosine A_1, A_{2A}, A_{2B}, 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_{2A}, A_{2B}, and A_3 receptors was present in both fibroblasts and HUVEC. In contrast, message for A_1 receptors was expressed in HUVEC but not in fibroblasts. Results of other in vitro studies with these cells indicated that occupancy of adenosine A_2 receptors, both A_{2A} and A_{2B} receptors, promoted migration of both fibroblasts and HUVEC into an artificial in vitro wound by a cAMP-dependent mechanism (data not shown).

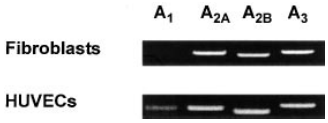

Figure 1. Endothelial cells (HUVEC) and a fibroblast cell line (CCD-25sk) express message for adenosine receptor subtypes. RNA was isolated from confluent monolayers of either HUVEC or CCD-25sk cells, as described. cDNA was generated from the isolated mRNA by reverse transcriptase and the message for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

Topical Application of an Adenosine A_{2A} Receptor Agonist Promotes Wound Closure In Vivo. The observation that both HUVEC and dermal fibroblasts express mRNA for both A_{2A} and A_{2B} receptors, the preliminary finding that adenosine A_2 receptor occupancy increases the rate of fibroblast migration, and the previous demonstration that agents binding to adenosine A_2 receptors may promote angiogenesis (3–6) all suggested that an adenosine A_2 receptor agonist might accelerate wound healing in vivo. To test this hypothesis we studied the effect of the topical application of CGS-21680, the specific A_{2A} agonist, on wound healing in healthy, young (6–8-wk-old) BALB/c mice. As shown in Fig. 2, wound closure was significantly more rapid in the CGS-21680–treated mice than the mice treated with carrier alone (50% wound closure by day 2 versus by day 6; $P < 0.00001$, $n = 10$ wounds per group). Topical application of the adenosine A_2 receptor antagonist DMPX (2.5 mg/ml) did not affect wound healing itself, but completely reversed the effects of CGS-21680 on the rate of wound closure (Fig. 2 *A*). Similarly, the more selective A_{2A} receptor antagonist CSC also completely reversed the effect of CGS-21680 on wound healing (Fig. 2 *B*). Upon histologic examination of the wounds, fibroblast infiltration, matrix density, and reepithelialization were markedly enhanced in the CGS-21680–treated animals as compared to controls (Fig. 3). Surprisingly, in contrast to the demonstrated anti-inflammatory effects mediated by adenosine A_2 receptor occupancy (2), CGS-21680 did not affect the inflammatory infiltrate in the wound until day 10 after wounding. The change in inflammatory infiltrate observed so late in the course of wound closure may have resulted from earlier wound closure rather than any direct effect of CGS-21680 on inflammatory infiltrate in the wound. Topical application of CGS-21680 to open wounds had no obvious toxic effect on the mice.

Topical Application of an Adenosine A_{2A} Receptor Agonist Promotes Wound Closure in Diabetic Rats. Patients with diabetes mellitus suffer from impaired wound healing, and poor wound healing is responsible for significant morbidity and mortality in these patients. To determine whether an adenosine A_{2A} receptor agonist might be useful in the promotion of wound healing in patients with diabetes, we studied the effect of topical application of the adenosine receptor agonist CGS-21680 to full-thickness wounds in rats rendered diabetic by a single injection of streptozotocin. As expected, streptozotocin-treated rats had high serum glucose concentrations (432 ± 25 mg/dl in the diabetic rats versus 156 ± 18.5 mg/dl in the nondiabetic; $P < 0.00001$) and the wounds of the diabetic animals healed more slowly

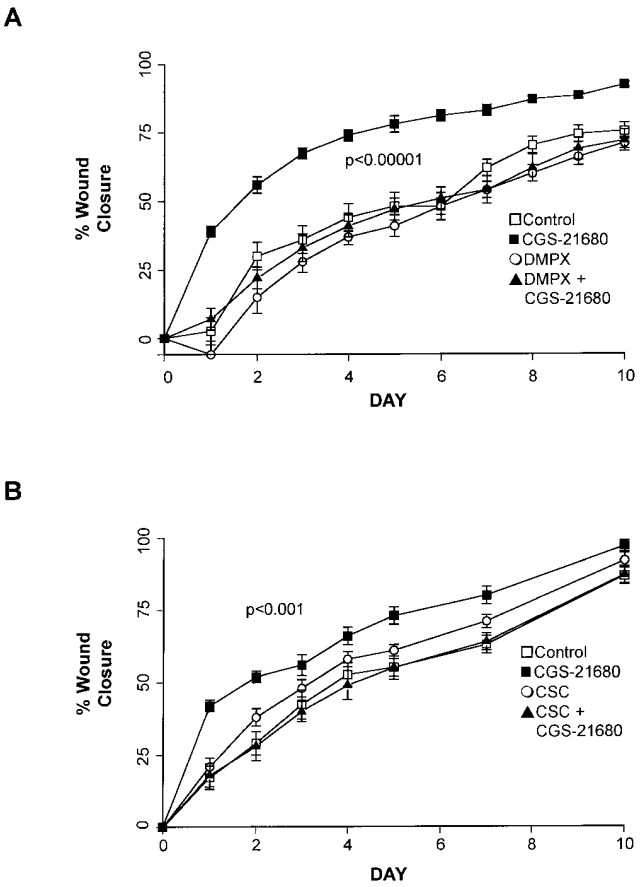

Figure 2. The effect of the adenosine A_{2A} agonist CGS-21680 (250 μg/ml) on wound closure. (*A*) Wounds were excised on the dorsum of mice and treated with carrier (1.5% methylcellulose), CGS-21680, the adenosine A_2 antagonist DMPX (2.5 mg/ml), or their combination, as described. Wounds were traced 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 A_{2A} antagonist CSC (250 μg/ml), or their combination, as described. Each point represents the mean (± SEM) of 10 wounds. Similar results were found in two other experiments.

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 = NS). 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 A_{2A} receptors increases the rate at which wounds

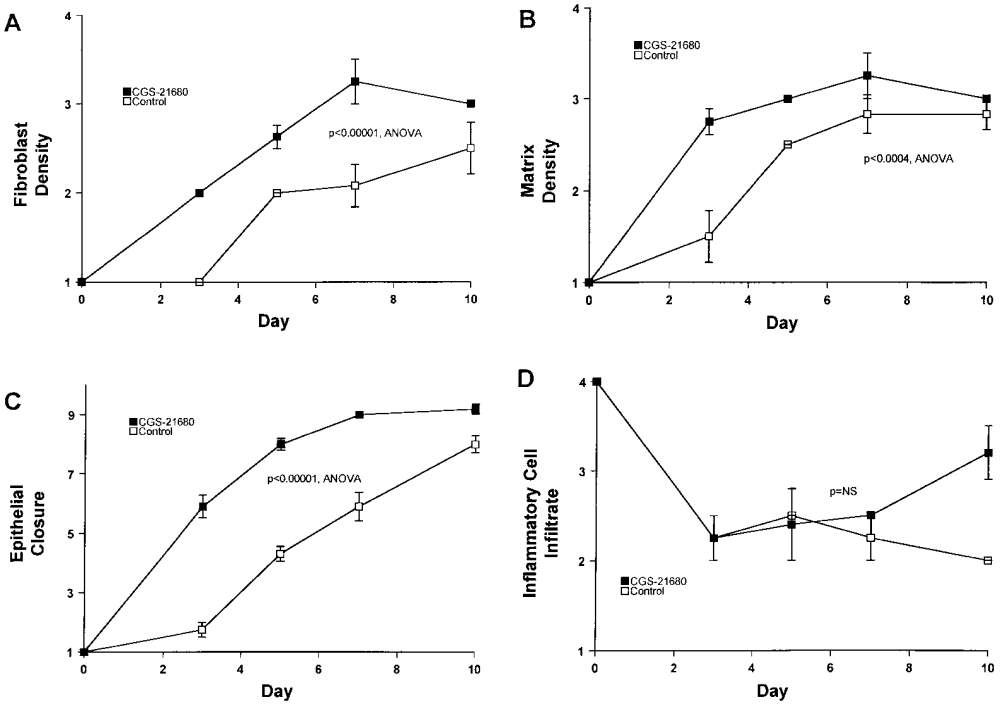

Figure 3. Histologic analysis of wounds in control and CGS-21680–treated mice. Wounds were excised and mice were treated with topical application either of carrier or CGS-21680 in carrier. Analysis of fibroblast density (*A*), matrix density (*B*), epithelial closure (*C*), and inflammatory cell infiltrate (*D*) was carried out blindly, as described. Each point represents the mean (± SEM) of six wounds on three mice.

heal in young, healthy mice and rats as well as in diabetic rats. 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 A_2 receptor occupancy, both A_{2A} and A_{2B}, contributes to enhanced fibroblast and endothelial cell migration. Signal transduction at adenosine A_{2A} and A_{2B} 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, Sexl et al. have reported that adenosine A_{2A} 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 A_{2A} 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 A_2 receptors, receptors linked to $G_{\alpha S}$ 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 A_{2A} 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-

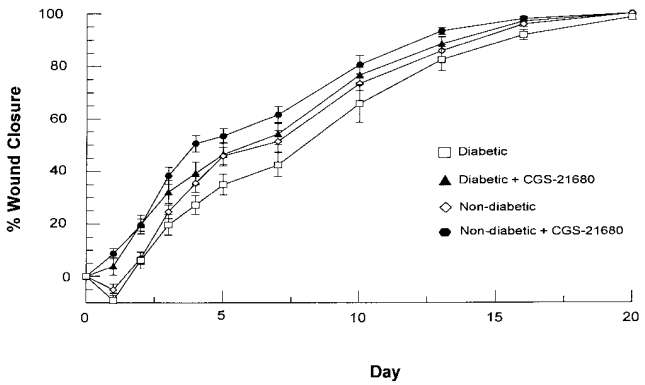

Figure 4. The effect of the adenosine A_{2A} agonist CGS-21680 on wound closure in normal and diabetic rats. Animals received a single injection of streptozotocin (60 mg/kg) followed 8 d later by excision of three wounds (2 cm in diameter) on the dorsum of each rat. Wounds were treated with carrier (1.5% methylcellulose/PBS, wt/vol) alone or CGS-21680 (250 μg/ml) in carrier. The wounds were traced at the indicated intervals and the area was determined after computer digitization of the wounds. Each point represents the mean (± SEM) of 9–21 wounds.

nosine receptor occupancy on wound healing is suggested by the work of Boyle et al. who reported that adenosine A_2 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 A_2 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 A_{2A} receptors promotes wound healing even in young, healthy animals (1). The observation that this same adenosine receptor agonist promotes wound healing in diabetic animals as well suggests an entirely novel approach for development of agents that promote wound healing in both normal individuals and individuals with impaired wound healing.

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