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Adenosine A_{2A} Receptor Blockade or Deletion Diminishes Fibrocyte Accumulation in the Skin in a Murine Model of Scleroderma, Bleomycin-induced Fibrosis

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Abstract

Peripheral blood fibrocytes are a newly identified circulating leukocyte subpopulation that migrates into injured tissue where it may display fibroblast-like properties and participate in wound healing and fibrosis of skin and other organs. Previous studies in our lab demonstrated that A_{2A} receptor-deficient and A_{2A} antagonist-treated mice were protected from developing bleomycin-induced dermal fibrosis, thus the aim of this study was to determine whether the adenosine A_{2A} receptor regulates recruitment of fibrocytes to the dermis in this bleomycin-induced model of dermal fibrosis. Sections of skin from normal mice and bleomycin-treated wild type, A_{2A} knockout and A_{2A} antagonist-treated mice were stained for Procollagen α 2 Type I and CD34 and the double stained cells, fibrocytes, were counted in the tissue sections. There were more fibrocytes in the dermis of bleomycin-treated mice than normal mice and the increase was abrogated by deletion or blockade of adenosine A_{2A} receptors. Because fibrocytes play a central role in tissue fibrosis these results suggest that diminished adenosine A_{2A} receptor-mediated recruitment of fibrocytes into tissue may play a role in the pathogenesis of fibrosing diseases of the skin. Moreover, these results provide further evidence that adenosine A_{2A} receptors may represent a new target for the treatment of such fibrosing diseases as scleroderma or nephrogenic fibrosing dermopathy.

Keywords

fibrocyte; adenosine A_{2A} receptor; scleroderma

INTRODUCTION

Adenosine and its receptors are important physiological and pharmacologic regulators of many different processes including fibrosis. Adenosine A_{2A} and A_{2B} receptors regulate proliferation, cytokine and collagen production and other functions by dermal, pulmonary and cardiac fibroblasts and hepatic stellate cells [1–10]. First shown to play a role in wound healing in normal and diabetic mice and rats [11], adenosine and its receptors have been

shown to play a central role in the pathogenesis of pulmonary and hepatic fibrosis and in a model of Scleroderma, diffuse dermal fibrosis following bleomycin treatment [2, 8, 12–16].

Although stimulation of local fibroblasts may account for fibrosis in some settings recent work suggests that fibrocytes, circulating bone marrow-derived cells, may be recruited to injured or inflamed areas where they play a central role in promoting wound healing and fibrosis (Recently reviewed in [17, 18]). These cells, which are recognizable in the circulation and in tissues as CD34+/procollagen 1 α 2+ cells, can differentiate into fibroblasts in the tissue after recruitment by chemokines produced at the inflamed site. Fibrocytes have been reported to play a role in such diseases as scleroderma, a disease of unknown etiology that is characterized by fibrosis of the skin and other organs, and nephrogenic fibrosing dermopathy, a recently described fibrosing syndrome related to gadolinium use [17–19].

As noted above, adenosine and adenosine A_{2A} receptors play a critical role in the development of dermal fibrosis following bleomycin treatment [2] and fibrocytes play a central role in fibrosing disorders [20, 21]. We therefore asked whether fibrocytes are present in the skin of bleomycin-treated mice and whether adenosine A_{2A} receptor deletion or blockade altered the recruitment of these cells to the skin. We report here that there is a marked increase in dermal fibrocyte number in bleomycin-treated mice, even before the onset of significant fibrosis. Moreover, significantly fewer fibrocytes accumulate in the dermis of mice treated with bleomycin when adenosine A_{2A} receptors are deleted or blocked. These results suggest that adenosine A_{2A} receptors regulate fibrocyte recruitment to the skin and may present a novel target for the treatment of scleroderma and other fibrotic disorders.

MATERIALS AND METHODS

Tissue Sections from Bleomycin-treated Mice

Paraffin-embedded, fixed skin sections from wild type or adenosine A_{2A} receptor knockout mice treated with bleomycin, and wild type mice treated with bleomycin plus an adenosine A_{2A} receptor antagonist (ZM241385) were used in these studies. The effects of adenosine receptor blockade and deletion on dermal fibrosis in these mice were previously reported by us [2] and sections from these same blocks, stained for hematoxylin and eosin or picrosirius red were also published previously [2].

Immunohistochemistry

Double immunohistochemistry was carried out on the Formalin-fixed, paraffin-embedded tissue sections. Antigen Retrieval was performed using Tris-EDTA buffer/pH 9.0 at 95–100° C for 20 min. Sections were blocked by incubation in normal rabbit serum—species same as secondary antibody and endogenous peroxidase was inactivated with H₂O₂. Tissue sections were first incubated with a rat anti-mouse CD34 mAb (30 min at room temperature) and secondly with a rabbit anti-Rat Biotinylated secondary antibody (Vector lab. inc. Burlingame, CA) in PBS for 30 min at room temperature. For detection, slides were incubated with ABC-Peroxidase Solution for 30 min at room temperature and developed in

DAB peroxidase substrate (brown chromagen; all from Vector Laboratories, Burlingame, CA, USA). Then, sections were pre-incubated with donkey serum, incubated with Goat anti-mouse type I procollagen (Vector) followed by incubation with alkaline phosphatase-conjugated Donkey anti-goat antibody (Vector). The color reaction was performed using the Fast Red substrate system (Dako). Slides were counterstained with Mayer's hematoxylin and mounted in glycerol gelatin. Controls included omission of the primary and/or secondary antibodies and substitution of the primary antibodies with irrelevant antibodies of the same species. Quantification of procollagen-positive and CD34-positive cells was based on the average cell number of ten high-power fields per slide using an $\times 10$ eyepiece and $\times 40$ objective lens and the results from three slides from three different mice in each group were counted.

Statistical Analysis

Statistical analyses were performed using SPSS (SPSS Inc, Chicago, IL, USA) statistical software. Experimental data are reported as means SD of triplicate independent samples.

RESULTS

Fibrocytes are Present in Normal Skin and the Skin of Mice Treated with Bleomycin

Some fibrocytes (CD34+/procollagen 1+ cells) were found in the skin of PBS treated-wild type mice although there was a marked increase in the skin of bleomycin-treated mice, as compared to PBS-treated mice (Figs. 1, 2, 3). The fibrocytes were found primarily in groups in the peri-vascular space and individually just under the epidermis (Fig. 1) and their presence preceded dramatic fibrosis of the skin since similar numbers and tissue localization of dermal fibrocytes were seen in mice treated with bleomycin for 1 or 3 weeks (Fig. 2).

Adenosine Receptor Deletion or Blockade Diminishes Dermal Fibrocyte Numbers After Bleomycin Treatment

In the skin sections studied here we had previously demonstrated that adenosine A_{2A} receptor blockade or deletion suppressed the development of fibrosis [2], thus we counted the number of fibrocytes in the skin of bleomycin-treated mice in which adenosine A_{2A} receptors were either deleted or blocked. We found that adenosine A_{2A} receptor blockade or deletion led to a significant reduction in the number of fibrocytes in the skin of bleomycin-treated mice associated with the previously reported reduction in fibrosis (Fig. 3).

DISCUSSION

We report here that fibrocytes accumulate in the skin of bleomycin-treated mice and fewer fibrocytes are recruited to the skin of bleomycin-treated mice when their adenosine A_{2A} receptors are deleted or blocked. These results are consistent with the hypothesis that fibrocytes play a role in dermal fibrosis in this model of scleroderma and that adenosine A_{2A} receptors are critical regulators of fibrocyte accumulation.

In contrast to our results Boban and colleagues [22] found no evidence of an increase in fibrocyte trafficking to the skin following bleomycin treatment. In our experiments mice

were treated every other day with bleomycin for only 3 weeks whereas Boban and colleagues treated their mice daily for 4 weeks and it is possible that either the high doses of the drug diminished production of these bone marrow-derived cells or that maximal fibrosis/fibrocyte recruitment occurs at earlier time points than that used by Boban *et al.* [22].

Prior studies have demonstrated that topical application of adenosine A_{2A} receptor agonists to wounds promotes wound healing by increasing both angiogenesis and matrix production [11, 23–25] and, conversely, deletion of adenosine A_{2A} receptors diminishes granulation tissue formation in wounds [23]. Although adenosine and its A_{2A} receptor stimulate mature dermal fibroblasts to produce increased matrix [2] and mature microvascular endothelial cells to form microtubules and produce angiogenic factors [26, 27] results of other studies indicate that adenosine A_{2A} receptors are required for optimal recruitment of circulating progenitor cells to wounds. Thus, Montesinos and colleagues observed that adenosine A_{2A} receptor agonists increase the recruitment of endothelial progenitor cells, bone marrow-derived mesenchymal cells, from peripheral blood to healing wounds [25] contributing to micro-vessel formation in healing wounds.

Bucala and colleagues first demonstrated the presence of fibrocytes in connective tissues scars in 1994 [28] and subsequent studies demonstrated that these cells traffic to skin wounds [29] and to the pulmonary interstitium in a model of pulmonary fibrosis induced by bleomycin [30]. Chemokines, primarily CCL21 and CXCL12 (SDF-1), and their receptors, CCR and CXCR4, respectively, have been shown to play an important role in recruiting fibrocytes to injured tissue and in models of pulmonary and renal fibrosis (reviewed in [17]). Consistent with this hypothesis is the recent report that adenosine, acting at A_{2A} and A_{2B} receptors, stimulates increased expression of CXCR4 and increased migration to CXCL12 by human carcinoma cells [31]. Fibrocytes may also mature from a population of CD14+ circulating monocytes that are recruited by the same chemokines to inflamed or injured sites (reviewed in [17]) and adenosine A_{2A} receptors have previously been shown to promote the differentiation of monocytes to an alternatively stimulated, pro-angiogenic phenotype *in vitro* [32]. Although the results of the studies reported here do not reveal whether adenosine and its A_{2A} receptor promote recruitment of fibrocytes or their monocyte precursors directly or stimulate differentiation of monocyte precursors to fibrocytes in the tissue it is possible that both mechanisms are operative at sites of tissue injury or fibrosis.

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Abbreviations

A2AKO adenosine A_{2A} receptor knockout

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Fig. 1. Double immunohistochemical staining for CD34 and procollagen 1 expression in mouse skin. Shown is a representative section of dermis from a bleomycin-treated mouse stained for CD34 and procollagen 1, originally described in [2], and counterstained with Mayer's hematoxylin, as described in "Materials and Methods." In this section several CD34+ (*brown stain*), procollagen 1+ (*red stain*) cells are seen surrounding a vessel (*arrow*). Original magnification $\times 400$.

Fig. 2. Double immunohistochemical staining for CD34 and procollagen 1 expression in murine skin. Representative sections of skin from mice treated as described in [2], stained for CD34 and procollagen 1 were prepared as described in “Materials and Methods.” **A** Skin from a normal PBS-treated mouse. **B** Skin from a bleomycin-treated wild type mouse treated with bleomycin ($1 \text{ mg kg}^{-1} \text{ week}^{-1}$ for 3 weeks). **C** Skin from a bleomycin-treated A_{2A} receptor knockout mouse. **D** Skin from a bleomycin- and ZM241385-treated mouse. Original magnification $\times 200$.

Fig. 3.

The number of CD34/procollagen 1 double-stained cells/hpf in mice. Mice were treated as previously described in [2], and the number of fibrocytes/high powered field were counted, as described in “Materials and Methods.” Asterisk, $P < 0.05$ compared to 3 weeks bleomycin treatment.