Blockade of PDGF Receptors by Crenolanib Has Therapeutic Effect in Patient Fibroblasts and in Preclinical Models of Systemic Sclerosis

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Systemic sclerosis (SSc) is a multi-organ fibrotic disease with few treatment options. Activated fibroblasts are the key effector cells in SSc responsible for the excessive production of collagen and the development of fibrosis. Platelet-derived growth factor (PDGF), a potent mitogen for cells of mesenchymal origin, has been implicated in the activation of SSc fibroblasts. Our aim was to examine the therapeutic potential of crenolanib, an inhibitor of PDGF receptor signaling, in cultured fibroblasts and in angiotensin II-induced skin and heart fibrosis. Crenolanib effectively inhibited proliferation and migration of SSc and healthy control fibroblasts and attenuated basal and transforming growth factor-β–induced expression of CCN2/CTGF and periostin. In contrast to healthy control fibroblasts, SSc fibroblasts proliferated in response to PDGFAA, whereas a combination of PDGFAA and CCN2 was required to elicit a similar response in healthy control fibroblasts. PDGF receptor α mRNA correlated with CCN2 and other fibrotic markers in the skin of SSc patients. In mice challenged with angiotensin II, PDGF receptor α-positive cells were increased in the skin and heart. These PDGF receptor α-positive cells co-localized with PDGF receptor β, procollagen, and periostin. Treatment with crenolanib attenuated the skin and heart fibrosis. Our data indicate that inhibition of PDGF signaling presents an attractive therapeutic approach for SSc.

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INTRODUCTION

Systemic sclerosis (SSc) is a devastating multi-organ disease with few treatment options. Prominent skin and organ fibrosis is a hallmark feature of SSc and is accompanied by fibroproliferative vasculopathy and immune dysfunction (Allanore et al., 2015). Activated fibroblasts are the key effector cells in SSc responsible for the excessive production of collagen and a subsequent development of fibrosis. A large number of soluble paracrine mediators have been implicated in fibrosis; in particular, the transforming growth factor (TGF)-β signaling pathway plays a central role in inducing profibrogenic cellular programs (Lafyatis, 2014).

In addition to soluble mediators, several matricellular proteins have also been shown to contribute to the development of SSc. For example, CCN2 (also known as connective tissue growth factor, or CTGF), which is present at elevated levels in SSc serum and fibrotic skin, has been associated with fibrosis in multiple organs (Igarashi et al., 1995; Leask and Abraham, 2006; Sato et al., 2000). CCN2 has been shown to cooperate with TGF-β to induce persistent fibrosis (Mori et al., 1999). Furthermore, fibroblast-specific ablation of CCN2 prevented development of dermal fibrosis in the bleomycin injection model (Liu et al., 2011). It has also been suggested that CCN2 may contribute to myofibroblast recruitment during bleomycin-induced skin fibrosis (Liu et al., 2011). Recent studies have also implicated periostin in the process of fibrosis (Huang et al., 2015; Lorts et al., 2012). Elevated levels of periostin are present in the lesional skin and serum of SSc patients (Yamaguchi et al., 2013). Periostin was shown to induce fibroblast proliferation and a myofibroblast phenotype in hypertrophic scarring (Crawford et al., 2015). Periostin-deficient mice were protected from the bleomycin-induced dermal fibrosis (Yang et al., 2012).

Platelet-derived growth factors (PDGFs), the primary mitogens for cells of mesenchymal origin, mediate their biological effects through the activation of two structurally related tyrosine kinase receptors, PDGF receptor (PDGFR)-α and PDGFRβ (Heldin and Lennartsson, 2013). Although PDGF-A is a relatively weak mitogen for dermal fibroblasts

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Abbreviations: Akt, protein kinase B; Ang II, angiotensin II; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HC, healthy control; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; SSc, systemic sclerosis; TGF-β, transforming growth factor-β

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compared with PDGF-B, recent studies have provided evidence for the important role of activated PDGFRα signaling in the development of organ fibrosis, including skin fibrosis (Olson and Soriano, 2009). On the other hand, activation of PDGFRβ leads to increased immune activation but not fibrosis (Olson and Soriano, 2011). Furthermore, administration of PDGF-A promotes atrial fibrosis, and neutralizing PDGFRα suppresses atrial fibrosis (Liao et al., 2010). Analyses of SSc skin biopsy samples also support the involvement of PDGF-A/PDGFRα in SSc. PDGF-A is overexpressed in SSc lesions (Yamakage et al., 1992), and increased expression of PDGFRα and PDGFRβ on spindle cells correlates with collagen deposition in SSc biopsy samples (Daoussis et al., 2012). Increased PDGF-A has also been reported in the dermal interstitial blister fluid of SSc patients (Clark et al., 2015). In addition, agonistic anti-PDGFRα autoantibodies have been detected in the serum of SSc patients (Moroncini et al., 2015).

Crenolanib besylate is an orally bioavailable, well-tolerated, selective inhibitor of type III tyrosine kinases (PDGFRα, PDGFRβ, and FMS-like tyrosine kinase 3) (Galanis et al., 2014). Crenolanib has higher sensitivity for PDGFRα (half maximal inhibitory concentration = 0.4 ng/ml) versus PDGFRβ (half maximal inhibitory concentration = 0.8 ng/ml) and preferentially binds to phosphorylated active kinases. Crenolanib has been clinically evaluated in phase I and II settings for treatment of solid tumors, including glioma and gastrointestinal stromal tumors (Heinrich et al., 2012). Given the key role of PDGFR signaling in the development of SSc fibrosis, the goal of this study was to evaluate the potential efficacy of crenolanib as a potential therapeutic agent for SSc using patient-derived dermal fibroblasts and a murine model of angiotensin II (Ang II)-induced skin and heart fibrosis.

RESULTS

PDGFR signaling contributes to expression of periostin and CCN2 in healthy control (HC) and SSc dermal fibroblasts

In an initial experiment, we examined the effect of crenolanib on the activation of PDGFRs in human skin fibroblasts stimulated with PDGFAA or PDGFBB. Crenolanib (25−100 nmol/L) dose-dependently blocked phosphorylation of PDGFRα upon stimulation with PDGFAA or PDGFBB (see Supplementary Figure S1a and b online) and PDGFRβ upon stimulation with PDGFBB (see Supplementary Figure S1b). Crenolanib did not affect cell viability.

Although TGF-β is the principal inducer of the extracellular matrix (ECM) proteins, PDGF has also been shown to contribute to matrix production (Horikawa et al., 2015). Therefore, we examined the effect of crenolanib on type I collagen and other selected ECM proteins in healthy dermal (HC) and SSc fibroblasts at the basal level and after TGF-β stimulation. Crenolanib (100 nmol/L) significantly reduced basal and TGF-β−induced periostin and CCN2 protein expression, whereas only modest inhibition was observed for type I collagen (Figure 1a). This dose of crenolanib is comparable with the dose used in human preclinical trials (Lewis et al., 2009). Crenolanib also partially reduced basal and TGF-β−induced mRNA levels of type I collagen, periostin, and CCN2 in HC and SSc fibroblasts (see Supplementary Figures S2 and S3 online). Depletion of PDGFRα and PDGFRβ in HC fibroblasts reproduced the inhibitory effects of crenolanib on periostin, CCN2, and type I collagen protein levels (Figure 1b). We next assessed whether PDGF ligands induce expression of periostin, CCN2, and type I collagen in HC fibroblasts. PDGFAA, but not PDGFBB, up-regulated periostin protein levels; however, CCN2 and type I collagen were not affected (Figure 1c). Together, these results suggest that PDGFR signaling contributes to the expression of selected matrix proteins in dermal fibroblasts.

Crenolanib inhibits proliferation and migration of fibroblasts

PDGF is a potent mitogenic and chemotactic agent for fibroblasts; therefore, we evaluated the effect of crenolanib on proliferation and migration in HC and SSc fibroblasts using the IncuCyte system (Essen BioScience, Ann Arbor, MI). Crenolanib was very effective in inhibiting HC and SSc fibroblast proliferation in response to 10% fetal bovine serum (FBS) or PDGFBB (Figure 2a). PDGFAA up-regulated proliferation only in SSc fibroblasts (Figure 2a), and crenolanib blocked this response. We also examined the mitogenic effect of CCN2, which has previously been reported to indirectly induce fibroblast proliferation (Xu et al., 2015). Although CCN2 alone had no effect, addition of PDGFAA together with CCN2 induced proliferation of HC fibroblasts with similar potency to that of PDGFBB (Figure 2b). The co-stimulatory effect of CCN2 on mitogenic effects of PDGFAA was not present in SSc fibroblasts (Figure 2b). The addition of CCN2 did not influence stimulatory effects of PDGFBB.

We also investigated the effects of crenolanib on HC and SSc fibroblast migration in response to 10% FBS or PDGF ligands. Cell migration was measured as relative wound density over time. The 10% FBS and both PDGF ligands stimulated migration of HC and SSc fibroblasts, and crenolanib blocked these responses (see Supplementary Figure S4a online). CCN2 did not induce cell migration and did not cooperate with either PDGFAA or -BB in inducing cell migration (see Supplementary Figure S4b). The effects of crenolanib on cell proliferation and migration in response to 10% FBS may also include the inhibitory effects on other agonists, which signal via PDGFRs such as vascular endothelial growth factor (Ball et al., 2007). We also cannot exclude off-target effects of crenolanib.

To further investigate the cooperation between PDGFAA and CCN2 in HC fibroblast proliferation, we focused on extracellular signal-regulated kinase (ERK) 1/2 and protein kinase B (Akt), the main intracellular signaling pathways activated by PDGFRs. Both PDGFAA and -BB induced phosphorylation of ERK1/2 and Akt, with PDGFBB eliciting a stronger response (Figure 2c), and as expected, crenolanib blocked these responses. CCN2 had no effect on ERK1/2 and Akt phosphorylation; however, addition of CCN2 together with PDGFAA significantly increased ERK1/2, but not Akt, phosphorylation compared with PDGFAA alone in HC fibroblasts (Figure 2d). The cooperation between PDGFAA and CCN2 did not occur in SSc fibroblasts. These results are consistent with the

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