αv Integrin Depletion Inhibits Profibrotic Cell Activation And Skeletal Muscle Fibrosis

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Introduction: Fibrotic disease is a major healthcare burden worldwide, and currently the only effective therapy is organ transplantation. Following injury profibrotic myofibroblasts generate scar tissue that eventually impairs organ function. Perivascular cells expressing PDGFRβ are known to be important in liver, lung and kidney fibrosis. However, the origin of myofibroblasts in skeletal muscle remains unclear. Secreted transforming growth factor-β (TGFβ) is a major pro-fibrogenic cytokine, however, pan-blockade of TGFβ or the TGFβ receptor has the potential for adverse effects due to the wider role of TGFβ in immunity and carcinogenesis. A large proportion of TGFβ is bound to the extracellular matrix in an inactive form, and αv integrins are major regulators of TGFβ activity by facilitating the activation of latent TGFβ to its active form. We therefore hypothesized that PDGFRβ-Cre labels profibrotic cells in skeletal muscle, and that targeting αv integrins in these cells reduces skeletal muscle fibrosis.

Methods: Mice: The contribution of PDGFRβ+ perivascular cells to skeletal muscle fibrosis was assessed in double-flourescent reporter (mTmG) mice under the control of PDGFRβ-Cre. PDGFRβ+ cells in these mice express eGFP with all other cells expressing TdTomato. To investigate whether loss of αv integrins on PDGFRβ+ cells influences the development of skeletal muscle fibrosis in vivo we used Itgavflox/flox;PDGFRβ-Cre mice (null for αv in PDGFRβ+ cells). C57BL/6 mice were used for pharmacologic inhibition of αv integrin studies. Skeletal muscle fibrosis model: Skeletal muscle fibrosis was induced by intramuscular (IM) injection of cardiotoxin (CTX). Mice were culled 21 days after CTX injection and fibrosis was assessed in skeletal muscle by quantification of picrosirius red (PSR) staining in 15 random microscopic fields. Cell sorting and qPCR: PDGFRβ+ cells were isolated from skeletal muscle of mTmG;PDGFRβ-Cre mice by fluorescent activated cell sorting (FACS) for eGFP with all other cells expressing TdTomato. To investigate whether loss of αv integrins on PDGFRβ+ cells influences the development of skeletal muscle fibrosis in vivo we used Itgavflox/flox;PDGFRβ-Cre mice (null for αv in PDGFRβ+ cells). C57BL/6 mice were used for pharmacologic inhibition of αv integrin studies. Skeletal muscle fibrosis model: Skeletal muscle fibrosis was induced by intramuscular (IM) injection of cardiotoxin (CTX). Mice were culled 21 days after CTX injection and fibrosis was assessed in skeletal muscle by quantification of picrosirius red (PSR) staining in 15 random microscopic fields. Cell sorting and qPCR: PDGFRβ+ cells were isolated from skeletal muscle of mTmG;PDGFRβ-Cre mice by fluorescent activated cell sorting (FACS) for eGFP. PDGFRβ+ cells were isolated from Itgavflox/flox;PDGFRβ-Cre and C57BL/6 mice by FACS using anti-PDGFRβ antibodies. Profibrotic gene expression in cells was determined by qPCR. Expression of a number of these mRNAs was also confirmed at the protein level. Pharmacologic inhibition of αv integrins: To determine whether pharmacological blockade of αv integrins could attenuate fibrosis in vivo and in vitro we used a small-molecule inhibitor of αv integrins CWHM12. Alzet minipumps containing CWHM12 or control (CWHM96; R-enantiomer of CWHM12) were inserted subcutaneously into C57BL/6 mice, prior to CTX injection (prophylactic model) and 10 days after CTX injection (therapeutic model). Mice were culled and tissues analyzed 21 days after CTX injection. FACSort sorted PDGFRβ+ cells from C57BL/6 mice were treated with CWHM12 or control in vitro for 5 days and then analyzed by qPCR. Statistical analysis: All data are presented as mean ± S.E.M. Statistical significance was calculated using a 2-tailed Student’s t test. Differences with a P value of less than 0.05 were considered statistically significant.

Results: The specificity of recombination in PDGFRβ+ perivascular cells using PDGFRβ-Cre was confirmed by immunostaining of uninjured skeletal muscle from mTmG;PDGFRβ-Cre mice. At 21 days following CTX
injury, PDGFRβ+ (eGFP+) cells were distributed in a manner characteristic of skeletal muscle myofibroblasts (n=10 mice) (Figure 1A). PDGFRβ+ (eGFP+) cells sorted from injured muscles of mTmG;PDGRβ-Cre mice showed marked induction of genes associated with transition to a myofibroblast phenotype (n=6 per group) (Figure 1B), and higher expression of a number of these mRNAs was also confirmed at the protein level. PDGFRβ+ (eGFP+) cells sorted from uninjured mTmG;PDGRβ-Cre skeletal muscle demonstrated marked induction of αSMA (p<0.0001), Col1A1 (p<0.0001) and TIMP1 (p<0.0001) following activation in vitro for 14 days, confirming their ability to differentiate into myofibroblasts (n=6 per group). Itgavflox/flox;PDGFRβ-Cre mice were significantly protected from CTX induced fibrosis, as determined by PSR staining for collagen (5.69±0.57% vs 9.27±0.62%, n=8 mice per group, p<0.01) (Figure 2). Furthermore, FACs sorted αv-null (itgavflox/flox;PDGFRβ-Cre) PDGFRβ+ cells demonstrated significant reduction in Col1A1 expression compared to controls (relative expression, n=5 per group, p<0.05). CWHM12 treatment significantly reduced skeletal muscle fibrosis when delivered from the time of CTX (prophylactic model: 5.87±0.56% vs 9.13±0.92%, n=15 mice in each group, p<0.01) and when delivered from day 10 post injury fibrotic (therapeutic model: 6.56±0.51% vs 8.70±0.67%, n=14 mice in each group, p<0.01) (Figure 3).

Discussion: In conclusion, we have demonstrated that PDGFRβ-Cre labels profibrotic cells in skeletal muscle and depletion of αv integrins in these cells using this genetic strategy reduces skeletal muscle fibrosis. Most importantly from a treatment standpoint, we have shown that pharmacologic inhibition of αv integrins using a small molecule inhibitor may have utility in the prevention and treatment of established skeletal muscle fibrosis. Heterogeneity within skeletal muscle perivascular cells is increasingly recognised and the discovery of distinct “specialist” subpopulations responsible for each of the main biological roles such as fibrosis and tissue regeneration should facilitate the discovery of highly targeted therapies in future.

Significance: These data demonstrate that PDGFRβ-Cre labels and allows gene manipulation in skeletal muscle pro-fibrotic cells. Furthermore, we have shown that αv integrins expressed on this population of cells are key regulators of skeletal muscle fibrosis, and pharmacologic inhibition of αv integrins by the small molecule CWHM12 has potential clinical utility in the treatment and prevention of skeletal muscle fibrosis.
Figure 1. **PDGFRβ+** perivascular cells become activated myofibroblasts *in vivo* and *in vitro*. (A) Immunofluorescence micrographs of skeletal muscle sections harvested from mTmG:PDGFRβ-Cre reporter mice 21 days following control (PBS) or CTX IM injection. Scale bars, 30 μm. (B) Gene expression profile of freshly sorted eGFP+ (PDGFRβ+) cells from skeletal muscle at day 10 following control (PBS) or CTX IM injection. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***p < 0.001, ****p < 0.0001 (Student’s t-test).
**Figure 2.** Deletion of αv integrins on PDGFRβ+ perivascular cells protects mice from CTX-induced skeletal muscle fibrosis. (A) Representative images of PSR stained sections from control and αv Cre mice 21 days following CTX injection (images are x40). (B) Quantification of PSR staining in muscle tissue after PBS or CTX treatment of control and itgav(lox/lox)/PDGFRβ-Cre mice. Data are mean ± SEM. **P < 0.01 (Student’s t test).

**Figure 3.** Blockade of αv integrins by the small molecule CWHM12 attenuates skeletal muscle fibrosis. (A) Quantification of PSR staining in the prophylactic model. (B) Quantification of PSR staining in the therapeutic model. Data are mean ± SEM. **P < 0.01 (Student’s t test).