Stem Cell Research 17 (2016) 161–169

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Short Report

Pharmacological blockage of fibro/adipogenic progenitor expansion and suppression of regenerative fibrogenesis is associated with impaired skeletal muscle regeneration

Daniela Fiore [a,c,1], Robert N. Judson [a,b,1], Marcela Low [a,b], Sunny Lee [a], Erica Zhang [a], Claudia Hopkins [a], Peter Xu [a], Andrea Lenzi [c], Fabio M.V. Rossi [a,b,2], Dario R. Lemos [a,b,*,2]

[a] The Biomedical Research Centre, University of British Columbia, 2222 Health Sciences Mall, Vancouver, BC, V6T 1Z3, Canada
[b] Faculty of Medicine, The University of British Columbia, 317-2194 Health Sciences Mall, Vancouver, BC, V6T 1Z3, Canada
[c] Department of Experimental Medicine, Section of Medical Pathophysiology, Food Science and Endocrinology, Sapienza University of Rome, Viale Regina Elena, 324, Rome 00161, Italy

ARTICLE INFO

Article history:
Received 20 November 2015
Received in revised form 16 June 2016
Accepted 20 June 2016
Available online 26 June 2016

Keywords:
Fibro/adipogenic progenitors
Satellite cells
Fibrosis
Myogenesis
Regeneration
Protein-tyrosine kinase inhibitors

ABSTRACT

Acute skeletal muscle injury triggers an expansion of fibro/adipogenic progenitors (FAPs) and a transient stage of fibrogenesis characterized by extracellular matrix deposition. While the perpetuation of such phase can lead to permanent tissue scarring, the consequences of its suppression remain to be studied. Using a model of acute muscle damage we were able to determine that pharmacological inhibition of FAP expansion by Nilotinib, a tyrosine kinase inhibitor with potent antifibrotic activity, exerts a detrimental effect on myogenesis during regeneration. We found that Nilotinib inhibits the damage-induced expansion of satellite cells *in vivo*, but it does not affect *in vitro* proliferation, suggesting a non cell-autonomous effect. Nilotinib impairs regenerative fibrogenesis by preventing the injury-triggered expansion and differentiation of resident CD45$^-$:CD31$^-$:α7integrin$^-$:Sca1$^+$ mesenchymal FAPs. Our data support the notion that the expansion of FAPs and transient fibrogenesis observed during regeneration play an important trophic role toward tissue-specific stem cells.

1. Introduction

Acute muscle damage triggers the activation and expansion of Pax7$^+$ tissue-specific stem cells called satellite cells (myogenic progenitors, MPs). Satellite cell proliferation gives rise to a population of CD31$^-$:CD45$^-$:Sca1$^-$:α7 integrin$^+$ myoblasts, that embark into a stepwise process characterized by the sequential upregulation of myogenic regulators such as MyoD, myogenin and MRF4 to eventually lead to differentiation (Le Grand and Rudnicki, 2007). Upon differentiation, myoblasts fuse and rebuild the damaged myofibers, regenerating the architecture of the muscle.

Acute skeletal muscle damage also triggers the activation of a population of CD45$^-$:CD31$^-$:α7$^-$ integrin:Sca1$^+$ mesenchymal progenitors that reside in the interstitial space between muscle fibers. Based on their ability to originate adipocytes and fibroblasts both *in vivo* and *in vitro*, these cells have been named fibro/adipogenic progenitors (FAPs) (Joe et al., 2010; Uezumi et al., 2010; Heredia et al., 2013). FAPs proliferate early during the response to acute damage, and they transiently synthesize extracellular matrix (ECM). Such fibrogenic stage is brief and its end is marked by both a decline in the number of FAPs and clearance of the collagen deposited in the extracellular space (Joe et al., 2010; Uezumi et al., 2010; Lemos et al., 2015). Recent data indicate that besides their fibrogenic activity, FAPs support developmental (Mathew et al., 2011) and regenerative myogenesis through the release of promyogenic cytokines, including IL6 and IL10 (Joe et al., 2010; Lemos et al., 2012). In order to confirm their relevance in muscle regeneration, experiments involving genetic ablation of FAPs have been attempted, reporting somewhat detrimental effects to the regenerative process (Murphy et al., 2011). However, these studies have been somewhat limited by the inability to fully ablate FAPs in skeletal muscle. An alternative strategy is to pharmacologically inhibit FAP expansion following acute damage.

Abbreviations: FAPs, fibro/adipogenic progenitors; ECM, extracellular matrix; MP, myogenic progenitor cells; TA, tibialis anterior muscle; RTK, receptor tyrosine kinase; TKIs, tyrosine kinase inhibitors; (NTX), notexin; (MyoG), Myogenin.

* Corresponding author at: Biogen, 12 Cambridge Center, Cambridge, MA 02142, United States.
E-mail addresses: daniela.fiore@uniroma1.it (D. Fiore), robert.judson@stemcell.com (R.N. Judson), mlow@brc.ubc.ca (M. Low), leesunny.96@gmail.com (S. Lee), zxy_ez_lz@hotmail.com (E. Zhang), claudia.i.hopkins@gmail.com (C. Hopkins), z.xu@alumni.ubc.ca (P. Xu), andrea.lenzi@uniroma1.it (A. Lenzi), fabio@brc.ubc.ca (F.M.V. Rossi), dario@brc.ubc.ca, dlemavo@gmail.com (D.R. Lemos).

[1] These authors contributed equally to this work.
[2] These authors are co-senior authors.

http://dx.doi.org/10.1016/j.scr.2016.06.007

Tyrosine kinase inhibitors (TKIs) have been effectively used in the treatment of the most common and progressive forms of human fibrosis (Rosenbloom and Jiménez, 2008; Beyer and Distler, 2013). Nilotinib (Tasigna®, AMN107; Novartis), a second-generation TKI, has been rationally designed to overcome Imatinib resistance in Chronic Myeloid Leukemia (Saglio et al., 2010) and is characterized by better bioavailability, tolerability and lacks the toxic effects commonly seen with Imatinib treatment, such as fluid retention, edema, and weight gain (Kantarjian et al., 2006). Nilotinib showed a more potent antifibrotic effect than Imatinib in liver and lung fibrosis (Rhee et al., 2011; H. Liu et al., 2011; Y. Liu et al., 2011; Shaker et al., 2011). Nilotinib simultaneously targets PDGFR and TGFβ pathways, which explains its potent antifibrotic effects. These data suggest that TKI, could be effectively used as antifibrotic agents (Rokosz et al., 2008).

Taking advantage of the fact that both PDGFR –a tyrosine kinase family member receptor- and TGFβ receptor drive FAP activity *in vitro* and *in vivo* (Uezumi et al., 2011; Lemos et al., 2015), here we used Nilotinib to pharmacologically block FAPs in the context of skeletal muscle regeneration. Our results show that Nilotinib reduces FAP proliferation and expansion, dampening transient fibrogenesis during muscle regeneration, an effect that is associated with a reduction of fibrogenic gene expression and collagen deposition. We also show that this effect correlates with poor regeneration after acute muscle damage, due to non-cell autonomous reduced myoblast expansion. These results suggest that while tyrosine kinase inhibitor-based therapies could prove useful to reduce excessive fibrosis associated with degenerative pathologies, such therapies could also have a detrimental effect on the overall regenerative capacity of healthy patients.

2. Materials and method

2.1. Animals

All mice were maintained in pathogen-free facility, and all experiments were performed in accordance with University of British Columbia Animal Care Committee regulations. C57BL/6 and PDGFRα-H2B::EGFP were purchased from The Jackson Laboratory. Col1a1*3.6-eGFP mice were a gift from D.W. Rowe (Center for Regenerative Medicine and Skeletal Development, University of Connecticut Health Center). Mice were treated by intraperitoneal (i.p.) injection with vehicle (DMSO) or Nilotinib (20 mg/kg/day) in DMSO (concentration 5 mg/ml). Muscle damage was induced by intramuscular injection of 0.15 μg notexin (NTX) snake venom (Latoxan), into the tibialis anterior muscle (TA).

2.2. Skeletal muscle cells preparation

TA muscle was carefully dissected and gently torn with tissue forceps until homogeneous. Collagenase type 2 (Sigma; 250 μl of 2.5 U/ml), in 10 mM $CaCl_2$, was added to each sample, and the preparation was placed at 37 °C for 30 min. After washing, a second enzymatic digestion was performed with Collagenase D (Roche Biochemicals; 1.5 U/ml) and Dispase II (Roche Biochemicals; 2.4 U/ml), in a total volume of 250 μl per each sample, at 37 °C for 60 min. Preparations were passed through a 40-μm cell strainer (Becton Dickenson), and washed. Resulting single cells were collected by centrifugation at 1600 rpm for 5 min.

2.3. Flow cytometry/FACS

Cell preparations were incubated with primary antibodies for 30 min at 4 °C in supplemented PBS containing 2 mM EDTA and 2% FBS at ~3×10^7 cells/ml. We used the following monoclonal primary antibodies: anti-CD31 (clones MEC13.3, Becton Dickenson, and 390, Cedarlane Laboratories), anti-CD45 (clone 30-F11, Becton Dickenson), anti-Sca-1 (clone D7, eBiosciences) and anti-α7 integrin (produced in-house). Typical antibody dilutions used were: antiCD31, 1:100–400; anti-CD45, 1:200–400, 1:200–400; anti-Sca-1, 1:2000–5000; anti-α7 integrin, 1:100–400. For all antibodies we performed fluorescence minus one controls by staining with appropriate isotype antibodies. Cells were stained Hoechst 33342 (2.5 μg/ml) and resuspended at ~1×10^7 cells/ml immediately before sorting or analysis. Analysis was performed on LSRII (Becton Dickenson) equipped with three lasers. Data were collected using Facs DIVA software. Biexponential analysis was performed using FlowJo X (Treestar) software. Sorts were performed on a FACS Vantage SE (Becton Dickenson) or FACS Aria (Becton Dickenson), both equipped with three lasers. Sorting gates were strictly defined based on isotype control (fluorescence minus one) stains.

2.4. FAP cell culture

FAPs were FACS sorted from either wildtype or transgenic mice expressing EGFP under a collagen1a1 enhancer (Collagen1a1 3.6-EGFP) and grown in high–glucose Dulbecco's modified eagle medium (DMEM) (Invitrogen), supplemented with 10% FBS and 2.5 ng/ml bFGF (Invitrogen) at density of 10.000 cell/well in a 48 well-plate. For TGFβ treatment experiments, after 72 h in culture the cells were stimulated with 1 ng/ml TGFβ (eBioscience) along with different concentrations of Nilotinib (0.5, 1 and 3 mM). Cells were trypsinized and resuspended in PBS containing 2 mM EDTA, 2% FBS and Hoechst 33342 (2.5 μg/ml). Col1-GFP levels in FAPs were evaluated by FACS after 72 h of treatment.

2.5. Gene expression analysis

RNA isolation was performed using RNeasy mini kits (Qiagen) and reverse transcription was performed using the Superscript Reverse Transcriptase (Applied Biosystems). The cDNA was diluted ten times in TE buffer and 5 μl was used in a reaction mix containing Droplet Digital PCR Supermix (Bio-Rad), 1. TaqMan assay and H_2O. Droplets were generated with a QX100 droplet generator (Bio-Rad), after mixing 20 μl of reaction mix and 70 μl of droplet generator oil (Bio-Rad). The emulsified samples were loaded onto 96-well plates and endpoint PCRs were performed in C1000 Touch thermal cycler (Bio-Rad) at the following cycling conditions: 95 °C for 10 min, followed by 45 cycles at 94 °C for 30 s and 60 °C for 1 min, followed by 98 °C for 10 min. The droplets from each sample were read through the QX100 droplet reader (Bio-Rad). Resulting PCR-positive and PCR-negative droplets were counted using QuantaSoft software (Bio-Rad). Expression levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT). A similar approach was taken to assess the expression of *Tgfb1* and *TNF* in single cells. Briefly, single macrophages were sorted directly into a 96-well plates containing 5.5 μl of lysis buffer (CellsDirect Resuspension & Lysis Buffer, Life Technologies). After RNA isolation and reverse transcription (High Capacity cDNA Reverse Transcription Kit, Life Technologies), diluted cDNA was used for droplet generation, endpoint PCRs, and droplet reading as discussed above. Resulting PCR-positive and PCR-negative droplets were counted to calculate the absolute number of transcripts per cell.

2.6. Isolation, culture and immunostaining of single myofibers

Single myofibers were isolated and cultured *ex vivo* as previously described (Collins and Zammit, 2009). Briefly, whole extensor digitorum longus muscles from 6 to 8 week old C57BL6 mice were removed and digested in 0.025% collagenase I for 1 h 45 min at 37 °C. Liberated single myofibers and their associated satellite cells were then maintained in myofiber media (DMEM, 20% v/v FBS and 1% v/v chicken embryo extract, 1% v/v pren-strep) for up to 72 h. For immunostaining, single myofibers were fixed in 4% paraformaldehyde (PFA) for 10 min and then permeabilized with 0.5% v/v Triton X-100 in PBS for 6 min. Fibers were incubated in blocking buffer (10% v/v goat serum and 10% v/v