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Anti-fibrotic effect of pirfenidone in muscle derived-fibroblasts from Duchenne muscular dystrophy patients

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ABSTRACT

Aims: Tissue fibrosis, characterized by excessive deposition of extracellular matrix proteins, is the end point of diseases affecting the kidney, bladder, liver, lung, gut, skin, heart and muscle. In Duchenne muscular dystrophy (DMD), connective fibrotic tissue progressively substitutes muscle fibers. So far no specific pharmacological treatment is available for muscle fibrosis. Among promising anti-fibrotic molecules, pirfenidone has shown anti-fibrotic and anti-inflammatory activity in animal and cell models, and has already been employed in clinical trials. Therefore we tested pirfenidone anti-fibrotic properties in an in vitro model of muscle fibrosis.
Main methods: We evaluated effect of pirfenidone on fibroblasts isolated from DMD muscle biopsies. These cells have been previously characterized as having a pro-fibrotic phenotype. We tested cell proliferation and migration, secretion of soluble collagens, intracellular levels of collagen type I and fibronectin, and diameter of 3D fibrotic nodules.
Key findings: We found that pirfenidone significantly reduced proliferation and cell migration of control and DMD muscle-derived fibroblasts, decreased extracellular secretion of soluble collagens by control and DMD fibroblasts, as well as levels of collagen type I and fibronectin, and, in DMD fibroblasts only, reduced synthesis and deposition of intracellular collagen. Furthermore, pirfenidone was able to reduce the diameter of fibrotic-nodules in our 3D model of in vitro fibrosis.
Significance: These pre-clinical results indicate that pirfenidone has potential anti-fibrotic effects also in skeletal muscle fibrosis, urging further studies in in vivo animal models of muscular dystrophy in order to translate the drug into the treatment of muscle fibrosis in DMD patients.

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1. Introduction

Tissue fibrosis is a complex, not completely understood, progressive process that arises in inflammatory chronic conditions. It is characterized by excessive deposition of extracellular matrix (ECM) that severely impairs tissue structure and function. Fibrosis affects various organs, including the liver [10], kidney [23], myocardium [16,25], skin [13] and lung [34]. Fibrosis has become the principal cause of death in the developed world and few therapeutic options are available. Activated fibroblasts/myofibroblasts, are the key effector cells in fibrosis, and TGF-β1 is the most potent pro-fibrotic cytokine, both in vitro and in vivo, inducing the myofibroblasts phenotype, through up-regulation of α-smooth muscle actin (α-SMA) and collagens [20].

Duchenne muscular dystrophy (DMD), the most frequent genetic disease of muscle in children, is due to mutations in the dystrophin gene. In DMD muscle fibers undergo continuous degeneration, regeneration is unable to keep pace with the requirements of new fibers, and chronic inflammation and massive connective tissue deposition with marked increases in levels of ECM proteins (fibrosis) occur.

The fibrotic process in DMD muscle, involve also (1) an imbalance of matrix metalloproteinases (MMPs) and their specific tissue inhibitors (TIMPs) [41], (2) the altered expression of proteoglycans and structural proteins [45], and (3) the release of fibrogenic cytokines such as TGF-β1. This cytokine is highly expressed in the dystrophic muscle of DMD patients and mdx mice, where it stimulates fibroblasts to produce ECM proteins like collagen and fibronectin [28,44]. In DMD and in severe congenital muscular dystrophies, fibrosis represents a negative prognostic trait associated with the primary genetic defect. In addition to impeding fiber regeneration, fibrosis might hinder the targeting of specific therapies (drugs or cells) to muscle and worsen disease progression by obstructing nutrient delivery and severely limiting patient biomechanics [21].

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone; PFD) is a small molecule that has demonstrated important anti-fibrotic and anti-inflammatory activities in vitro, on a wide range of cell types [6,11,38],

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and in vivo, in a variety of animal models of lung, kidney, hepatic and cardiac fibrosis [32]. PFD has already been employed in clinical trials, in particular in the treatment of idiopathic pulmonary fibrosis [3,22].

PFD is known to regulate key fibrotic cytokines (TGF-β and TNF-α) and growth factors (EGF, PDGF and FGF), involved in the initiation and maintenance of the fibrotic process, to inhibit several inflammatory mediators, and to restore immune response balance [29]. Furthermore, in hepatic stellate cells, PFD has been shown to reduce cell proliferation and collagen production and to modulate ECM homeostasis through regulation of MMP-2 and TIMP-1 [8]. Finally, in models of lung and hepatic fibrosis, PFD has also been demonstrated to reduce markers of oxidative stress [27]. Oxidative stress is often associated with fibrogenesis, and reactive oxygen species are implicated in cell damage and apoptosis and mediate TGF-β-induced myofibroblast proliferation, ECM production and contractility.

The purpose of this study was to test the potential anti-fibrotic activity of PFD, in an in vitro model of muscle fibrosis based on primary human fibroblasts isolated from muscle biopsies of patients affected by DMD [42,43]. These cells have been previously characterized as having a pro-fibrotic phenotype [41,42].

PFD has effects on multiple signalling cascades, but it remains unclear whether PFD has a single key target, or its effects derive from a broad spectrum of action. Relatively few pre-clinical in vitro studies are available on effects of PFD, or its pharmacological counterpart nintedanib, in fibrosis-related mechanisms. In this context the present study investigates the pre-clinical effects of PFD on DMD muscle-derived fibroblasts.

2. Materials and methods

2.1. Cell cultures

Primary cell cultures were derived from quadriceps muscle biopsies obtained after informed parental consent from three DMD patients (aged 2–4 years) and three controls (age 3–4 years). Control cells were from subjects that had undergone muscle biopsy because suspected of neuromuscular disease, but resulted to have normal muscle on biopsy and no evident muscle weakness on clinical examination. Investigations on human tissue were approved by our institutional review board and were in accordance with Italian law. DMD was diagnosed by dystrophin testing and gene analysis. Fibroblasts were isolated by immunomagnetic selection from primary muscle biopsy-derived cell cultures as described previously [41] and cultured in Dulbecco's modified Eagle's medium (DMEM, Cambrex Corporation, East Rutherford, NJ), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Cambrex), 1% penicillin–streptomycin (Cambrex) and 2 mM L-glutamine (Cambrex). Experiments were performed on early passages (3–10 passages) of normal and dystrophic fibroblasts. Comparisons were always between DMD and control fibroblasts at similar passages.

2.2. Chemicals

Pirfenidone (PFD) was purchased from Sigma Aldrich (Spruce St., St. Louis, MO, USA) and dissolved in DMSO to produce 20 μg/μl stock solution; working solution was obtained by diluting stock solution with DMEM (DMSO final concentration in the cell culture was kept below 0.1%). In untreated cultures an equivalent amount of vehicle (DMSO) was added. No cytotoxic effect was observed after DMSO exposition.

2.3. MTT assay

The toxicity of PFD on fibroblasts was evaluated by the MTT assay in basal conditions and after drug treatment. Briefly, DMD and control fibroblasts (2×10^4) were grown in 96-well plates in DMEM supplemented with 10% FBS for 24 h. The cells were then washed three times with serum-free DMEM and were serum-starved for 24 h to make cells quiescent. Subsequently, PFD (0, 50, 100, 150, 200 and 400 μg/ml in DMEM with 10% FBS) was added to each well for either 24 or 48 h. After washing with PBS, 20 μl of 5 mg/ml MTT was added to each well and the plate incubated for 3 h at 37 °C. After washing with PBS, 150 μl MTT solvent solution (containing 4 mM HCl, and 0.1% Nonidet P-40 in isopropanol) was added to each well. After 15 min, absorbance was read at 570 nm on a Victor Wallac 1420 multi-label reader (Perkin-Elmer, Waltham, MA, USA).

2.4. BrdU proliferation assay

The effect of PFD on cell proliferation was evaluated by measuring the incorporation of 5-bromo-2′deoxyuridine (BrdU). Briefly, DMD and control fibroblasts (2×10^4) were grown in 96-well plates in DMEM supplemented with 10% FBS for 24 h. The cells were then serum-starved for 24 h, to make cells quiescent, and then treated for 24 or 48 h with different concentration of PFD (0, 50, 100, 150, 200 and 400 μg/ml in DMEM with 10% FBS). After washing with PBS, 100 μl of 100 μM BrdU staining solution was added to each well and cells cultured for 24 h. After washing with PBS, cells were fixed in 3.7% formaldehyde in PBS, permeabilized with 0.1% Triton-X100 in PBS and denatured with 2N HCl. BrdU incorporation was detected with mouse monoclonal anti-BrdU antibody (Invitrogen Life Technologies, Carlsbad, CA, USA, diluted 1:100), followed by secondary GAM-biotinylated antibody (Jackson ImmunoResearch, Westgrove, PA, USA, diluted 1:1000) and alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, diluted 1:500) incubation. Signals were detected using alkaline phosphatase substrate kit (Biorad, Hercules, CA, USA) and reading the absorbance at 405 nm on a Victor Wallac 1420 multi-label reader.

2.5. Cell wound assay

Fibroblasts were harvested with buffered EDTA, re-suspended in serum-free DMEM with 0.1% BSA, and plated into 12-well culture plates (3×10^4 cells/well). At 24 h, the cell monolayers were gently scratched with a 200-μl pipette tip, to create a linear wound, and were treated either with PFD (200 μg/ml), TGF-β1 (10 ng/ml, Peprotech, London) or PFD plus TGF-β1. Digital pictures (4×) were taken immediately after scratching, and again after 24 and 48 h, and the amount of wound closure was measured in mm using Image J 1.43 software (http://rsbweb.nih.gov/). Cell migration was calculated as the mean percentage of the cell migrated distance compared with the initial wound distance. Percentage of closed wound was calculated after 24 and 48 h after image analysis.

2.6. Quantification of soluble/extracellular collagen

The total soluble (non cross-linked) collagen was measured in culture supernatants from control and DMD fibroblasts before and after treatment with PFD (200 μg/ml), TGF-β1 (10 ng/ml), or PFD plus TGF-β1 for 48 h, by the quantitative dye-binding Sircol assay (Biocolor, Belfast, N. Ireland), according to the manufacturer's instructions.

2.7. Intracellular collagen quantification

Four thousand cells/well were cultured in 96-well plates for 24 h in complete medium. After washes in PBS, the cells were treated with PFD (200 μg/ml), TGF-β1 (10 ng/ml), or PFD plus TGF-β1 for 48 h. The cells were then fixed in methanol at −20 °C overnight, washed twice with PBS, and incubated in 0.1% picrosirius red (Sigma) staining solution (100 μl/well) at room temperature for 3 h. The staining solution was removed, and the cells were washed three times with 0.1% acetic acid. Absorbed stain was solubilized in 0.1 M sodium hydroxide (200 μl/well) for 1 h with agitation, and absorbance was read at

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