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Tumor-derived cytokines impair myogenesis and alter the skeletal muscle immune microenvironment

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ABSTRACT

Muscle wasting is a decline in skeletal muscle mass and function that is associated with aging, obesity, and a spectrum of pathologies including cancer. Cancer-associated wasting not only reduces quality of life, but also directly impacts cancer mortality, chemotherapeutic efficacy, and surgical outcomes. There is an incomplete understanding of the role of tumor-derived factors in muscle wasting and sparse knowledge of how these factors impact *in vivo* muscle regeneration. Here, we identify several cytokines/chemokines that negatively impact *in vitro* myogenic differentiation. We show that one of these cytokines, CXCL1, potently antagonizes *in vivo* muscle regeneration and interferes with *in vivo* muscle satellite cell homeostasis. Strikingly, CXCL1 triggers a robust and specific neutrophil/M2 macrophage response that likely underlies or exacerbates muscle repair/regeneration defects. Taken together, these data highlight the pleiotropic nature of a novel tumor-derived cytokine and underscore the importance of cytokines in muscle progenitor cell regulation.

1. Introduction

Skeletal muscle maintenance requires balancing opposing forces of muscle breakdown and muscle growth. In healthy individuals, muscle breakdown occurs continuously as a consequence of acute muscle injury, exercise, and daily activity. This breakdown is counteracted by continuous myofiber repair and regeneration. In the context of muscle wasting, however, disease-associated and/or host-derived factors interfere with both muscle breakdown and growth/regeneration processes, ultimately leading to the loss of muscle mass and function. Muscle wasting is a serious concern when present in cancer patients, where the degree of skeletal muscle atrophy is tightly linked to cancer mortality, morbidity, therapeutic prognoses, and quality of life [50]. Although the molecular basis of cancer-associated muscle wasting is unclear, several key mediators are known. Pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α, and IFN-γ, are produced by host immune cells in response to the tumors and/or by the tumor itself and can directly impact skeletal muscle biology in several ways [3,16]. One way is by suppression of protein synthesis pathways [15]. This occurs through either reduced amino acid uptake or by direct suppression of mRNA transcription and/or translation. Second, wasting may also occur due to enhanced or accelerated protein breakdown. Indeed, the ubiquitin/proteasome system is one of the most rigorously studied processes associated with muscle wasting, with regulation of ubiquitin, 26S proteasome subunits, and muscle-specific E3 ligases all implicated as mediators or drivers of muscle protein breakdown [4,33,36]. Importantly, while much attention has focused on muscle protein disequilibrium in mature muscle/myofibers, fewer studies have examined how muscle regeneration is impaired in the wasting state [48].

Skeletal muscle regeneration is linked to a pool of muscle stem cells, termed satellite cells SCs), that activates, expands, and self-renews to repair and maintain tissue mass [12]. Loss or functional suppression of this adult stem cell compartment is implicated in diverse muscle pathologies including age-associated sarcopenia [47], some muscular dystrophies [6,44], and cancer-associated cachexia [26,35]. Furthermore, SC dysfunction/impaired muscle regeneration is observed in multiple muscle atrophy contexts including burn injury [9,19], chronic kidney disease [53], diabetes [2,31], and disuse atrophy [38]. Therapies that boost SC regenerative capacity can improve gross muscle mass decline and rescue functional deficits, suggesting that the SC pool is a viable therapeutic target to limit muscle atrophy. In this study, we identified secreted, tumor-derived cytokines able to suppress myogenesis. *In vivo* evaluation of CXCL1 – a prominent tumor derived factor not previously implicated in muscle wasting – revealed impaired muscle regeneration and satellite cell fusion, defects that may, in part, be attributable to aberrant CXCL1-mediated immune cell regulation. Taken

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together, our data underscore the complexity of cancer-associated muscle wasting and highlight the utility of using both cell culture and animal models to identify and evaluate novel muscle wasting factors.

2. Results

2.1. Tumor-derived factors interfere with myoblast homeostasis and differentiation

We used an established C2C12 myoblast differentiation model to screen a panel of lung cancer cell line supernatants for the ability to impair myogenic differentiation. *In vitro* myotube formation was assessed by exposing differentiating myoblasts to conditioned media from exponentially growing $Kras^{LA1/+}$;$p53^{R172H/\Delta g/+}$ lung adenocarcinoma cells and quantifying myotube maturation after 4 days in differentiation/conditioned media. C2C12 myoblasts cultured in the presence of control media exhibited extensive myogenic differentiation, as evidenced by the formation of Myosin Heavy Chain (MyHC)-positive, multi-nucleated myotubes. In contrast, C2C12 cells cultured using conditioned media (CM) from 307P or 531LN2 lung cancer cells formed fewer MyHC + myotubes (control, 307P, 531LN2 mean values = 2.56, 1.40, 1.15 respectively) that failed to mature into elongated, multi-nucleated structures (Fig. 1A and B). Importantly, we did not observe statistically significant myogenic suppression with all lung adenocarcinoma cell lines tested (344SQ, Fig. 1A and B), indicating that CM from 307P and 531LN2 lung cancer cells contains factors that actively suppress differentiation as opposed to simply depleting essential nutrients from base media. To determine whether this differentiation block was associated with defects in cell cycle progression, we performed flow cytometry based cell cycle/S-phase transit assays using 5-ethynyl-2′-deoxyuridine (EdU). We observed a significant decrease in S-phase (EdU) incorporation in 531LN2-CM C2C12 cultures (~28% in control compared to ~20% 531LN2-CM) whereas we observed slight, but insignificant deficits in 307P-CM C2C12 cultures (Fig. 1C and D). Cultured primary myoblasts similarly treated with either 531LN2 or 307P CM also exhibited minor reductions in Edu incorporation compared to control cells, whereas we observed statistically significant reductions in myogenin (a marker of late myogenic differentiation) expressing cells, compared to control myoblasts (Supplementary Fig. S1). To evaluate tumor-induced changes to mitochondria biogenesis in muscle cells, we measured mitochondria abundance using a Mitotracker Green dye in our lung cancer (LC)-CM C2C12 cultures. We labeled mitochondria in C2C12 cells cultured in the presence of LC-CM for 24 hours and found a ~15–20% decrease in Mitotracker Green mean fluorescence intensity (MFI), indicating a decrease in mitochondria mass in the presence of 307P and 531LN2 conditioned media (Fig. 1E and F). Taken together, these data show that secreted factors in LC-CM can potently block myogenic differentiation and that these changes may, in part, result from cell line- (or cancer type-) specific defects in cell cycle progression or alterations in mitochondria biogenesis.

2.2. Identification of tumor derived secreted cytokines

We next profiled over 100 cytokines/chemokines in 307P, 531LN2 and 344SQ lung cancer line supernatants using antibody arrays to identify secreted factors linked to suppression of myogenic differentiation. We were able to detect 26 proteins above background in at least one of three lung cancer line supernatants. Of these proteins, IGFBP3, LIX/CXCL6, SerpinE1, Osteopontin, and CXCL1/KC/GRO1 exhibited the highest relative signal intensities in our lung cancer supernatants (Fig. 2A). We then performed hierarchical clustering analyses based on normalized log2 transformed relative expression values and found two cytokine clusters ($344SQ^{low}/307P^{low/med}/531LN2^{high}$ and $344SQ^{low}/307P^{med/high}/531LN2^{high}$) that exhibited expression patterns consistent with the ability of these supernatants to suppress C2C12 differentiation (ref to Fig. 2B). Based on these expression patterns, we then tested the hypothesis that individual recombinant cytokines can suppress myogenesis. Of the eight cytokines tested (IGFBP3, IGFBP6, CXCL1, CXCL6, CCL2, CCL17, CCL20, Endostatin), three – IGFBP3, CXCL1, and CCL2 – displayed a statistically significant ability to impair *in vitro* myogenesis, as evidenced by suppression of multi-nucleated MyHC + myotube maturation (Fig. 2C and D). Reverse phase protein array (RPPA) analyses of CXCL1-treated C2C12 cells revealed down-regulation of several signal transduction pathways linked to myogenic differentiation, including PI3K-AKT [21], ErbB [18,24], and HIF-1/AMPK [20] signaling pathways (Supplemental Fig. S2). Importantly, while we did not observe suppressive activity with most cytokines tested, we cannot rule out the possibility that specific doses, lots, local concentrations, or cytokine combinations are required for these cytokines to impact myogenic differentiation. Taken together, these experiments revealed two cytokine clusters positively associated with suppression of myogenesis and provide evidence that several individual cytokines, including CXCL1, can directly impact myogenic differentiation.

2.3. Tumor bearing mice exhibit weight loss and elevated CXCL1 levels

Given the ability of tumor-derived cytokines to suppress myogenic differentiation and the importance of muscle regeneration/repair in maintenance of muscle mass, we next asked (1) if we could detect elevated CXCL1, the most differentially and highly expressed cytokine identified in our cytokine arrays, in serum and skeletal muscle of tumor-bearing mice, and (2) whether tumor-bearing mice exhibited evidence of weight loss. We used a transplant-based approach, previously established using related isogenic lung cancer cell lines [23], to initiate xenograft tumors in recipient mice. Prior to necropsy, we subjected mice to whole body dual-energy X-ray absorptiometry (DEXA) to quantify total and lean mass in the whole animal. DEXA analyses of mice bearing subcutaneous lung tumors revealed an overall reduction in total body weight excluding the tumor (mean = 24.3 g) compared to non-tumor control mice (mean = 27.0 g) (Fig. 3A and D). This decline in overall weight appeared largely attributable to loss of lean mass (mean = 19.0 g vs. 22.4 g). (Fig. 3A and E). Serum analysis of 531LN2-tumor bearing mice revealed a nearly 10-fold increase in serum CXCL1 levels compared to non-tumor bearing healthy control mice (Fig. 3B). Furthermore, hindlimb TA CXCL1 levels in tumor bearing mice were ~20-fold higher than control muscle samples (Fig. 3C). Interestingly, we observed a striking up-regulation (> 200 fold) of *cxcl1* mRNA in C2C12 myoblast or myotube cultures exposed to 531LN2 media (Supplemental Fig. S3), suggesting that elevated CXCL1 in the skeletal muscle of tumor bearing mice may be the combined result of both tumor-derived CXCL1 as well as tumor-induced CXCL1 production in skeletal muscle. Upon necropsy, we confirmed the DEXA-determined decline in lean mass with *tibialis anterior* (TA) and *gastrocnemius* (GR) wet weight measurements (Fig. 3F and G). Morphometric evaluation of myofiber size further revealed a statistically significant downward shift in the distribution of myofiber feret diameters (Fig. 3H). Taken together, our data show that lung tumor xenotransplantation results in overall weight loss, a decline in lean mass, and is associated with elevated CXCL1 levels in serum and skeletal muscle.

2.4. CXCL1 modulates skeletal muscle regeneration and in vivo satellite cell homeostasis

Skeletal muscle regeneration requires the activation, expansion, and differentiation of satellite cells, a resident adult muscle stem cell population. Given the antagonistic effect of CXCL1 on *in vitro* myogenesis, as well as increased CXCL1 abundance in lung tumor bearing mice, we next tested the hypothesis that elevated CXCL1 would limit *in vivo* muscle regeneration. We injured wildtype TA muscle with 1.2% barium chloride ($BaCl_2$) in either a 0.9% saline vehicle or vehicle containing