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Muscle Oxidative Phenotype in the Progression of Cancer-Cachexia

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in the College of Education and Health Professions

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Abstract:

Cancer is one of the leading causes of death across the world. Cancer-cachexia is a serious complication induced by cancer resulting in whole body muscle wasting and is responsible for up to 40% of cancer related deaths. Underlying mechanisms of cancer-cachexia are not well understood; however, a loss in oxidative metabolism in skeletal muscle is apparent in cancer-cachexia. PURPOSE: To examine oxidative phenotype of skeletal muscle in tumor bearing mice during progression of cancer-cachexia. METHODS: Mice were implanted with Lewis Lung Carcinoma (LLC) or sham phosphate buffered saline (PBS) at 8 weeks of age. The tumor was allowed to progress for four weeks, with cohorts harvested weekly. Tibialis Anterior (TA) muscle cross sections were stained for Succinate dehydrogenase (SDH) to analyze the percent of oxidative muscle fibers and cross-sectional area (CSA) of SDH positive and negative fibers was analyzed. Immunoblotting was utilized to examine markers of mitochondrial content and biogenesis, specifically peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and Cytochrome c oxidase-IV (COX-IV). RESULTS: TA weight was approximately 10% lower in 4 week LLC mice when compared to PBS control mice. In the PBS group, 65% of fibers were SDH positive compared to only 40% SDH positive in 4 week LLC mice with no other differences among groups. COX-IV was significantly reduced in 4 week LLC mice compared to PBS with no other differences among groups. No significant differences in PGC-1α were observed. CONCLUSION: LLC in mice may promote a cellular energy crisis leading to a decrease in skeletal muscle oxidative phenotype. Based on my findings, promoting the oxidative phenotype and hence the mitochondrial network could be a potential therapeutic target to treat cancer-cachexia.

Introduction:

Cancer is one of the leading causes of death across the world with the World Health Organization reporting 8.2 million deaths and 14.1 million new cancer cases in 2008 (6). Cancer-cachexia is a serious complication induced by cancer resulting in whole body muscle wasting. It is a multifactorial syndrome characterized by continuous and quick skeletal muscle mass degeneration which cannot be reversed by standard nutritional intervention (6). In fact, cancer-cachexia occurs in ~80% of cancer patients and is the primary cause of death for 22%-30% of all individuals that have cancer (6, 16). Overall, metabolic derangements that induce cancer-cachexia are not fully elucidated in scientific literature and underlying mechanisms of cancer-cachexia are not well understood; however, a loss in oxidative metabolism in skeletal muscle is apparent in late-stage cancer-cachexia (22).

The irreversible rapid skeletal muscle-wasting effects of cancer-cachexia are enhanced by a combination of abnormal metabolism and reduced intake of food induced by the cancer (20). The current therapy for cancer-cachexia patients includes increasing the caloric intake by modifying their diet or using pharmacological interventions, however, this therapy is inadequate because other factors besides hypophagia contribute to the loss of skeletal muscle during cancer-cachexia (19). These factors result in increased breakdown of muscle protein as well as a reduction in muscle protein synthesis, both of which lead to reduced skeletal muscle mass (4, 14, 15). Understanding the underlying mechanisms that induce cancer-cachexia is critical to the development of therapies to ensure survival.

The progressive muscle wasting effects developed in cancer-cachexia may be related to muscle metabolism since several diseases including cancer-cachexia show specific susceptibility

of glycolytic muscle fibers (6). Previous studies that include tumor implantation in mice to induce cancer-cachexia have shown that the gastrocnemius and tibialis anterior muscles undergo an atrophy of primarily fast-type muscle fibers (1, 6). The mitochondria are responsible for energy production in muscle cells; therefore, mitochondria are directly related to muscle oxidative capacity and muscle mass loss (6). During wasting conditions such as cancer-cachexia, the mitochondrial content of the muscle is compromised seemingly due to a suppression of mitochondrial biogenesis and/or a decreased regeneration of mitochondrial proteins (6). The primary regulator of mitochondrial biogenesis in skeletal muscle is the transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (9). PGC-1α is also directly related to modifications of muscle mass (6) and studies have shown that implantation of tumors in mice results in a decline in PGC-1α mRNA expression (18). Thus, PGC-1α is correlated to cancer-induced muscle atrophy. Cytochrome c oxidase (COX-IV) is also believed to be correlated to cancer-induced muscle atrophy because it is a major regulation site for oxidative phosphorylation and plays an important role in the final step of the mitochondrial electron transfer chain (10). In a prior study mice with tumors implanted subcutaneously and progression to a cachexic state after four weeks, the gastrocnemius and soleus muscles showed a drastic decrease in the oxidative mitochondrial content as well as an inhibition of the PGC-1α and COX-IV expression (6). In fact, impaired oxidative metabolism may instigate cancer-cachexia (2, 12). This is because the mitochondria are the primary adenosine triphosphate (ATP) producer, provide maintenance of protein balance that is energetically expensive, and are the number one free radical producer that may activate protein degradation. Therefore, mitochondrial degeneration in muscle cells may be an underlying cause of cancer-cachexia that ultimately leads to patient death.

The end result of cancer-cachexia is clearly problematic, but unfortunately there is a gap in current knowledge regarding the progression of the disease. Current literature suggests a need to focus on the prevention of cancer-cachexia (11). Unfortunately, there is a sparsity of work attempting to examine alterations in the progression of the disease. Narsale et al. (14) has implied progression from varying degrees of cachexia in mice spontaneously developing colorectal cancer, the Apc (Min/+) mouse. However, there is no known prior work to assess a direct time course of changes in the progression of cancer-cachexia. Therefore, the triggering events in the muscle leading to cachexia and whether these are related to altered oxidative phenotype, are currently unknown.

The purpose of this experiment was to examine oxidative phenotype of skeletal muscle in tumor bearing mice with cancer-cachexia and establish a time course of the changes in skeletal muscle oxidative phenotype throughout the progression of cancer-cachexia. Current studies surrounding cancer-cachexia do not focus on the progression of the cancer, but rather the end-point (17, 21, 22), therefore using a time course experiment provides valuable information about development of the condition which can aid in development of new therapies to treat cancer-cachexia. With this study, I observed not only how the phenotype of the muscle cells changed following development of the cachectic phenotype, but also what happened to them during tumor development. I hypothesized a decrease in skeletal muscle size and weight as well as a decrease in the number of muscles with an oxidative phenotype in the Lewis Lung Carcinoma (LLC)-induced mice when compared to a control group after four weeks. I also expected to observe a gradual progression of the loss of oxidative phenotype during the four weeks. My thesis will fill gaps in scientific literature and provide insight on the progression of cancer-cachexia, as well as how the oxidative phenotype in skeletal muscle cells changes.

Methods:

Experimental Animals:

All animal methods were approved by the University of Arkansas Institutional Animal Care and Use Committee. Male C57BL/6J mice were acquired from Jackson Laboratory (Bar Harbor, ME). Phosphate buffer saline (PBS) was injected into the hind flank of one group of mice to serve as sham control and LLC cells at 1×10^6 diluted in PBS were implanted into the hind flank of each remaining mouse at 8 weeks of age (17). To assess the time course of cachectic development, the tumor was allowed to develop for 1, 2, 3 or 4 weeks in separate experimental groups. Hind limb muscles from mice from each week were collected, weighed and processed for further assessment. I used LLC because it allowed me to control the time course of cachexia development and is known to cause numerous cytokines to become up-regulated during cancer-cachexia in mice, and those cytokines take part in a crucial role in the progression of muscle atrophy that results from cachexia (17). To validate cancer-cachexia phenotype, muscle size was assessed by muscle wet weights. For microscopy meas ures the tibialis anterior (TA) muscle was extracted and snap frozen in optimum cutting temperature compound (OCT, Tissue-Tek, Sakura). 10 µM cross sections were cut and stained for Succinate dehydrogenase (SDH) to analyze the percent of oxidative muscle fibers. To assess muscle protein contents relative to mitochondrial content and biogenesis gastrocnemius muscles were excised and snap frozen in Liquid Nitrogen before storage at -80°C until furthered processing.

SDH Staining:

The protocol used for SDH staining is based on Nachlas et al. (13) for staining skeletal muscle cross-section to determine if there is a change of mitochondrial SDH activity as an index

of oxidative phenotype. The skeletal muscle was cut in cross-sections (10 µm) using a cryostat and mounted on a positive charged glass slide, and stored at -80°C until ready for use. Next, the slides with muscle section were placed in a chamber, and incubated in a 20 mL, pre-warmed (37°C) incubation solution (50 mM sodium succinate [Sigma S2378], 50 mM phosphate buffer, 0.5 mg/mL [Sigma N5514]) for 40 minutes. The phosphate buffer stock solution was 100 mL (0.12M potassium dihydrogen phosphate [Fisher P380], 0.88M sodium hydrogen phosphate [Fisher S374]). After 40 minutes, samples were washed in distilled water three times for a minute each. Finally, the water was wiped off of the slide glass without touching the muscle section and mounted with a drop of toluene mounting media, and then sealed. Slides were imaged using Nikon TiS inverted microscope at 20X objective and Nikon Basic Research software (Melville, NY). SDH positive fibers were manually analyzed by counting SDH positive (purple) and SDH negative (white) muscle fibers by a blinded investigator. The counting of the fibers was cross-verified by a second blinded investigator and there was no difference between investigators. Data are expressed as percent SDH positive fibers to total fibers. Next, SDH positive and negative fibers were independently assessed for muscle cross-sectional area (CSA) using the same Nikon Basic Research software to determine specific atrophy of fibers by oxidative phenotype. Data are expressed by mean CSA $\pm$ SEM.

Immunoblotting:

Gastrocnemius muscles were prepared for immunoblotting as previously described by our laboratory (8). Subsequently, 40µg total protein was separated by SDS-PAGE, transferred to PVDF membrane, and blocked in 3% w/v Bovine Serum Albumin in TBS with 0.2% Tween 20 (TBST). Membranes were then probed overnight for primary antibodies specific to PGC-1α (sc-13067, Santa Cruz Biotechnologies, Santa Cruz, CA) and COX-IV (4844s, Cell Signaling

Technologies, Danvers, MA), followed by incubation in mouse anti-rabbit secondary antibody (L27A9) from Cell Signaling for 1 hour at room temperature. Membranes were imaged on Protein Simple FluorChem (Minneapolis, MN) and analyzed using Alpha View software. All bands were normalized to the 45 kDa Actin band of Ponceau S stain as a loading control.

Statistical Analysis:

The independent factors in this study were PBS and the number of weeks the tumor was allowed to progress. One-way ANOVA was employed as the global analysis for each dependent variable and the differences among means was determined by Student-Newman-Keuls Post Hoc Analysis. The comparison-wise error rate, α, was set at 0.05 for all statistical tests.

Results:

Phenotypic description of experimental animals:

Phenotypic data are presented in Table 1. Total body weight was 9% greater in four week LLC mice compared to PBS control mice. However, umor free body weight (BW-Tumor) was 5% lower in four week LLC mice compared to PBS. Tibialis Anterior (TA) and gastrocnemius muscle weights were approximately 10% and 12% lower in four week LLC mice when compared to PBS, respectively. The weight of the spleen was 352% higher in the four week LLC mice than the PBS, further indicating a cachectic state. Figure 1 A/B displays weights of the TA and gastrocnemius throughout the progression of cancer-cachexia. Tumor growth by weight across all cohorts of tumor-bearing mice exhibited an exponential growth curve with $R^2 = 1.00$.

Variable	PBS (n=24)	1 Week (n=16)	2 week (n=16)	3 week (n=12)	4 week (n=14)
Body Weight (g)	24.85±0.333a	23.925±0.328a	23.8±0.629a	24.35±0.811a	27.007±0.628b
Tumor Weight (g)	N/A	0.03±0.007a	0.167±0.029a	0.771±0.148b	3.501±0.361c
BW – Tumor (g)	24.85±0.333	23.898±0.328	23.665±0.645	23.579±0.714	23.506±0.561
TA (mg)	45.144±0.855a	43.803±1.163a	44.544±1.158a	42.883±1.64a	38.343±1.312b
Gastrocnemius (mg)	134.552±2.07a	129.494±2.459a	130.006±4.088a	125.163±3.47ab	118.393±4.059b
Spleen (mg)	81.164±3.214a	81.856±2.775a	85.713±2.599a	222.858±31.087b	366.564±31.8c

Table 1. Body weight, tumor weight and skeletal muscle wet weights at time of harvest. Muscle fibers were significantly smaller following tumor implantation. Different letters denote significance at an alpha set at $p<0.05$.
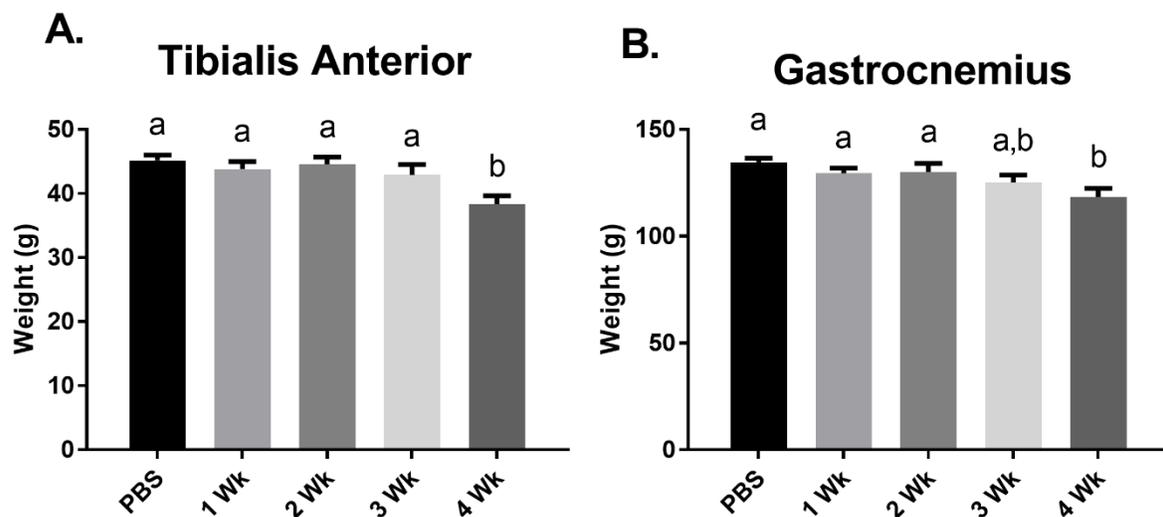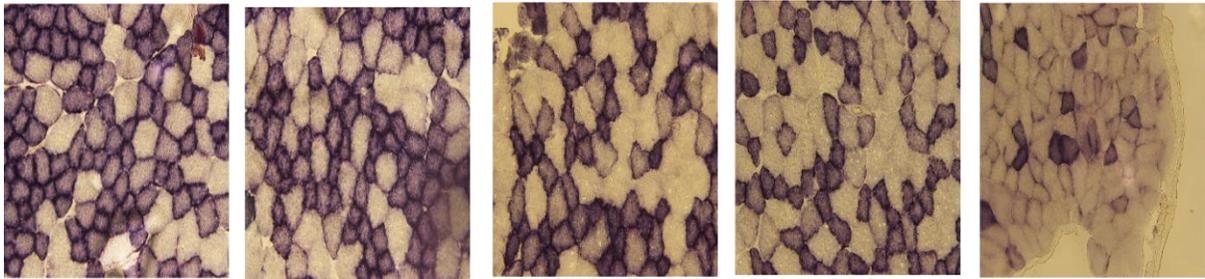

Figure 1. (A) The TA weight was significantly lower in four week LLC mice than PBS. (B) The gastrocnemius weight was also significantly lower in four week LLC mice compared to PBS. Differing letters denote significance at an alpha set at $p<0.05$.

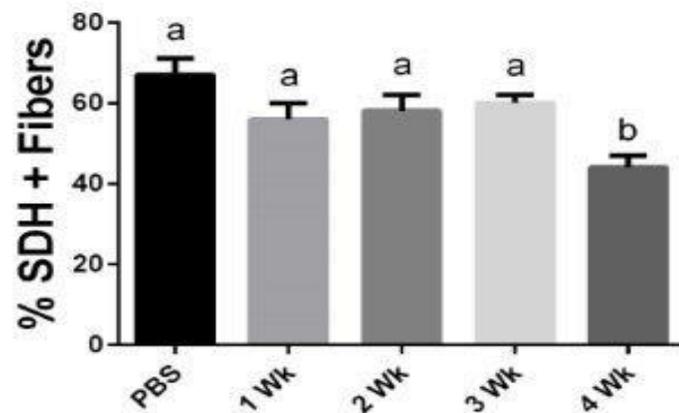*Late stage cancer-cachexia is associated with reduction in oxidative myofibers:*

We observed 65% SDH positive oxidative phenotype in the PBS group compared to a 40% SDH positive oxidative phenotype in the muscle fibers in 4 week LLC with no other statistical differences among groups (Figure 2 A/B), an apparent lower number of oxidative phenotype muscle fibers in other groups of tumor-bearing mice did not reach statistical

significance. To examine whether oxidative fibers were more or less susceptible to wasting, next I measured the CSA of SDH positive and SDH negative fibers. I found that the CSA was significantly different between the PBS and four week LLC mice for SDH negative and SDH positive fibers. CSA frequency distributions demonstrated more small muscle fibers and fewer large muscle fibers in four-week tumor bearing mice than all other conditions for both SDH positive and SDH negative fibers, however, the rise in number of smaller fibers was more prominent in SDH negative muscle fibers (Figure 2 C/D).
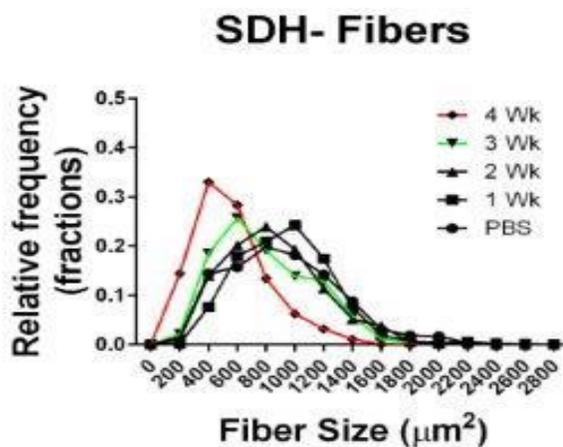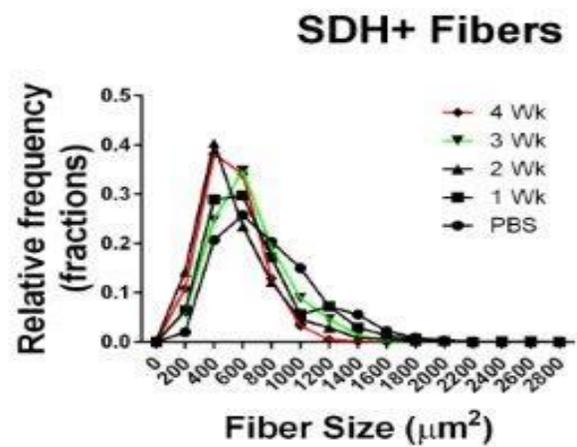
Figure 2. (A/B) Succinate Dehydrogenase Staining of the TA muscle. Significant loss of oxidative muscle fibers is observed at four weeks following tumor implantation. We observed 65% SDH positive oxidative phenotype in PBS mice and 40% SDH positive oxidative phenotype in the muscle fibers in four-week tumor bearing mice. (C/D) The CSA of both the non-oxidative and oxidative fibers in four week LLC mice significantly dropped. Significance is indicated by red and green lines.

Markers of mitochondrial content and biogenesis

My data did not show any significant differences associated with mitochondrial biogenesis as measured by content of PGC-1α across the time course of cancer-cachexia progression (Figure 3 B). Simultaneously, my data demonstrates significantly lower mitochondrial content measured by COX-IV in four week LLC mice compared to PBS mice, with no other differences among conditions (Figure 3 C). Figure 1 A demonstrates a visual of the immunoblotting for COX-IV and PGC-1α.
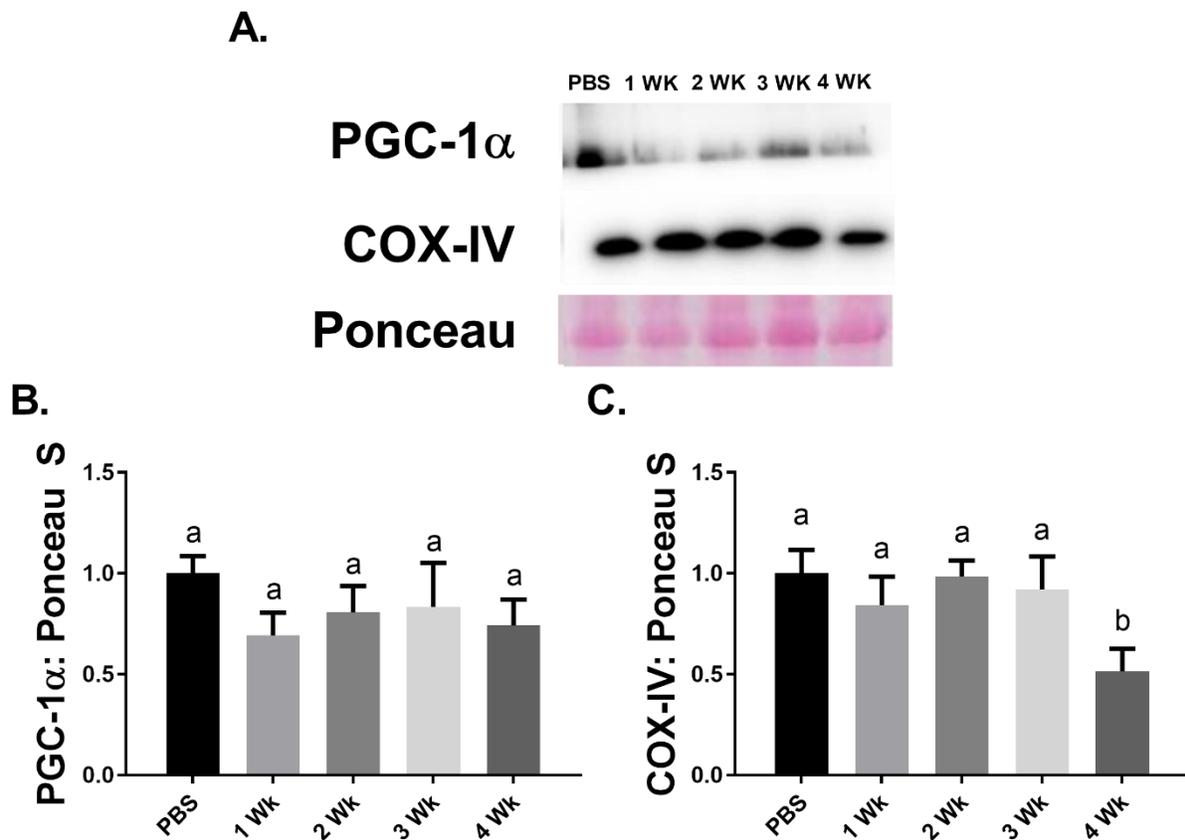

Figure 3. (A) Results of immunoblotting for PGC-1α and COX-IV compared to standard. (B) The mitochondrial content measured by PGC-1α in mice is shown to stay the same throughout the progression of cancer-cachexia. (C) The mitochondrial content measured by COX-IV presents to be significantly reduced in four week LLC mice compared to PBC mice.

Discussion:

I am the first to examine the deterioration of the oxidative phenotype during time course progression of cancer-cachexia in tumor-bearing mice. The results are clinically significant because of the improved survival and quality of life associated with the treatment of a cachectic phenotype during progression of cancer. My data shows that a decrease in the portion of SDH positive muscle fibers as well as the size of muscle fibers, specifically SDH negative (non-oxidative), does not become statistically significant until the fourth week. These data are also the first to show that mitochondrial content and the oxidative phenotype are not significantly impaired until cachectic muscle wasting develops. These results demonstrate that loss of oxidative phenotype occurs concomitantly with muscle wasting in cancer-cachexia, suggesting maintenance of the oxidative capacity of skeletal muscle might represent a viable approach to prevent the onset of muscle wasting.

Development of cachectic phenotype

Evidence of decreased muscle size was not present until the fourth week post-tumor implantation, which is in accordance with previously published data (17). This could be due in part to the exponential increase in tumor growth and the associated release of inflammatory factors. These inflammatory factors can negatively affect the balance of protein synthesis and destruction in muscle, or proteostasis. Finally, the development of muscle wasting can be a side-effect of decreased physical activity in cancer-bearing patients along with associated decreases in caloric intake (5).

Oxidative phenotype and fiber type-specific atrophy

In the SDH stain of four week LLC mice, I observed a shift away from oxidative (SDH positive) fibers toward non-oxidative (SDH negative; most likely glycolytic) fibers alongside a shift in CSA toward more smaller myofibers, specifically of the non-oxidative four week LLC fibers when compared to PBS mice (Figure 2). While the CSA of both the non-oxidative and oxidative fibers in four week LLC mice appeared lowered compared to PBS, the non-oxidative fibers observed are shown to be more prone to wasting than are oxidative fibers. A contributing factor to this might be the oxidative demand placed on the muscle cells and the lack of energetic capacity demonstrated by glycolytic muscle cells (9).

Mitochondrial content and biogenesis markers

Mitochondrial biogenesis assists in the synthesis of new mitochondria and the main regulator of this is PGC-1α (21). Our data did not show any significant changes associated with mitochondrial biogenesis as measured by PGC-1α across the time course of cancer progression. Simultaneously, I measured a significantly lower content of mitochondria at the four week timepoint in LLC mice using the marker COX-IV. This demonstrates that the change in the content of mitochondria is likely not a result of changes in the synthesis of new mitochondria but due to an increase in the breakdown of old mitochondria. This process of selective destruction of cellular components is called autophagy and when it is destroying mitochondria, it is called mitophagy (3). I find it likely that in later stage cancer-cachexia mitophagy is enhanced leading to a greater loss of mitochondria and a resultant loss in mitochondrial content. This speculation requires further examination which is beyond the scope of the current investigation.

Conclusion:

Loss of oxidative phenotype in skeletal muscle may induce skeletal muscle atrophy leading to cancer-cachexia. It appears that oxidative phenotype is lost concomitantly with loss in skeletal muscle size four weeks following tumor implantation because skeletal muscle size was lower four weeks following tumor implantation and there was a 35% lower number of oxidative muscle fibers in the TA at this same point. LLC in mice may promote a cellular energy crisis induced by a decrease in skeletal muscle oxidative phenotype likely tied to a loss in muscle mitochondrial content. Energy stress promotes atrophic signaling in myofibers, leading to cachexia. Since PGC-1α was not altered during the progression of cachexia, mitochondrial biogenesis is likely not affected during a cachectic state and unrelated to muscle atrophy during cancer-cachexia. However, the lowered mitochondrial content indicated by COX-IV in cancer-cachexia alongside the lack of change in PGC-1α suggests breakdown of mitochondria is enhanced leading to loss of mitochondrial content and a malfunction in oxidative phosphorylation (9). Based on these findings, promoting the oxidative phenotype and the mitochondrial network could be a potential viable therapeutic target to treat cancer-cachexia.

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