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Changes in Hepatic Extracellular Matrix During the Development of Cancer-Cachexia in Mice

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Introduction:

Cancer is one of the most widespread and deadly diseases of the modern medical age (Siegel, Miller, & Jemal, 2017). In 2017, it is estimated that 600,920 Americans will die from cancer, which translates to about 1,650 deaths due to cancer every day (Siegel et al., 2017). One of the major causes of cancer related death is cancer-cachexia (Hauser, Stockler, & Tattersall, 2006; Petruzzelli & Wagner, 2016). Cancer-cachexia is a condition characterized by uncontrollable weight and skeletal muscle loss, loss of appetite, and lethargy; and it affects up to 80% of patients with advanced stage cancers (Corie, 2015; Petruzzelli & Wagner, 2016). Cachexia is a complex, multi-organ, metabolic disorder that greatly disrupts the patient's energy balance, and may be irreversible at the first obvious signs of weight loss (Petruzzelli & Wagner, 2016).

Furthermore, hyper-caloric diets do not appear to be an effective treatment for cancer-cachexia in animals or humans (Corie, 2015; Petruzzelli & Wagner, 2016). In studies that force fed high-calorie diets to rats with cancer-cachexia, the diet is able to attenuate some weight loss, however, other signs and symptoms remained unchanged (Petruzzelli & Wagner, 2016). Also, severity of cancer-cachexia is often not correlated with the size or stage of the tumors, further complicating treatment options (Petruzzelli & Wagner, 2016).

As cancer-cachexia is said to be largely irreversible, recent calls have focused on efforts to prevent this condition (Fearon, 2016). Unfortunately, to date, knowledge of early stage events prior to cachectic development are severely lacking. Therefore, more research is necessary to understand the underlying mechanisms that drive cancer-cachexia and its associated mortality (Argiles, Stemmler, Lopez-Soriano, & Busquets, 2015; Petruzzelli & Wagner, 2016).

One area that is notably underrepresented in cancer-cachexia literature is liver physiology, and its relationship to cancer associated muscle wasting (Petruzzelli & Wagner, 2016). The liver is a key regulator of systematic metabolism and fuel utilization (Argiles et al., 2015). Therefore, it would be reasonable to suggest with a systemic disorder, driven by problems in energy balance, that the liver likely plays a predominant role in the progression of this pathology. However, currently, very little research has examined the extent of liver damage, or the liver's role in metabolic dysfunction during cancer-cachexia (Petruzzelli & Wagner, 2016).

In addition to cachexia, many cancer patients may concurrently exhibit liver fibrosis (Pinter, Trauner, Peck-Radosavljevic, & Sieghart, 2016). Liver fibrosis is the accumulation of excess collagen-rich extracellular matrix (ECM), and may lead to functional impairment of the liver, and eventually, even death (Pinter et al., 2016; Swathi et al., 2016). This fibrotic phenotype is caused by the liver's natural healing response to chronic liver injury and inflammation (Pinter et al., 2016; Swathi et al., 2016). Hepatic stellate cells (HSC) are the primary agents that cause liver fibrosis. When liver damage occurs, HSCs activate, replacing normal hepatic tissue with collagen-rich ECM (Swathi et al., 2016). Liver fibrosis is a complex process mediated by many different factors including other cell types within the liver, cytokines, and regulatory enzymes such as matrix metalloproteases (MMP) and tissue inhibitors of MMPs (TIMP) (Poole & Arteel, 2016; Zhou, Zhang, & Qiao, 2014).

MMPs are proteolytic enzymes that are responsible for cleavage and degradation of extracellular matrix collagen, and TIMPs are their inhibitors (Ricard-Blum, 2011). MMPs and TIMPs are constantly acting to regulate the ECM, and the relative upregulation or downregulation of these enzymes can contribute to collagen accumulation and fibrosis (Poole & Arteel, 2016; Zhou et al., 2014).

Preliminary Sirius Red histological staining data (Figure 1) shows that collagen deposition increased exponentially over time ($r^2 = 0.78$) in tumor-bearing mice. However, this histological data has limitations, as the Sirius red stain measures the deposition of both Collagen I and III, with no differentiation between them. Collagen I and III, while they are both fibrillar proteins, are functionally different (Kim, Kasukonis, Brown, Washington, & Wolchok, 2016). Collagen I is a mechanically stronger fiber, with more covalent interlinking, while Collagen III is a more elastic fiber (Kim et al., 2016). The ratio of Collagen I to Collagen III is widely utilized in ECM studies, with a high ratio indicating stiffer overall tissue, and a low ratio indicating a more distensible tissue (Kim et al., 2016). A more detailed examination of the expression of extracellular matrix proteins is needed in order to more closely observe the nature of the collagen deposition process, so that future studies may be able to identify and parse out the roles of specific ECM proteins in this pathology.

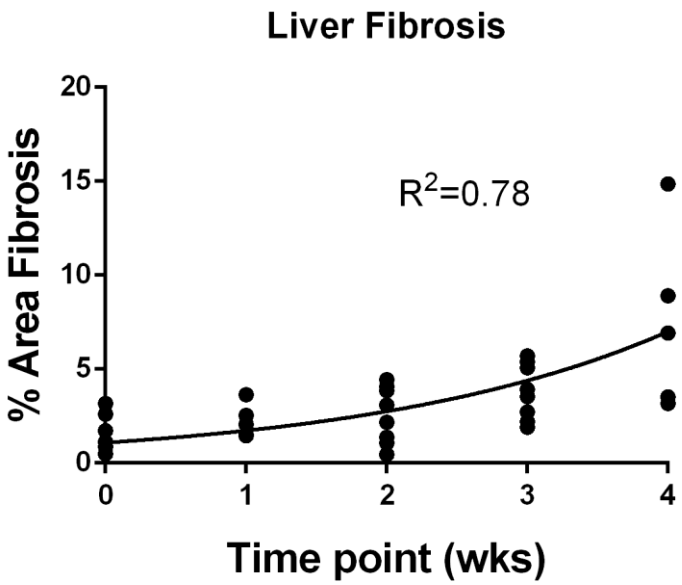

Figure 1: Exponential regression analysis of Sirius Red histological data. Percent area fibrosis vs Time. $R^2 = 0.78$

The purpose of this study was to characterize hepatic fibrosis by measuring mRNA content of Collagen I, Collagen III and MMP-9 during the development of cancer-cachexia.

Methods:

Experimental Design

The University of Arkansas Institutional Animal Care and Use Committee has approved all methods used for this experiment. Animals were housed at the University of Arkansas animal care facility in a secure, temperature and humidity controlled environment on a 12:12 light—dark cycle. All animals had free access to chow and water throughout the course of the study. Briefly, at 8 wks of age C57BL/6J mice were anesthetized with isoflurane and injected with $1X10^6$ Lewis Lung Carcinoma cells in the left hind flank. This commonly used method for inducing cancer cachexia in mice has previously been demonstrated to induce a cachectic phenotype (Choi et al., 2013; Iwata et al., 2016; Wang, Lai, Chan, Li & Wu, 2011). Control animals were injected with PBS as a sham control. Cancer was allowed to progress and cohorts of animals were humanely euthanized and tissues collected for analysis. Cohorts included PBS, 1wk, 2wk, 3wk, and 4wk of cancer progression (n=10-12/group). PBS animals were harvested with 4wk animals.

RNA isolation, cDNA synthesis, and RT-PCR

RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY). Liver samples were homogenized in a tissue homogenizer with Trizol. Total RNA was isolated using Ambion Purelink RNA minikit according to manufacturer instructions (Life Technologies, Grand Island, NY), then treated with DNase to remove impurities as previously described (Greene et al., 2015; Washington et al., 2013). Purity and concentration were tested using BioTek Take3 micro-volume microplate with a BioTek microplate reader (BioTek Instruments Inc., Winooski, VT). cDNA was reverse transcribed from 1µg of total RNA using Quanta qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD), as previously described (Greene et al., 2015; Washington et al.,

2013). Real Time Polymerase Chain Reaction (RT-PCR) was performed and amplification analyzed by StepOne Real-Time PCR system (Life Technologies, Applied Biosystems, Grand Island, NY). cDNA was amplified in a 25 µL reaction containing TaqMan Universal Mastermix (Applied Biosystems), and the fluorescence probes for Collagen I (Mm00801666_g1), Collagen III (Mm01254476_m1), MMP9 (Mm00442991_m1), and 18s (Mm03928990_g1) (Applied Biosystems). Samples were incubated at 95°C for 4 minutes, then cycled through denaturation (95°C), annealing (55°C), and extension (72°C) steps for 40 cycles. Cycle threshold (C_t) was determined and ΔC_t was calculated using the equation ($C_{t\,(sample)} - C_{t\,(18s)}$). Cycle threshold for 18s rRNA did not differ between experimental conditions. Final quantification of gene expression was calculated using the equation $\Delta\Delta C_t = (\Delta C_{t\,(calibrator)} - \Delta C_{t\,(sample)})$. Relative quantification was then calculated using $2^{-\Delta\Delta Ct}$.

Statistical Analysis

The independent variable was cancer progression with five different levels (PBS v. 1wk v. 2wk v. 3wk v. 4wk). Dependent variables included mRNA contents of Collagen I, Collagen III and MMP-9, as well as the ratio of Collagen I: Collagen III. Results were analyzed by one-way analysis of variance (ANOVA), with α set at 0.05. When significant F ratios were found, a Tukey's post-hoc test was used to determine difference among means. All data was analyzed using the Statistical Analysis System (SAS, version 9.3, Cary, NC) and expressed as mean ± SEM.

Results

Liver weights, normalized to tibia length, were ~30% larger in 4 wks animals compared to all other groups (Figure 2; $p<0.05$). Sirius Red histological staining data (Figure 1) shows that collagen deposition was exponentially greater with longer tumor development times ($r^2 = 0.78$), with 4wks

having statistically greater collagen deposition than all other groups ($p<0.05$). Collagen I mRNA content was 2.5 fold higher at 4wks than all other groups, with no other differences noted (Figure 3A; $p<0.05$). Collagen III mRNA content was 3.5 fold higher at 4wks than all other groups, with no other differences noted (Figure 3B; $p<0.05$). MMP-9 mRNA content was 6.5 fold higher at 4wks than all other groups, with no other differences noted (Figure 3C; $p<0.05$). The Collagen I:III ratio showed no significant differences between groups (Figure 4, $p>0.05$).

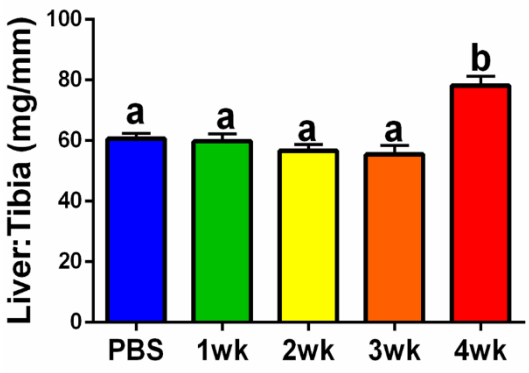

Figure 2: Liver weight normalized to tibia length vs time point. Different lowercase letters represent a difference of $p<0.05$

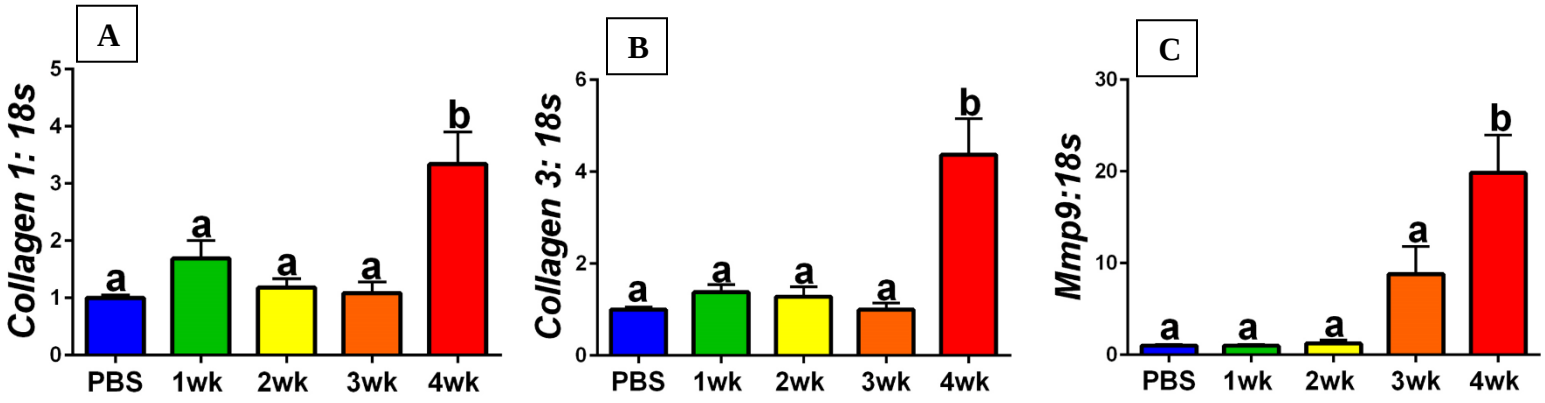

Figure 3: mRNA contents normalized to 18s vs time point. Different lowercase letters represent a difference of $p<0.05$. A: Collagen I:18s. B: Collagen III:18s. C: MMP-9:18s.

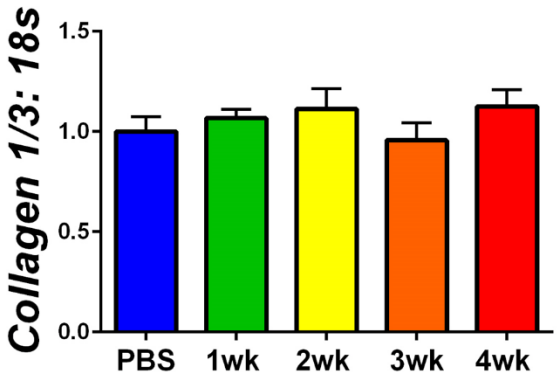

Figure 4: Collagen I: Collagen III mRNA content ratio normalized to 18s. $p>0.05$

Discussion

The liver is a notably underrepresented organ in the field of cancer-cachexia research, and to our knowledge, no one else has studied liver ECM during the cancer-cachexia pathology. Therefore, this research is a novel examination of the time course of events that happen in the liver ECM during cancer-cachexia progression.

In cancer-cachexia, we have noted an increase in liver size that occurs concomitantly with increased collagen deposition (Figure 1, Figure 2). In this examination, across all variables measured for hepatic mRNA related to ECM, the greatest change occurs between 3 and 4 wks of cancer progression. At 4wks of cancer progression, mRNA content for Collagen I, Collagen III, MMP-9, as well as liver weight and actual collagen deposition are significantly higher than all other groups, likely resulting in decreased liver function and negatively affecting the health of the organism. This timeline echoes our lab's findings from the skeletal muscle of these same mice,

that demonstrate muscle wasting (cachexia), lower mitochondrial content (via COX IV and VDAC), and loss of oxidative phenotype in muscles of 4 wks mice (Brown et al., 2017).

One important finding is that although mRNA content of the ECM regulatory enzyme MMP-9 increases even more than Collagen I and III, (6.5 fold increase compared to 2.5 and 3.5, respectively), the actual deposition of collagen is still higher at 4wks compared to all other groups. One explanation for this could be that MMP-9 does not directly cleave collagen I and III, as it instead cleaves collagen IV and denatured collagens (Ricard-Blum, 2011). However this is very likely not the entire picture, and it emphasizes the need for future studies to examine mRNAs of a more diverse group of collagens, MMPs, and TIMPs.

Another thing to note when interpreting this data is that the measure of mRNA content is a good measure for determining when a cell 'decides' to produce certain proteins, however, it does not necessarily reflect the actual phenotype of the liver ECM. Many post transcriptional and epigenetic factors may impact the expression of proteins.

This research is a cursory look into the physiology of the liver ECM during the development of cancer-cachexia and is meant to be built upon before any specific conclusions regarding cause and effect relationships can be parsed out. More thorough examinations are needed understand the mechanisms triggering hepatic ECM changes and their functional role during cancer cachexia progression.

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