Metalloprotease expression is altered in cardiac and skeletal muscle in cancer cachexia

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Devine RD, Bicer S, Reiser PJ, Velten M, Wold LE. Metalloprotease expression is altered in cardiac and skeletal muscle in cancer cachexia. Am J Physiol Heart Circ Physiol 309: H685–H691, 2015. First published June 19, 2015; doi:10.1152/ajpheart.00106.2015.—Cardiac and skeletal muscle dysfunction is a recognized effect of cancer-induced cachexia, with alterations in heart function leading to heart failure and negatively impacting patient morbidity. Cachexia is a complex and multifaceted disease state with several potential contributors to cardiac and skeletal muscle dysfunction. Matrix metalloproteinases (MMPs) are a family of enzymes capable of degrading components of the extracellular matrix (ECM). Changes to the ECM cause disruption both in the connections between cells at the basement membrane and in cell-to-cell interactions. In the present study, we used a murine model of C26 adenocarcinoma-induced cancer cachexia to determine changes in MMP gene and protein expression in cardiac and skeletal muscle. We analyzed MMP-2, MMP-3, MMP-9, and MMP-14 as they have been shown to contribute to both cardiac and skeletal muscle ECM changes and, thereby, to pathology in models of heart failure and muscular dystrophy. In our model, cardiac and skeletal muscles showed a significant increase in RNA and protein levels of several MMPs and tissue inhibitors of metalloproteinases. Cardiac muscle showed significant protein increases in MMP-2, MMP-3, and MMP-14, whereas skeletal muscles showed increases in MMP-2, MMP-3, and MMP-14. Furthermore, collagen deposition was increased after C26 adenocarcinoma-induced cancer cachexia as indicated by an increased left ventricular picrosirius red-positive-stained area. Increases in serum hydroxyproline suggest increased collagen turnover, implicating skeletal muscle remodeling. Our findings demonstrate that cancer cachexia-associated matrix remodeling results in cardiac fibrosis and possible skeletal muscle remodeling. With these findings, MMPs represent a possible therapeutic target for the treatment of cancer-induced cachexia.

CANCER is a rapidly growing health concern worldwide, with 25% of all deaths attributed to cancer in the United States (31).

NEW & NOTEWORTHY

Our findings represent a significant expansion in the current field of cancer cachexia with an emphasis on targeted therapy. Increased matrix metalloproteinase expression results in cardiac fibrosis and skeletal muscle remodeling in response to C26 adenocarcinoma cell injection. These findings expand the field and introduce potentially new therapeutic strategies.

During disease progression ~30–80% of cancer patients develop cachexia, a syndrome characterized by marked wasting of adipose tissue and skeletal muscle (13). Cachexia not only increases the likelihood of morbidity from cancer but also is directly responsible for 20–40% of deaths in cachectic patients (37). Cachexia is a detrimental syndrome, negatively affecting patient quality of life and the potential for recovery (14, 41). Understanding the molecular mechanisms of cancer cachexia could assist in creating effective therapies to improve survival and quality of life in these patients.

Increased serum levels of proinflammatory cytokines, including IL-6 and TNF-α, play pivotal roles in tumor-induced muscle wasting (3, 4, 42). These cytokines increase activity of the ubiquitin proteasome and autophagy systems, promoting muscle protein breakdown (10, 19, 24). Ubiquitin activity may play a role in early skeletal muscle wasting; however, the ubiquitin pathway of degradation does not completely explain cachexia-associated pathology. For example, studies in human cancer patients have not correlated increased ubiquitin mRNA levels with weight loss (36). Therefore, other degradation systems likely contribute to skeletal muscle wasting in cancer cachexia.

In addition to influencing skeletal muscle mass and function, tumor burden also promotes cardiac dysfunction. CD2F1 mice injected with C26 adenocarcinoma cells (5) are a recent established model to study cancer cachexia. In this model, tumor development is directly correlated with reduced cardiac function as measured by ejection fraction and fractional shortening (35). Although in vivo cardiac function was reduced in C26 adenocarcinoma-induced tumor cachexia, in vitro analyses of isolated cardiomyocytes did not indicate cardiac dysfunction at the cellular level (43), suggesting the involvement of the extracellular matrix (ECM) in the development of cardiac dysfunction. Heart samples from human cancer cachexia patients show evidence of myocardial fibrosis (32), potentially associated with matrix metalloproteinase (MMP)-mediated alterations in the ECM.

MMPs degrade several components of the ECM, resulting in tissue remodeling processes including angiogenesis (23). In addition, MMPs play active roles in cell proliferation and migration. MMPs are secreted in an inactive form that requires the removal of an inhibitory domain for activation (26). Additionally, endogenous inhibitors of MMPs [tissue inhibitors of metalloproteinases (TIMPs)] inhibit MMP activity by directly binding to the MMP active site. MMPs are secreted by tumor cells, and this facilitates angiogenesis for tumor growth and survival as well as metastasis (11). However, the involvement
of MMP activity in muscle wasting or dysfunction in cancer cachexia remains unknown. The purpose of the present study was to investigate MMP and TIMP production in skeletal and cardiac muscle in a model of cancer cachexia. We show that tumor-bearing mice have increased MMP levels in both cardiac and skeletal muscle compared with control mice, which may contribute to altered heart and skeletal muscle function associated with cachexia. These findings provide insights into the contribution of MMPs to skeletal muscle wasting and cardiac dysfunction in cancer cachexia.

MATERIALS AND METHODS

Mice. Adult (10 wk old) female CD2F1 (BALB/c × DBA/2 F1) mice weighing 20–22 g were obtained from Charles River Laboratories. Mice were housed with 1–3 mice/cage and maintained at 25°C under a 12:12-h light-dark cycle with ad libitum access to water and standard rodent chow. All animal care and procedures were approved by the Institutional Animal Care and Use Committee of the Ohio State University.

Mouse model of tumor growth. The C26 cell line was maintained in culture and prepared for injection as previously described (34). Mice were randomly selected to receive the cell injection subcutaneously between the scapulae with 5 × 10⁵ cells in 0.2 ml of PBS, whereas control mice were injected with PBS alone. Tumor growth was usually palpable by day 12 postinjection, and mice became moribund typically by day 24 postinjection. In this study, mice were euthanized by day 21 postinjection. Mice were euthanized using a ketamine (10 mg/ml)-xylazine (1 mg/ml) cocktail at a volume of 0.01 ml/g body wt typically by day 24 postinjection, and mice became moribund usually by day 21 postinjection. Mice were euthanized using a ketamine (10 mg/ml)-xylazine (1 mg/ml) cocktail at a volume of 0.01 ml/g body wt (~0.2 ml), and blood was withdrawn from the brachial artery. The heart, hindlimb muscles, and diaphragm were dissected, snap frozen in liquid nitrogen, and stored at −80°C until biochemical analyses.

Samples and preparation. The free wall of the left ventricle (LV), diaphragm, and soleus and extensor digitorum longus (EDL) muscles of control and tumor-bearing mice were used for electrophoretic gel and Western blot analyses. Muscles were dissected and homogenized (model PRO200 homogenizer, PRO Scientific, Monroe, CT) in sample buffer (8) (30 μl/mg muscle) for 15 s. Homogenates were then centrifuged at 14,000 rpm for 4 min. Supernatants of the samples were collected, heated at 65°C for 2 min, and maintained on ice for 5 min. Samples were maintained in a −40°C freezer until analyzed.

Gel electrophoresis. Separating (12% acrylamide with a 200:1 cross-linking ratio) and stacking (4% acrylamide with a 50:1 cross-linking ratio) gels were prepared as previously described (6). Gels were run at constant current (30 mA/gel) for 3 h at 18°C in Hoefer SE600 units (Hoefer Scientific, San Francisco, CA). Gels were stained with Coomassie blue (6). Stained gels were used to determine the loading amount, which was determined by the visualization of actin. Images of the gels were obtained with a digital camera (Spot Insight Firewire, model 18.2, Diagnostic Instruments, Sterling Heights, MI). Gels were analyzed with ImageJ software (National Institutes of Health).

Western blot analyses. Gels for immunoblot analysis were run, and proteins were transferred onto nitrocellulose membranes at constant voltage (100 V) for 1 h. Membranes were incubated with 1% BSA in Tris-buffered saline with Tween 20 (TBST) for 1 h at 25°C or overnight at 4°C. Membranes were incubated with primary antibodies for 2 h at 25°C and washed with TBST with gentle shaking three times for 10 min per wash. Membranes were then incubated with secondary antibodies for 1 h at 25°C. Membranes were washed with TBST for 30 min (three 10-min washes with gentle shaking). Color was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium color development substrate (S3771, Promega, Madison, WI). Finally, membranes were air dried, and digital images were captured as described above for gel imaging. Images were analyzed with ImageJ software. Primary antibodies were anti-MMP-2 clone 2C1 (1:100 diluted), anti-MMP-3 clone F-1 (1:100), anti-MMP9 clone E-11 (1:200), anti-MMP-14 clone C-9 (1:200), anti-TIMP-1 clone H-150 (1:100), and anti-TIMP2 clone 3A4 (1:200). All primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Primary antibodies were diluted with 0.3% BSA in TBST. Secondary antibodies were anti-mouse IgG1 (S3721, Promega, Madison, WI).

Fig. 1. RNA expression changes of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in left ventricle (A), diaphragm (B), extensor digitorum longus (EDL; C), and soleus (D) of control and C26 tumor-bearing mice. RNA expression levels were determined using the Livak method with GAPDH serving as the internal control. Cc, threshold cycle. *P < 0.05 was considered statistically significant.
luted 1:10,000) and anti-rabbit IgG (S3731, Promega, diluted 1:10,000). Secondary antibodies were diluted with 1% BSA in TBST.

RNA isolation and real-time quantitative PCR. RNA was isolated from the free wall of the LV, EDL, soleus, and diaphragm using a TissueLyser system in TRIzol reagent (Sigma-Aldrich, St. Louis, MO). RNA was extracted using RNeasy spin column purification (Qiagen, Valencia, CA). RNA was quantitated, and 300–500 ng of RNA were reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). Quantitative PCR was performed using a three-step protocol on the CFX96 Real Time System (Bio-Rad), as previously described (43). Briefly, cDNA was amplified using real-time quantitative PCR and was normalized based on reference cDNA (GAPDH). Data were analyzed using the $2^{-\Delta\Delta C_t}$ algorithm (where $C_t$ is threshold cycle) (18).

Picrosirius red quantification. Hearts from control and tumor-bearing mice were fixed in paraformaldehyde and then embedded in paraffin. Paraffin blocks were cut in 10-μm sections, mounted on glass slides, and rehydrated. After rehydration, slides were placed in 0.1% sirius red and diluted in saturated picric acid for 90 min. After being washed, slides were dehydrated with graded alcohols before being cleared and then mounted in synthetic media (9).

For histological analyses, slides from the area below the papillary muscles were identified, and images were taken of the septum and posterior, free, and anterior walls at a magnification of ×200 using a BX 41 microscope (Olympus, Hamburg, Germany). Quantification and planimetric analyses were performed using Analysis (Olympus). The picrosirius red positive-stained area was quantified as a percentage of the total myocardial area (12).

Hydroxyproline assay. Serum from control and tumor-bearing mice was used to perform a hydroxyproline assay (Sigma-Aldrich). Briefly, samples were hydrolyzed with hydrochloric acid and then spun at 13,000 g. Samples were then treated with chloramine-T to oxidize hydroxyproline, and perchloric acid was then added to remove chloramine-T. Oxidized samples were incubated with 4-(dimethylamino)benzaldehyde (DBMA) to produce a photometric product at 560 nm. Results were acquired using a Bio-Tek Power Wave (Bio-Tek, Winooski, VT) plate reader and calculated using the standard curve of hydroxyproline provided with the assay kit.

Statistical analyses. Values from six control mice and six tumor-bearing mice are reported as means ± SE. The two groups were compared using a Student’s t-test, where $P < 0.05$ was used to indicate statistical significance.

RESULTS

Tumor burden increased MMP and TIMP mRNA expression in striated muscle. mRNA expression of MMPs and TIMPs was determined in skeletal and cardiac muscles of tumor-bearing mice to test whether they were elevated in association with cancer cachexia (Fig. 1). In the LV, MMP-2, MMP-3, and MMP-14 were significantly increased in tumor-bearing mice compared with control mice (Fig. 1A). The skeletal muscle response for tumor-bearing mice differed from that of cardiac muscle. In the diaphragm, MMP-2 and MMP-9 were significantly increased (Fig. 1B), whereas both EDL and soleus muscles showed significant increases in MMP-3 (Fig. 1, C and D).

Certain striated muscles of tumor-bearing mice had increased levels of TIMP-1 (Fig. 1) compared with those from control mice. The LV had significantly increased TIMP-1 expression, but TIMP-2 was unchanged (Fig. 1A). In skeletal muscle, the diaphragm had significantly increased expression of TIMP-1 but not TIMP-2, whereas the EDL and soleus muscles showed no increases in either TIMP-1 or TIMP-2 (Fig. 1, B–D).

MMP and TIMP protein levels were increased in tumor-bearing mice. In the LV, protein levels of MMP-2, MMP-3, MMP-9, and MMP-14 were significantly increased compared

![Fig. 2. Quantification of MMP and TIMP protein expression levels in left ventricle (A), diaphragm (B), EDL (C), and soleus (D) of control and C26 tumor-bearing mice. Protein levels are expressed as the ratio between the target protein (MMP or TIMP) and actin, the reference protein. *$P < 0.05$ was considered statistically significant.](http://ajpheart.physiology.org/)

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with control values (Fig. 2A). The EDL, soleus, and diaphragm demonstrated similar changes in MMP protein levels. The diaphragm, EDL, and soleus had significantly increased expression of MMP-2, MMP-3, and MMP-14 without changes in MMP-9 (Fig. 2, B–D). Protein levels of TIMP-2 were increased in tumor-bearing mice. The LV had significantly increased TIMP-2 but not TIMP-1 protein levels (Fig. 2A). The diaphragm, EDL, and soleus showed significant increases in TIMP-2 but not TIMP-1 in a manner similar to cardiac muscle (Fig. 2, B–D). Representative immunoblots of MMPs and TIMPs are shown in Figs. 3 and 4.

Collagen deposition was increased in the LV of tumor-bearing mice. To assess increased collagen deposition in the LV, we analyzed picrosirius red-stained sections. There were
significantly increased amounts of collagen deposition in the LV of tumor-bearing mice compared with control mice (Fig. 5). Collagen deposition, as a percentage of the total area, was significantly increased in tumor-bearing mice.

Hydroxyproline was increased in the serum of tumor-bearing mice. To assess increased collagen turnover, we analyzed the serum of mice for the collagen byproduct hydroxyproline. There was a significant increase in the amount of hydroxyproline in the serum of tumor-bearing mice (Fig. 6), indicating increased MMP activity and collagen turnover.

DISCUSSION

In the present study, we found that tumor burden increased the mRNA expression and protein amount of MMPs and TIMPs in striated muscle. Upregulation of the ubiquitin proteasome pathway has been established in cancer-induced cachexia (22), but other proteolytic or degradation pathways have not been extensively studied. This study is the first to demonstrate that cancer-induced cachexia in a mouse model is associated with increased expression of MMPs and TIMPs in skeletal and cardiac muscle. This increased expression of MMPs could contribute to the cardiovascular and skeletal muscle pathologies observed in the tumor-burdened mouse. The cardiac phenotype in the cachectic mouse model used in this study exhibited decreased in vivo function but not decreased isolated cardiomyocyte contractility (43). The increases in muscle atrophy F-box (MAFbx)/muscle RING finger 1 (MuRF-1) found in our model are expected to negatively affect cardiomyocyte contractility (1, 18). The action of MAFbx/MuRF-1 was demonstrated in a rat model of chronic heart failure, where increases in MAFbx and MuRF-1 resulted in contractile dysfunction that was abrogated by treatment with MAFbx/MuRF-1 small interfering RNA (1). However, we did not observe cardiomyocyte dysfunction 21 days after tumor cell injection in our current model (43). While there are increases in MAFbx/MuRF gene and protein expression in the heart of cachectic mice (34, 43), the lack of changes in cardiomyocyte contractile properties indicates that the functional changes are not entirely due to the ubiquitin system.

Increases in MMPs have been implicated in heart failure and morbidity in heart failure patients (15a). The gelatinases MMP-2 and MMP-9 (7, 30) are most commonly associated with alterations of the ECM in heart failure. We observed significant upregulation in both enzymes at the protein level. The collagenase MMP-9 was not increased at the RNA level but was increased at the protein level. MMP-9 protein is often present at a basal level and activated posttranscriptionally during inflammatory states, possibly explaining the discrepancy in RNA levels compared with protein levels. MMP-3 and MMP-14 (muscle type 1-MMP) have not been directly implicated in cardiac remodeling but more often regulate the expression and activity of other MMPs (40). MMP-3 may regulate MMP-2 and MMP-9 in heart failure models, whereas MMP-14 activates MMP-2. TIMP-1 was significantly increased at the RNA level but showed no change in protein expression, whereas TIMP-2 showed no RNA increase but a significant protein increase. TIMP-1 RNA regulation shares some transcription factors with MMP-9 (39), which also showed no RNA increase, possibly indicating certain transcription factors may not be activated in the tumor-burdened mouse. The increases in collagen deposition, determined by both picrosirius red staining and hydroxyproline assay, demonstrate that MMPs are actively remodeling the ECM and increasing collagen deposition. This MMP activity and matrix remodeling are most likely responsible for the decreases in wall thickness during contraction, contributing to decreased cardiac function.

Skeletal muscle loss has been an intense area of focus within the cachectic field due to the wasting present in both human cancer patients and animal models of cancer. This wasting is due to activation of the ubiquitin proteasome system, primarily from MAFBx and MuRF-1. These two E3 ubiquitin ligases...
have been heavily studied and regarded as a primary cause of wasting in cachexia. Treatment with the proteasome inhibitor MIG132 has been shown to alleviate cachexia by inhibiting MAfBx and MuRF-1, showing their involvement in wasting (44). The wasting phenotype does not involve a reduction in the total number of muscle fibers or altered force generation per unit of cross-sectional area (25, 29), although others have reported a significant reduction of force/cross-sectional area in the murine C26 model (28) and in human patients (38). Skeletal muscles in some cachectic models are unable to generate new muscle cells due to the inability of satellite cells to mature into myoblasts (16). MMPs can contribute to the differentiation of satellite cells (27). In canine and murine models of muscular dystrophy, MMPs promote the release of satellite cells as well as the release of growth factors that stimulate proliferation (15, 20). There is evidence that MMP-2 and MMP-9 activities contribute to the differentiation of satellite cells in mdx mice. However, MMP-2 and MMP-9 have been shown to degrade the ECM and to prevent muscle regeneration in inflammatory myopathies (20). The extent of MMP upregulation appears to play a role in their specific activity; a small upregulation can release satellite cells and permit differentiation, whereas a more profound increase can disrupt the ECM and prevent differentiation. At the RNA level, only MMP-3 was upregulated in the soleus and EDL muscles, whereas at the protein level, MMP-2, MMP-3, MMP-14, and TIMP-2 were significantly increased. There is posttranslational modification occurring; however, the reason remains unknown. For MMP and TIMP-2, it is possible that skeletal muscle has higher basal amounts of RNA, negating the need for a prolific gene response while maintaining a robust protein response. This aberrant increase in MMPs in the EDL and soleus muscles in the cachectic mouse model may contribute to the inability of satellite cell proliferation by breaking down the surrounding matrix and disrupting normal cell survival signals transmitted through the matrix and between cells.

Diaphragm strips from tumor-bearing mice generate decreased peak specific force compared with normal mice (25, 28). Cachectic patients undergo respiratory complications due to failure of the diaphragm (28). The decreased peak specific force of the diaphragm in tumor-bearing mice could be the mechanism by which human patients experience respiratory complications. Increased MMPs in the diaphragm could change the underlying architecture of the ECM, contributing to changes in the physical and mechanical properties of the diaphragm. Additionally, while MMPs perform most of their proteolytic actions extracellularly, they have been shown to localize to sarcomeric proteins in the heart (2), and it is possible that they are able to localize to sarcomeric proteins in other muscle types. In a mouse model of mdx, there was decreased specific force of the diaphragm (17, 33). A separate study (21) showed that decreased force production could be ameliorated by the MMP inhibitor batimastat. However, Kumar et al. (21) investigated several MMPs as well as a disintegrin and metalloproteinases, making the contributions of specific MMPs in respiratory pathology difficult to evaluate. The roles of MMPs in diaphragm and respiratory dysfunction warrant further investigation.

In the present study, we demonstrated a significant increase of specific MMPs in cardiac and skeletal muscles at the RNA and protein levels. Currently, the only other studies that have reported changes in MMPs in both cardiac and skeletal muscles were performed in muscular dystrophy models. In the mdx model, increases in MMP-2 and MMP-9 correlated with cardiomyopathy in mice. This cardiac dysfunction is also coupled with skeletal muscle dysfunction. As mentioned above, treatment of mdx mice with batimastat, a broad MMP inhibitor, improved skeletal muscle function and significantly reduced fibrosis (21). MMP-2 and MMP-9 have been most heavily studied in cardiac and skeletal muscles; however, it is known that MMP-3 can regulate the activity of other MMPs, and MMP-14 can activate MMP-2. These MMP-induced MMP expression and activity changes can account for some of the differences in gene expression compared with protein differences. MMPs could cleave other substrates, leading to a protranscription rate while not altering the amount of gene expressed. While we found increased protein expression of TIMP-2, which is capable of inhibiting all MMPs, from our histology and serum assays we can infer that TIMP-2 is no longer providing adequate MMP inhibition. TIMPs, while having a high affinity for MMPs, can bind the active site in a one-to-one ratio, and this can significantly limit their inhibitory activity in cases of marked increases in MMPs.

Further research is needed to elucidate the role of MMPs in the cardiac and skeletal muscle dysfunction in cancer cachexia. Determining to what extent MMPs contribute to the pathology will potentially lead to the development of effective treatments for cachectic patients, especially with respect to cardiac complications due to tumor burden.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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