Learning Objectives

- To understand the major mediators regulating muscle wasting in cancer cachexia;
- To understand the mechanisms by which the mediators of cancer cachexia induce muscle wasting.

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6. Summary

Key Messages

- Cachexia is a debilitating wasting syndrome that affects a majority of cancer patients and results from the depletion of both adipose tissue and skeletal muscle;
- Muscle wasting is regulated by tumor and host derived factors that activate selective protein degradation pathways;
- The ubiquitin proteasome system represents the major pathway regulating muscle wasting in cancer and other conditions associated with chronic inflammation;
- Cachexia is regulated by the loss of selective myofibrillar proteins;
- Much can be learned about cachexia from studying other conditions of muscle wasting.
1. Introduction

Cachexia derives from the Greek words, *kekos* that stands for “bad” and *hexis*, standing for “condition”. This syndrome is most pronounced in end stage diseases that tend to associate with chronic inflammation, such as in cancer, AIDS, chronic heart failure, sepsis, tuberculosis, and severe burns (1, 2). This disease state encompasses features of anorexia, anemia, lipolysis, acute phase response, and insulin resistance. Unlike simple starvation, which causes the depletion of fat while preserving protein content mainly from skeletal muscle, in cachexia neither tissue is spared (3) (Fig. 1). The chronic consumption or wasting of muscle protein, combined with an absence of compensatory production of new protein, culminates in conditions of asthenia, immobility, and eventual death (4). Cachexia is one of the most prevalent adverse effects of cancer. It is estimated that over 50% of cancer patients suffer from cachexia, and 20% of mortalities are thought to result from cachexia rather than the direct tumor burden (2, 5). Weight loss as small as 5% of the usual body weight can significantly worsen prognosis. The wasting condition can also drastically lower tumor responsiveness to chemo and radiotherapy. Patients diagnosed as cachectic must therefore receive lower doses of treatment, and even under such conditions often develop dose-limited toxicity (6).

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**CANCER CACHEXIA**

- Wasting condition characterized by extreme weight loss
- Unlike anorexia or simple starvation, in cachexia both fat and skeletal is lost
- Patients respond poorly to therapy and have poor quality of life
- Estimated that nearly 20% of mortalities result from cachexia rather than tumor burden

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Figure 1 Sequelae of cancer cachexia

Cancer cachexia is foremost a metabolic condition that ensues as a direct consequence of the growth and survival of tumor cells. Tumor development is fueled at the expense of the host, thus creating an energy imbalance that underlies the wasting state (7) (Fig. 2). Glucose is the major source of fuel, which tumor cells obtain from the breakdown of adipose and skeletal muscle. Fat wasting generates free fatty acids and glycerol which are directly used by the cancer cells, or otherwise converted to glucose to be subsequently reabsorbed by the tumor. Because of insufficient oxygen, the Kreb cycle and oxidative phosphorylation are not favored processes in tumor cells. Rather, glucose is converted to lactate, which is then transported to the liver. There, the carbon skeleton is metabolized to resthesize glucose only to be imported back to the tumor (the Cori cycle). Skeletal muscle is the most abundant tissue in the human body and it is utilized as a major fuel source for the benefit of a growing tumor. A minor fraction of this energy is provided by glucose, while the majority derives from amino acids resulting from the breakdown of myofibrillar proteins and from free pools that are not utilized due to an inhibition in protein synthesis. Of the amino acids transported from skeletal muscle, approximately 50% are represented by glutamine and alanine (8). Tumor cells utilize glutamine as a source of nitrogen for the synthesis of purine and pyrimidine bases, while alanine is used as a vehicle of nitrogen transport. Other amino acids are recycled via the liver and converted to glucose, which essentially insures the continuing supply of energy to the tumor at the expense of the host.
2. Cytokines as Mediators of Cancer Cachexia

Much of the work in the cancer cachexia community has been devoted in the past two decades to identifying the mediators of wasting, with the hope that this would lead to the development of more effective therapies. To date however, this search continues and whether one or more cachetic factors are sufficient to initiate or sustain the wasting condition in human malignancies remains a point of discussion. Nevertheless, active research in the field has discovered several key mediators of cachexia that fall into two general classes. The first class are factors that are believed to be strictly produced and secreted from tumor cells. Lipid-mobilizing factor (LMF) is one such example that can be detected in the urine of cachectic patients and acts through a beta3-adrenoceptor to mediate the lipolysis of adipose tissue. It is also identical to the plasma protein Zn-alpha-2-glycoprotein (ZAG) (2). A second example is the tumor-derived proteolysis-inducing factor (PIF), which is a 24 kDa sulfated glycoprotein capable of inducing cachexia through its induction of protein degradation in skeletal muscles. The second class are the pro-inflammatory cytokines, including TNF, IL-1, IL-6, IFN, which are predominantly synthesized from immune cells in response to the tumor (1) (Fig. 3). Of these cytokines, TNFa has been the most extensively studied, and in fact was given the name cachectin by one of the groups that had originally identified it.
Whether TNF alone is sufficient to induce the cachectic phenotype in cancer remains in question. Certainly with relevance to adipose tissue, both in vitro and in vivo studies support the role of TNF in fat wasting. In regards to muscle wasting however, the consequence of chronic TNF signaling is less clear. Although administration of TNF in animals leads to a reduction in muscle mass (9), other investigators were unable to demonstrate any depletion in muscle protein when cultured muscle explants were treated with the cytokine (10). TNF treatment of cultured myotubes was also seen to have little effect on the regulation of myosin heavy chain, a myofibrillar protein considered a standard marker of muscle wasting (11). In contrast, other investigators using similar myotube cultures have reported reductions of myosin levels by TNF (12). Why such an apparent discrepancy exist is not yet clear, but it suggests that other pro-cachectic factors may function in addition to, or in combination with, TNF to regulate muscle wasting. Like TNF, chronic injection of IL-1 in animals was also shown to cause a redistribution of body proteins (13). IL-6 has also been strongly implicated in regulating the cachectic phenotype in cancer. This cytokine is known to regulate the expression of hepatic acute phase proteins, which themselves are considered cachectic factors of skeletal muscle. A clear role for IL-6 in cachexia was demonstrated using a tumor model of cachexia in IL-6 knockout mice. In this study, investigators reported a marked attenuation in wasting in these animals as compared to tumor bearing mice deleted in TNF, IL-1, or IFN- genes (14). However, since inhibition of wasting was measured in body weight rather than in lean mass, it is difficult to interpret the essentialness of IL-6 for muscle wasting in this tumor model. In addition to these factors, tumors expressing IFN- also indicate a role of this cytokine in wasting, but whether these cytokines alone are sufficient to induce both fat and skeletal muscle breakdown remains enigmatic.

3. Is the Proteasome System the Master Driver of Skeletal Muscle Wasting in Cancer?

Muscle catabolism may also result from the activation of several proteolytic pathways (15). One is the lysosomal system, whereby endocytosed plasma proteins or membrane receptors undergo degradation by acid-activated cathepsin proteases B, H, and D. These enzymes may also be responsible for the degradation of cytosolic proteins that become taken up by autophagic vacuoles that are subsequently fused to lysosomes (16). The second of these pathways functions in the cytosol and is referred to as the calcium-dependent calpain proteolytic system (17). Calpains are calcium activated cysteine proteases which exist as m-calpain, µ-calpain, and the muscle specific form p94. The activity of these proteases is regulated by the inhibitory calpastatin. The third, and by far the best studied of these proteolytic systems, is the cytosolic, ATP-dependent ubiquitin proteasome pathway (Fig. 4). This system plays a prominent role not only in cancer cachexia, but has also been implicated in muscle wasting associated with sepsis, weightlessness, starvation, and denervation atrophy (18). In this pathway, proteins are marked for degradation through the covalent binding of ubiquitin, a small heat-stable polypeptide. Conjugation of ubiquitin is regulated by ubiquitin E1-activating, E-2 carrier, and E-3 ligase enzymes, which regulate the polyubiquination of proteins marked for degradation. Targeted degradation is regulated by the proteasome, a multimeric complex consisting of the central 20S catalytic core and the 19S terminal regulatory domains. Proteins entering the proteasome are cleaved by a cyclical “bite-chew” mechanism of proteolysis. These cleavage “bites” are regulated by the chymotrypsin like activity of the 20S core which is preceded by the “chew” of a caspase like activity (19).
A recent DNA microarray study was performed exploring the gene expression profiles of several conditions of muscle wasting, one of which included cancer (20). Results revealed significant increases in a subset of genes with restricted functional role in the ubiquitin proteasome system (Fig. 5). Such genes seen to increase were the 20S proteasome subunits and specific muscle E3 ubiquitin ligases, MuRF1 and Atrogin-1 or MAFbx. Remarkably, this pattern of expression from the ubiquitin proteasome system was consistent in all models of wasting that was explored. This strongly suggests that the ubiquitin proteasome system may be a general mechanism through which skeletal muscle proteins are degraded in response to non-physiological cues.
To confirm DNA microarray data, total RNA from muscles was collected following the subjection of mice to various wasting conditions. This RNA preparation was fractionated on an agarose gel and transferred to a nylon membrane, where it was probed for one of the highly expressed genes from the array analysis (in this case, the E3 ubiquitin ligase Atrogin-1). Results showed that in every case tested for muscle wasting, Atrogin-1 gene expression was pronouncedly elevated over the control samples (Fig. 6). Similar results were observed with another prominent muscle E3 ligase, MuRF1.

Figure 6 Increased expression of Atrogin-1 in skeletal muscle during the development of cancer cachexia

4. What are the Protein Targets of the Ubiquitin Proteasome System?

A general feature of cancer cachexia is that skeletal muscles rich in type II fibers are particularly susceptible to the effects of tumor factors (21). As demonstrated in the colon-26 (C-26) model of cancer cachexia, mice muscles lose approximately 50% of their original mass and type II fiber diameters (22) (Fig. 7).

Figure 7 Selective loss of type 2 muscle fibre diameter in cancer cachexia (22)

The major targets of degradation in muscle are thought to be the myofibrillar proteins. The four core myofibrillar proteins including, myosin heavy chain, actin, tropomyosin, and troponin, constitute the sarcomere or the basic contractile unit of skeletal muscle (23) (Fig. 8 and Fig. 9). Multiple adjoining sarcomeres form myofibrils which multimerizes to fill a skeletal muscle fiber. Myosin heavy chain alone consists of 40% of myofibrillar proteins. Elegant in vitro studies were
performed to demonstrate that each of core myofibrillar proteins served as substrates for ATP dependent proteasome degradation (24). However, it was also shown that pre-assembled myofibrils were resistant to this degradative activity, suggesting that disassembly of the sarcomere is the rate-limiting step of ATP-dependent proteolysis. More recent work showed that release of the myofibril and their subsequent disassociation is a calcium-calpain-dependent process (25). Isolated muscles from tumor-bearing animals have been shown to undergo proteolysis in an ATP-dependent fashion, which could be blocked by proteasome inhibitors. Taken together, these studies indicate that tumor regulated muscle wasting is largely dependent on the activity of the ubiquitin-proteasome pathway.

Figure 8 Structure of the sarcomere in skeletal muscle

Figure 9 Electromicrograph of skeletal muscle demonstrating structure of the sarcomere
5. What can be Learned about Cancer Cachexia from Studying Other Conditions of Muscle Wasting?

It is clear from the studies of Goldberg and co-workers that various conditions of skeletal muscle wasting are associated with the activation of ubiquitin proteasome system. From these works, we can estimate that, similar to the proteasome pathway, there are likely to be other shared mechanisms that underlie the pathogenesis of muscle wasting in cachexia. Recently, our laboratory elucidated the involvement of the dystrophin glycoprotein complex (DGC) as a potentially new contributor to the wasting state in cancer cachexia (22). The DGC is a multi-protein structure associated with skeletal muscle membranes (26). At the core of the DGC is the protein dystrophin, a large 427 kDa polypeptide that associates with the cytoskeleton through interactions with F-actin and beta-dystroglycan (b-DG) (Fig. 10). -DG is in turn bound to alpha dystroglycan (α-DG) which itself is linked to the extracellular matrix by binding with laminin-2. The DGC therefore forms a mechanical link between the cytoskeleton and the extracellular matrix protecting cells from contraction-induced injuries. Mutations in dystrophin or other members of the DGC disrupt the mechanical linkage resulting in membrane damage, necrosis, and eventual muscle wasting that are the hallmark features of muscular dystrophies (27).

![Figure 10: Structure of the dystrophin glycoprotein complex (DGC)](Image)

The discovery that a molecular link existed between cancer cachexia and muscular dystrophy came from morphological examination of skeletal muscle sections from control and C-26 tumor bearing mice. Examination of these sections by light microscopy (Fig. 11) and electron microscopy (Fig. 12) revealed severe abnormalities in the membrane structure from muscles of C-26 bearing mice. Specifically, as compared to the smooth and well-defined membrane bordering each myofiber in control muscles, membranes in cachectic muscles appeared wrinkled and irregular (Fig. 11 and Fig. 12).
The DGC plays a major role in regulating membrane integrity and mutations in this complex result in different forms of muscular dystrophies (27). Although the causative mechanisms of muscular dystrophies and cachexia are considered distinct, given the membrane irregularities observed in tumor bearing mice, we considered that the DGC might also be involved in the regulation of cancer cachexia. Indeed, results from immunoblot and immunofluorescence analysis revealed that the central player in the DGC, dystrophin, was strongly downregulated in muscles from tumor bearing mice (Fig. 13). As a way to confirm the validity of our results, we probed for the expression of the dystrophin homologue, utrophin. In muscular dystrophies utrophin is often over expressed to compensate for the absence of dystrophin (28). Similar to this dystrophy phenotype, we too observed the induction of utrophin expression in muscles from tumor bearing mice (Fig. 13).
Conditions where dystrophin expression is lost, such as in muscular dystrophies (27) or enterovirus-mediated cardiomyopathy (29), typically result in the concurrent downregulation of other DGC members. This was not the case in cancer cachexia since expression of α-DG, β-DG, alpha sarcoglycan (SG), β-SG, α-SG, and dysferlin were unaltered (Fig. 14). Prominently detected in muscles from tumor bearing mice however was the presence of a higher migrating band for both β-DG and β-SG, which we confirmed was due to hyperglycosylation (Fig. 14, see red arrowheads). These aberrant glycosylated forms were clearly induced during the progression of the wasting state suggesting they may be a contributing factor in the pathogenesis of cancer cachexia.

To ascertain whether similar regulation of dystrophin and the DGC was present in patients with cancer cachexia, we screened these proteins in muscle biopsies from patients with gastrointestinal cancers, since these patients often undergo excessive weight loss (30). Compared to weight-stable healthy controls, results showed that carcinoma patients with marked weight loss had dramatic
reductions in dystrophin (Fig. 15A). In addition, similar to the mouse model of cancer cachexia, dystrophin reduction in patients was tightly linked with aberrant glycosylation of DGC proteins (see arrowheads). Equally striking was that of 10 non-surviving cases, 10/10 (100%) were also positive DGC deregulation. Kaplan-Meier survival analysis demonstrated a statistically significant difference between normal and deregulated DGC cases (p=0.0096 by log rank test) (Fig. 15B). These results demonstrate that gastrointestinal cancer patients exhibit dysfunctional DGC that correlate positively with cachexia and inversely with survival. Furthermore, the findings provide important insight into the mechanism of cancer cachexia by revealing that tumor-induced DGC dysfunction is a contributing factor in muscle wasting.

Figure 15 Reduced dystrophin expression and hyperglycosylation of DGC proteins in skeletal muscle of cachectic cancer patients (A)
Reduced overall survival of cancer patients expressing abnormalities of the DGC complex in their skeletal muscle (B) (22)

6. Summary

Skeletal muscle wasting in cancer cachexia can be mediated by multiple factors derived from tumor and host cells;
The ubiquitin proteasome is a major intracellular system regulating skeletal muscle wasting in response to tumor factors and inflammatory cytokines.
Based on our findings that a dysfunctional DGC serves as a molecular link between muscular dystrophy and cancer cachexia, it is likely that additional information concerning cancer cachexia will be obtained from the study of other muscle wasting conditions.

References


