Altered expression of skeletal muscle myosin isoforms in cancer cachexia

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Diffee, Gary M., Katherine Kalfas, Sadeeka Al-Majid, and Donna O. McCarthy. Altered expression of skeletal muscle myosin isoforms in cancer cachexia. Am J Physiol Cell Physiol 283: C1376–C1382, 2002.—Cachexia is commonly seen in cancer and is characterized by severe muscle wasting, but little is known about the effect of cancer cachexia on expression of contractile protein isoforms such as myosin. Other causes of muscle atrophy shift expression of myosin isoforms toward increased fast (type II) isoform expression. We injected mice with murine C-26 adenocarcinoma cells, a tumor cell line that has been shown to cause muscle wasting. Mice were killed 21 days after tumor injection, and hindlimb muscles were removed. Myosin heavy chain (MHC) and myosin light chain (MLC) content was determined in muscle homogenates by SDS-PAGE. Body weight was significantly lower in tumor-bearing (T) mice. There was a significant decrease in muscle mass in all three muscles tested compared with control, with the largest decrease occurring in the soleus. Although no type IIb MHC was detected in the soleus samples from control mice, type IIb comprised 19% of the total MHC in T soleus. Type I MHC was significantly decreased in T vs. control soleus muscle. MHC isoform content was not significantly different from control in plantaris and gastrocnemius muscles. These data are the first to show a change in myosin isoform expression accompanying muscle atrophy during cancer cachexia.

CACHEXIA IS a life-threatening muscle wasting syndrome that is associated with chronic diseases such as acquired immunodeficiency syndrome (AIDS) and cancer. This progressive loss of skeletal muscle mass affects ~50% of cancer patients (13, 35). The decrease in muscle mass leads to generalized weakness, decreased mobility, and an overall decrease in quality of life. In addition, cachexia often interferes with the effectiveness of anticancer therapy (2, 39). Severe wasting may eventually compromise respiratory muscle and cardiac functions (5, 36). The specific mechanisms that underlie this muscle wasting are unknown, but recent evidence points to a role for proinflammatory cytokines (reviewed in Ref. 5) and various proteolytic pathways including the ubiquitin-proteosome pathway (8, 22). The activation of these pathways is thought to lead to perturbations in muscle protein metabolism such that protein degradation rates exceed protein synthesis rates (37).

Whereas little is known about the mechanisms underlying muscle wasting in cancer cachexia, even less is known about the impact of this wasting on muscle function. Although the significant loss of muscle mass leads to a general muscle weakness, little is known about the effect of this decrease in muscle mass on other aspects of muscle function such as force-velocity characteristics, power output, metabolic properties, and fatigability. Under other conditions in which significant loss of muscle mass occurs, these muscle functional properties have been shown to be significantly altered. These changes in muscle functional properties are most often associated with a shift in fiber type distribution or a shift in myosin isoform expression. Decreased use, whether through denervation of skeletal muscle (12, 21), spinal isolation or transection (29, 31), or reduced mechanical loading (6, 11, 14, 16, 34), has been shown to result in muscle atrophy as well as changes in the expression of myosin isoforms.

The purpose of this study was to examine changes in myosin isoform expression in skeletal muscles during cancer cachexia. Understanding the effect of cancer cachexia on muscle protein isoform expression will aid in determining how muscle function is likely to be affected by this syndrome, 2) may also provide important clues regarding the cellular mechanism(s) of this wasting process, and 3) may increase our understanding of the control of contractile protein isoform expression in skeletal muscle. We hypothesized that, similar to changes seen in some other models of muscle atrophy, the decrease in muscle mass during cancer cachexia would be accompanied by a shift in myosin heavy chain (MHC) and myosin light chain (MLC) expression characterized by a decrease in the phenotypic expression of “slow” myosin isoforms (type I MHC and slow forms of MLC) and an increase in the pheno-

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typic expression of the “fast” myosin isoforms (type II MHC and fast MLC isoforms).

METHODS

All experimental procedures were approved by the Research Animal Resources Committee at the University of Wisconsin. Twelve pathogen-free female CD2F1 mice (BALB/c × DBA/2; Harlan Sprague Dawley, Madison, WI) aged 7–8 wk were randomly divided into tumor-bearing (T) and control (C) groups (n = 6/group). All mice were housed individually in the Animal Care Facility at the University of Wisconsin Medical School. Mice were allowed free access to food and water and were maintained on a 12:12-h dark-light cycle.

Tumor model. The murine C-26 adenocarcinoma, generously provided by Dr. Yutaka Tanaka (Nippon Roche Center, Tokyo, Japan), was used to induce muscle wasting in the mice. This tumor cell line was previously shown to cause significant loss of mass in gastrocnemius and extensor digitorum longus muscles in mice without significant effects on food intake (3, 18, 32). Thus any muscle wasting associated with this tumor cannot be attributed to anorexia and altered energy intake. The C-26 cells were cultured in vitro with RPMI 1640 supplemented with fetal calf serum and 1% penicillin-streptomycin (Mediatech, Herndon, VA). Tumor cells were trypsinized in a subconfluent state and suspended in Hanks’ balanced salt solution at a concentration of 2.5 × 10⁵ cells/ml. Mice were weight matched and divided into T and C groups. Mice in the T group were inoculated subcutaneously between the scapulas with a 0.2-ml cell suspension containing 5 × 10⁶ cells. Mice in the C group were injected with 0.2 ml of phosphate-buffered saline. Body weight for each animal was recorded every 3 days for 21 days (Fig. 1).

Food intake was monitored by providing a premeasured amount of food each day and weighing the remaining food, including any visible food scattered in the cage, the next day. The amount consumed in 24 h was the difference between the weights. These data are shown in Fig. 2.

Muscle homogenization and protein determination. At 21 days after tumor inoculation, the mice were killed by cervical dislocation under anesthesia (0.03 mg/kg fentanyl and 30 mg/kg etomidate) and the tumor was removed and weighed. The gastrocnemius, plantaris, and soleus muscles from both hindlimbs were removed. The muscles were trimmed of connective tissue and fat, blotted dry, weighed, and then quick frozen in liquid nitrogen and stored at −80°C for later analysis. Muscles were homogenized on ice in a buffer consisting of (in mM) 50 Tris·HCl, 0.25 sucrose, and 5 EDTA with 1% (wt/vol) sodium dodecyl sulfate (SDS) with a Tekmar TissueMizer. The resulting homogenate was then centrifuged for 10 min at 3,000 rpm at 4°C in a Beckman bench top centrifuge (GS15R). Total muscle protein concentration was determined with a Bio-Rad protein assay kit (based on the Bradford method). Samples were read at 595 nm with a Shimadzu UV-2101PC spectrophotometer at room temperature according to kit instructions.

Analysis of MHC isoform content. MHC isoform content of muscle homogenates was determined with a modification of the SDS-polyacrylamide gel electrophoresis technique of Tal-madge and Roy (30). Muscle homogenates (4–10 μg protein/lane) were heated (3 min at 100°C), combined with sample buffer (8 M urea, 2 M thiourea, 0.05 M Tris pH 6.8, 75 mM dithiothreitol, 3% SDS, and 0.05% bromophenol blue), and loaded onto polyacrylamide gels. Stacking gels were composed of 5% bis-acrylamide (50:1), 25% glycerol, 87.5 mM Tris pH 6.7, 5 mM EDTA, and 0.5% SDS. Resolving gels were composed of 8% bis-acrylamide (50:1), 30% glycerol, 0.2 M Tris pH 8.8, 0.1 M glycine, and 0.4% SDS. Gels were run with SE 200 Tall (10 × 12 cm) Mighty Small Mini-Vertical Units (Hoefer) with 0.75-cm-thick spacers and an EPS 301 power supply (Amersham Pharmacia Biotech). The upper running buffer consisted of 100 mM Tris (base), 150 mM glycine, and 0.1% SDS. The lower running buffer consisted of 50 mM Tris (base), 75 mM glycine, and 0.05% SDS. The gels were run at 135 V (constant voltage) for 43–48 h at 4°C. Gels were silver stained with a Bio-Rad Silver Stain Plus kit according to kit instructions. Stained gels were dried down and scanned into bitmap file format (.bmp) with an Epson Perfection 1200 Photoscanner with its transparency adapter (back lit). Density of bands was quantified from the bitmap file with UnScan-It gel quantification software (Silk Scientific, Orem, UT). Density of the bands corresponding to each of the four MHC isoforms is expressed as a percentage of the total of all four bands. MLC content of soleus samples was determined with SDS-PAGE by using a modification of a previously described technique (19). Briefly, resolving gels were 18% acrylamide, 10% glycerol, and 0.7 M Tris (pH 9.3). Stacking
gels were 3% acrylamide, 10% glycerol, and 0.13 M Tris (pH 6.8). Sample preparation and loading were as described above. Gels were run at 40 V (constant voltage) for 24 h at room temperature. Staining and scanning were as described above.

Statistical analysis. All data are presented as means ± SD from n = 6 mice per group. The significance of differences between groups (T vs. C) was tested with Student’s t-test. Differences were considered significant at the 0.05 level of confidence.

RESULTS

Effect of tumor on body weight, muscle mass, and muscle protein concentration. The time course of changes in body weight of both T and C mice after tumor injection is given in Fig. 1. Initial body weight of the T mice declined over the course of 21 days, with most of the decrease occurring over the final 6 days. Final body weight at the time of death was 15.9 ± 0.6 g (mean ± SE) in T mice and 19.6 ± 0.5 g in C mice. This represents a decrease in body weight of 19% in T compared with C mice and is a statistically significant difference (P < 0.05). However, mean (±SE) initial tumor mass at the time of death was 2.1 ± 0.2 g. Thus the mean non-tumor body weight at the time of death was 13.7 ± 0.56 g in T animals. This represents a 30% decrease in non-tumor body weight compared with C mice. Mean values for daily food intake are shown in Fig. 2. There was no significant difference between the two groups at any time point during the 21 days. Mean (±SD) values for hindlimb muscle mass and protein concentration are given in Table 1. Gastrocnemius muscle mass from T mice declined to 62% of the mass of the gastrocnemius from C mice. Plantaris and soleus muscle mass values decreased to 61% and 49% of the mass from C mice, respectively. In T mice the protein concentration in crude homogenates from the gastrocnemius muscle declined to 76% of that in C mice, whereas in the plantaris and soleus muscles the protein concentration in T mice declined to 93% and 67%, respectively, of control values. All of the above differences in muscle mass and protein concentration between the T group and the C group, with the exception of the plantaris muscle protein concentration, were statistically significant (P < 0.05).

MHC isoform content. Figure 3 depicts a representative 8% polyacrylamide gel showing the distribution of MHC isoforms in gastrocnemius, plantaris, and soleus muscles from T and C mice. The gel demonstrates a shift in MHC expression in the soleus muscles from T mice characterized by an increase in the appearance of type IIb MHC and a decrease in the amount of type I MHC compared with soleus muscles from C mice. Figure 4 shows the mean data for soleus, gastrocnemius, and plantaris muscles from six animals in each group. In the control soleus muscle the average relative MHC isoform distribution was 26% type IIx, 22% type IIA, and 52% type I. There was no detectable presence of type IIb MHC in control soleus muscle. In the soleus muscles from T mice, the average relative distribution of MHC isoforms was 29% type IIx, 19% type IIA, 19% type IIb, and 33% type I. The relative amounts of type IIb and type I MHC isoforms were significantly different (P < 0.05) between the T and C groups. There were no significant differences in the MHC isoform content in gastrocnemius and plantaris muscles between T and C animals.

MLC content. A representative gel demonstrating the MLC content of control and tumor-bearing soleus muscles is shown in Fig. 5 (a plantaris muscle is shown in lane 1 for comparison). Little information is available in the literature regarding the relative positions on SDS-PAGE gels of the fast vs. slow isoforms of the MLC in mouse skeletal muscle. For the present study, MLCs were identified on the basis of their molecular weight, and the relative positions of the fast vs. slow isoforms of MLC1 and -2 were identified on the basis of comparisons between soleus and plantaris samples. In addition, the relative positions were assumed to be
As shown in Fig. 5, soleus muscles from tumor-bearing animals showed an increase in the relative amount of the fast isoforms of MLC1 and -2. In addition, although no MLC3f was found in control soleus, we detected a small amount in four of the six tumor-bearing soleus samples.

**DISCUSSION**

The decrease in body weight and hindlimb muscle mass in tumor-bearing mice observed in the present study was similar in magnitude to that described in other studies of cancer cachexia in mice using the C26 adenocarcinoma cell line (3, 18, 32). We found that the presence of the tumor did not significantly alter food intake, a result also seen in previous studies using this tumor cell line (3, 32). Thus the significant loss of muscle mass/body mass is likely not due to anorexia but is rather a specific response to the presence of the tumor in these animals. The decline in muscle mass was accompanied by a decrease in muscle protein concentration (Table 1), an effect that is similar to that seen in previous studies of cancer cachexia (3, 33). Although muscle protein loss can occur via a variety of proteolytic pathways, recent evidence has suggested that muscle wasting during cancer cachexia is associated with an increase in activity of the ubiquitin-proteosome pathway (8, 22).

The loss of muscle mass was also qualitatively and quantitatively similar to that seen in studies using a variety of interventions that produce muscle atrophy. In studies of atrophy resulting from decreased use (hindlimb unweighting, space flight, and spinal transection/isolation) it has been demonstrated that, although all hindlimb muscles are subject to loss of mass and decreased protein concentration, it is predomi-
nantly slow muscles, such as the soleus, that show the
most significant effects with regard to muscle mass,
protein concentration, and myosin isoform content
compared with the other ankle extensors, the plantaris
and gastrocnemius (reviewed in Ref. 7). These results
are similar to the results of the present study, in which
the tumor-bearing mice showed the greatest percent
loss of muscle mass in the soleus muscle compared
with the plantaris and gastrocnemius.

The most significant finding of this study is that the
loss of skeletal muscle mass that accompanies cancer
cachexia is associated with a shift in myosin isoform
content in the soleus muscle. This shift is characterized
by an increase in the relative amount of type Iib MHC
from undetectable levels of expression in control soleus
muscle to 19% of the total MHC in soleus from tumor-
bearing animals. This increase in type Iib MHC came
primarily at the expense of type I MHC expression.
Change in MLC isoform expression mirrors the change
in MHC content, with an increase in the amount of the
fast isoforms of MLC1 and MLC2 in soleus samples
from tumor-bearing animals. Although a number of
previous studies described the decrease in muscle mass
associated with the presence of a tumor, this is the first
report of alterations in contractile protein isoform ex-
pression accompanying this muscle wasting.

The differential expression of myosin isoforms in
skeletal muscle is known to be affected by a number of
diverse factors. Those factors with particular relevance
to the results of the present study include muscle
activity, sepsis, and caloric restriction. The effects with
regard to MHC isoform content presented in the
present study are qualitatively similar to those ob-
served in studies using other interventions that de-
crease muscle mass, such as decreased mechanical
loading (1, 11, 14, 16, 17), and spinal transection/spinal
isolation (20, 23, 29, 31). Atrophy associated with these
interventions has been shown to be associated with an
increase in the relative amount of type II MHC iso-
forms and a decrease in the relative amount of type I
MHC. This effect has been shown to be more prominent
in slow twitch muscles such as the soleus, a result
similar to that seen in the present study.

The similarity of the results of the present study to
changes in muscle mass and myosin isoform expression
observed with decreased muscle use suggests the pos-
sibility that changes in myosin expression associated
with cancer cachexia merely reflect decreased muscle
usage in the tumor-bearing mice. However, the large
increase in the relative amount of type Iib MHC seen
in the present study represents a much more substan-
tial isoform shift than has been reported in studies
involving decreased usage. These previous studies gen-
erally demonstrated that the decrease in type I MHC is
accompanied by an increase in type Ila/x MHC expres-
sion with little (11, 15) or no (29, 31) increase in type
Iib MHC content. Although we did not measure activ-
ity levels in these animals, it seems unlikely that
changes in the activity level of the mice could have
resulted in more rapid and significant changes in my-
osin isoform expression than complete removal of me-
chanical load or nerve activity. Thus it seems likely
that the changes in myosin expression are specific to
the presence of the tumor. It must be noted, however,
that very few studies of the effect of decreased muscle
activity have been carried out in mice and thus little is
known regarding possible species differences in the
magnitude of the effects of decreased use on myosin
isoform expression.

Cancer cachexia in both humans and animals is
often associated with anorexia, or reduced food intake
(35), although cachexia still occurs even in the absence
of significant effects on food intake (3, 32). Decreased
caloric intake is also known to effect the differential
expression of myosin in striated muscle. In the rat
heart, caloric restriction has been shown to increase
the expression of slow (β) MHC isoform (28) but re-
duced caloric intake has not been shown to have an
effect on skeletal muscle MHC isoform expression (4).
We measured food intake in the present study to de-
determine whether changes in muscle mass or myosin
expression could be attributed to decreased caloric in-
take. The C-26 adenocarcinoma tumors induced in the
present study were shown not to decrease food intake
(Fig. 2), a result similar to that seen in other studies
using this tumor cell line (3, 32). These results suggest
that the changes in myosin expression in the present
study were not due to changes in caloric intake.

Similar to cachexia, sepsis provokes rapid and sig-
nificant skeletal muscle atrophy along with effects on
other organ systems (40). In the case of sepsis, muscle
atrophy has been shown in some studies to be associ-
ated with shift in the relative expression of MHC iso-
forms. An increase in type I MHC gene expression
has been observed in both cardiac (25) and skeletal (24)
muscle. Although this response is opposite of our re-
sults showing a decrease in type I MHC and an in-
crease in type Iib MHC, comparison of these two atro-
phy-producing conditions might prove helpful in
determining the mechanism(s) of these changes in my-
osin expression. The mechanism of the effects of sepsis
on MHC gene expression is not understood at present,
but proinflammatory cytokines have been suggested to
play a role. Sepsis is known to be associated with the
release of various proinflammatory cytokines such as
tumor necrosis factor (TNF)-α, interleukin (IL)-1 and
IL-6, and interferon (IFN)-γ (38). Recently, these cyto-
kines have been shown to directly affect MHC gene
expression in the heart, with some cytokines appar-
ently acting to increase MHC expression and others
having a repressive effect on expression (26). Thus the
effect of sepsis to alter the relative expression of car-
diac MHC isoforms may depend on the balance of
particular cytokines present (26). There is a great deal
of evidence that a number of these cytokines also play
a substantial role in cancer cachexia, although there is
some disagreement about the precise role of particular
cytokines (reviewed in Ref. 5). This raises the possibil-
ity that altered cytokine levels associated with the
presence of the tumor may play a role in the changes in
MHC isoform expression observed in the present
study.
It is of course possible that the changes in myosin expression described in the present study do not result from changes in gene expression but rather are the result of translational or posttranslational modification of protein levels. For example, the change in the relative amounts of MHC isoforms may be due to differential susceptibility to protein degradation. The changes in myosin occur against the backdrop of overall decreases in muscle protein concentration. Previous studies implicated the ubiquitin-proteosome pathway in the muscle atrophy associated with cancer cachexia (8, 22) as well as in muscle atrophy induced by a variety of models of decreased muscle use (9). In the case of decreased muscle use, it has been demonstrated that changes in MHC isoform content are generally present at the mRNA level as well as the protein level (1, 11). This suggests that, even as overall muscle protein concentration is decreasing as a result of ubiquitin-proteosome activity, the change in MHC content is the result of increased transcription of the type II MHC genes, and decreased transcription of type I MHC, rather than a differential susceptibility to protein degradation. The de novo appearance of type Iib MHC in tumor-bearing soleus muscle makes it seem likely that the altered MHC isoform content seen in the present study is the result of altered gene expression. We are currently investigating whether MHC isoform mRNA levels are altered in the soleus muscles of tumor-bearing mice.

Besides the effects of muscle wasting on overall muscle strength, the effects of cancer cachexia on other properties of muscle function have not been described. The results of the present study would suggest that contractile function is likely altered along with changes in muscle mass. Because it is known that shortening velocity is affected by changes in both MHC and MLC isoforms (10), it is likely that the myosin isoform shift observed in the present study would increase the velocity of shortening of the soleus muscle. Studies on unweighting indicate that the shift in MHC/MLC content increases shortening velocity at both the single-fiber and whole muscle level (11, 14, 16, 17), whereas maximal force and power output are decreased due to the muscle atrophy.

In conclusion, we have demonstrated for the first time that the decline in muscle mass in cancer cachexia is associated with a shift in the myosin isoform content in the soleus muscle but not the plantaris or gastrocnemius muscles. This shift is characterized by an increase in the relative amount of type Iib MHC and a decrease in type I MHC relative to other MHC isoforms along with an increase in fast MLC1 and MLC2 and a decrease in the relative amount of slow MLC1 and MLC2. These changes are likely to have a significant impact on the functional properties of muscles during cachexia. The mechanism of this effect on myosin isoform expression is unknown at present, but it may be related to proinflammatory cytokines associated with the muscle wasting process.

REFERENCES


