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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. First published June 26, 2002; 10.1152/ajpcell.00154.2002.—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.

myosin heavy chains; myosin light chains; muscle atrophy

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 *1*) 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 adenocarcinaoma, 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 2.5×10^5 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 5×10^5 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 two 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.

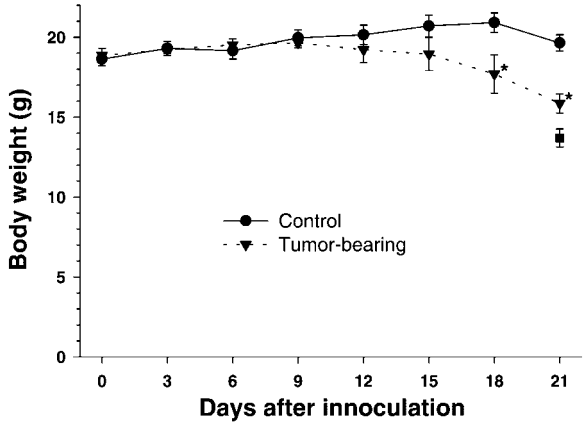

Fig. 1. Changes in body weight in control and tumor-bearing mice over 21 days. Means (±SE) for 6 animals in each group are shown. Animals were weighed every 3 days after injection of tumor cells into tumor-bearing animals. Animals were killed on *day 21*. *Significant difference ($P < 0.05$) between control and tumor-bearing groups. ■, Mean body weight of tumor-bearing animals after removal of tumor mass.

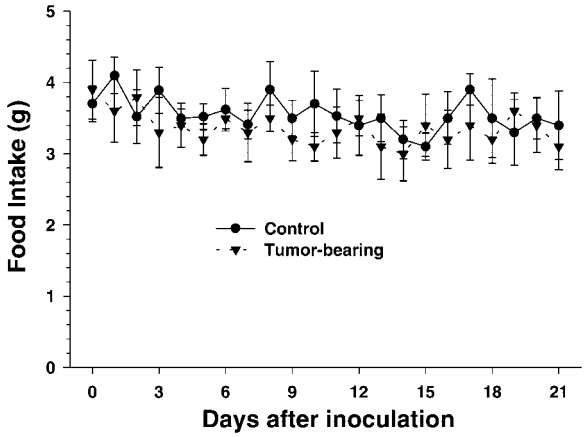

Fig. 2. Daily food intake in control and tumor-bearing mice over 21 days. Food intake was measured as described in METHODS. Values are means ± SE for 6 animals/group. There were no significant differences in food intake between control and tumor-bearing mice at any time point.

Analysis of MHC isoform content. MHC isoform content of muscle homogenates was determined with a modification of the SDS-polyacrylamide gel electrophoresis technique of Talmadge 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

Table 1. *Effect of tumor bearing on muscle mass and protein concentration in hindlimb muscles of mice*

	Gastrocnemius		Plantaris		Soleus	
	Muscle mass, mg	Protein concentration, mg/ml	Muscle mass, mg	Protein concentration, mg/ml	Muscle mass, mg	Protein concentration, mg/ml
Control	102.5 ± 12.8	15.58 ± 0.68	11.1 ± 2.61	9.23 ± 1.82	6.9 ± 1.48	7.34 ± 1.38
Tumor bearing	61.6 ± 8.86*	11.89 ± 2.12*	6.68 ± 1.49*	8.57 ± 2.45	3.4 ± 1.61*	4.95 ± 1.11*
Tumor bearing as % of control	60.1%	76.3%	60.2%	92.8%	49.7%	67.4%

Results are presented as means ± SE for $n = 6$ mice per group. *Significant difference ($P < 0.05$) compared with control.

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. Body weights 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) tumor mass at the time of death was 2.1 ± 0.2 g. Thus the mean nontumor body weight at the time of death was 13.7 ± 0.56 g in T animals. This represents a 30% decrease in nontumor 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

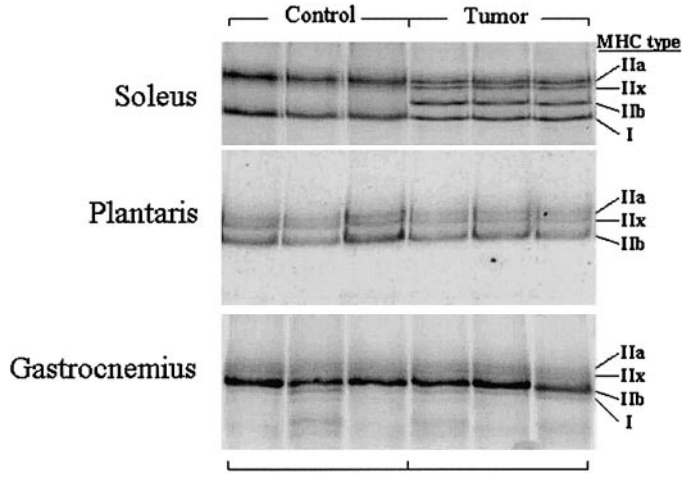

Fig. 3. Representative 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels showing the distribution of myosin heavy chain (MHC) isoforms in gastrocnemius, plantaris, and soleus muscles from 3 control and 3 tumor-bearing mice. Identification of MHC isoforms was from Ref. 30.

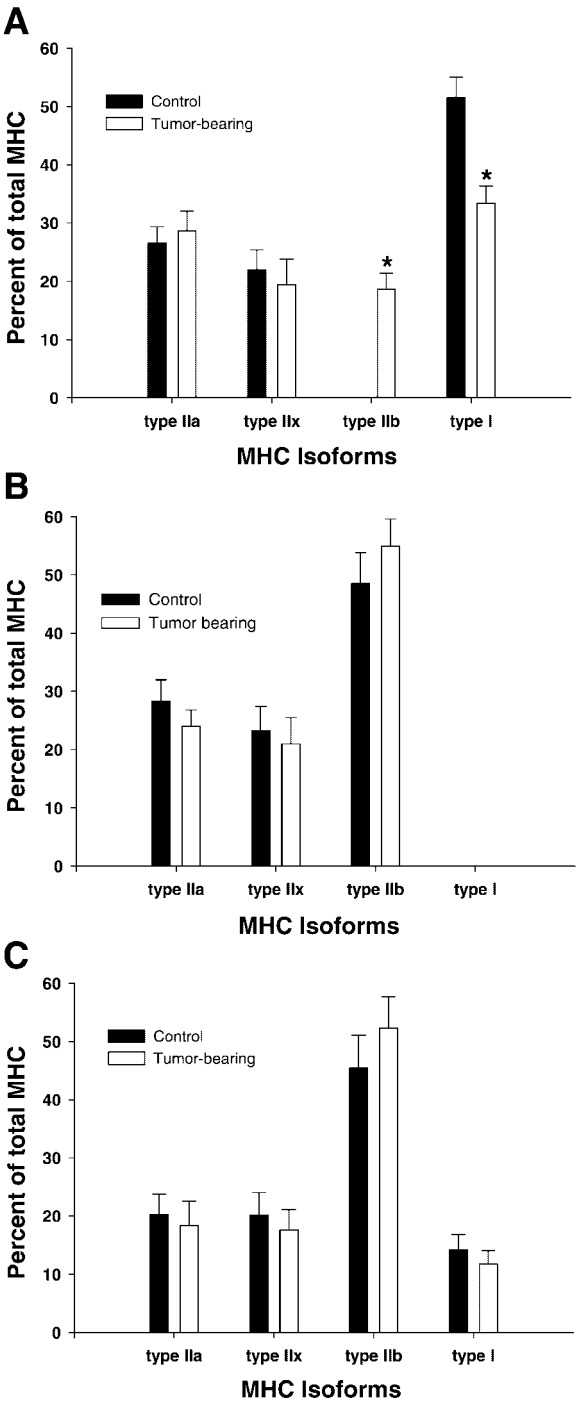

Fig. 4. Quantification of MHC distribution in soleus (*A*), plantaris (*B*), and gastrocnemius (*C*) muscles from control and tumor-bearing animals. SDS-PAGE gels were scanned, and the relative content of each of the MHC isoforms was determined as described in METHODS. The amount of each MHC isoform is presented as % of the total MHC pool on the gel for that sample. Data are means ± SE from $n = 6$ animals/group. *Significantly different from control ($P < 0.05$).

similar to those observed in rat skeletal muscle (14). As shown in Fig. 5, soleus muscles from tumor-bearing muscles 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-

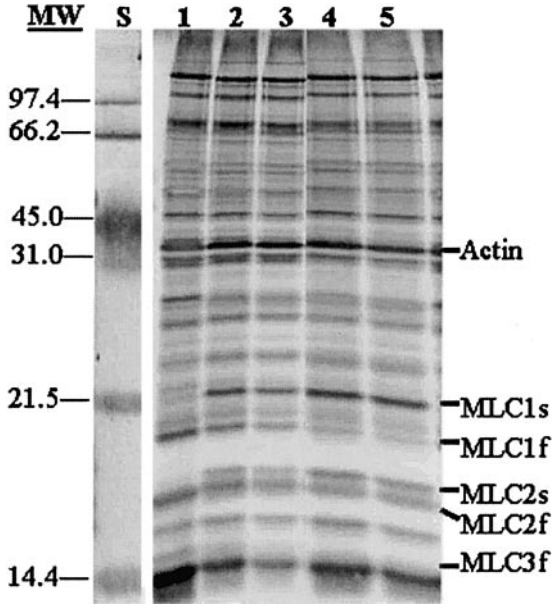

Fig. 5. Representative 18% SDS-polyacrylamide gels showing the distribution of myosin light chain (MLC) isoforms in soleus muscles from control and tumor-bearing mice. *Lane S* contains molecular weight standards with molecular weights (MW) given at *left*; *lane 1* contains a sample from control plantaris muscle for reference to positions of fast MLC isoforms. *Lanes 2* and *3* are soleus samples from tumor-bearing mice, and lanes *4* and *5* are soleus samples from control mice.

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 expression 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 observed in studies using other interventions that decrease 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 isoforms 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 possibility 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 substantial isoform shift than has been reported in studies involving decreased usage. These previous studies generally demonstrated that the decrease in type I MHC is accompanied by an increase in type IIa/x MHC expression with little (11, 15) or no (29, 31) increase in type IIb MHC content. Although we did not measure activity 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 myosin isoform expression than complete removal of mechanical 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 reduced 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 determine whether changes in muscle mass or myosin expression could be attributed to decreased caloric intake. 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 significant 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 associated with shift in the relative expression of MHC isoforms. 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 results showing a decrease in type I MHC and an increase in type IIb MHC, comparison of these two atrophy-producing conditions might prove helpful in determining the mechanism(s) of these changes in myosin 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 cytokines have been shown to directly affect MHC gene expression in the heart, with some cytokines apparently 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 cardiac 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 possibility 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.

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