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A Key Role for Leukemia Inhibitory Factor in C26 Cancer Cachexia

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Background: Cachexia is the widespread loss of muscle and fat that causes death in many cancers. Secretion of leukemia inhibitory factor (LIF) by C26 cancer cells activates differential gene expression that results in muscle cell atrophy.

Results: Secretion of leukemia inhibitory factor (LIF) by C26 cancer cells activates differential gene expression that results in muscle cell atrophy.

Conclusion: LIF produced by cancer cells acts on muscle to cause atrophy.

Significance: LIF and its signaling pathway are new targets in fighting cancer cachexia.

Cachexia is an exacerbating event in many types of cancer that is strongly associated with a poor prognosis. We have identified cytokine, signaling, and transcription factors that are required for cachexia in the mouse C26 colon carcinoma model of cancer. C2C12 myotubes treated with conditioned medium from C26 cancer cells induced atrophy and activated a STAT-dependent reporter gene but not reporter genes dependent on SMAD, FOXO, C/EBP, NF-κB, or AP-1. Of the gp130 family members IL-11, IL-6, oncostatin M (OSM), and leukemia inhibitory factor (LIF), only OSM and LIF were sufficient to activate the STAT reporter in myotubes. LIF was elevated in C26 conditioned medium (CM), but IL-6, OSM, TNFα, and myostatin were not. A LIF-blocking antibody abolished C26 CM-induced STAT reporter activation, STAT3 phosphorylation, and myotube atrophy but blocking antibodies to IL-6 or OSM did not. JAK2 inhibitors also blocked C26 CM-induced STAT reporter activation, STAT3 phosphorylation, and atrophy in myotubes. LIF at levels found in the C26 CM was sufficient for STAT reporter activation and atrophy in myotubes. In vivo, an increase in serum LIF preceded the increase in IL-6 in mice with C26 tumors. Overexpression of a dominant negative Stat3Cβ-EGFP gene in myotubes and in mouse muscle blocked the atrophy caused by C26 CM or C26 tumors, respectively. Taken together, these data support an important role of LIF-JAK2-STAT3 in C26 cachexia and point to a therapeutic approach for at least some types of cancer cachexia.

Cancer cachexia is the systematic loss of body weight that cannot be reversed by diet and is a burden often determining the prognosis of the disease. In up to 30% of all cancers it leads to death through a combination of extreme weakness, decreased resistance to infection, and the inability to tolerate needed therapy (1–3). Cachexia is especially prevalent in cancers of the colon, lung, and pancreas and involves a significant loss of both fat stores and muscle mass throughout the body (1, 4). To study cancer cachexia, animal tumor models have been used as systems in which the factors responsible for muscle and adipose loss can be isolated and tested more easily than in cancer patients. One of the most studied models is C26 colon adenocarcinoma in mice (5–7). Several different transcription factors, signaling pathways, and triggering ligands have been implicated in C26-induced skeletal muscle atrophy. In particular, there is evidence that the JAK/STAT, SMAD, FOXO, and ERK families are involved in muscle wasting during C26 cancer cachexia (8–13), and the cytokines most commonly linked to muscle atrophy from C26 tumors include IL-6 and myostatin (10, 12, 14). However, our understanding of the cell biology underlying cachexia is still in its early stages.

In this work, we identified a key cytokine and its signaling and transcription factor pathways responsible for C26 cancer cachexia. A C26 conditioned medium (CM) model was used to identify the cytokines secreted and the signaling and transcription factors activated by C26 tumor cells that produce myotube atrophy. The main observations were confirmed in mice with C26 tumors. We found that JAK/STAT (8, 12) and ERK (11) signaling was stimulated by CM from C26 tumor cells. Moreover, we show that leukemia inhibitory factor (LIF) is the cytokine inducing both JAK2/STAT3 and ERK signaling as well as atrophy in myotubes treated with medium from C26 cancer cells. This is the first direct evidence that LIF is an essential regulator of cachexia from C26 tumors.

Experimental Procedures

Biochemicals—The cytokines used were as follows: mouse IL-11, mouse OSM, mouse IL-6, and myostatin were from R&D Systems (Minneapolis, MN). Mouse LIF was from eBioscience (San Diego, CA). Blocking antibodies for LIF and OSM were obtained from R&D Systems, and blocking antibody for IL-6 was obtained from eBioscience. When antibodies were used to...
block cytokine activity, normal IgG of the same species as the blocking antibody was added to controls. The inhibitors used were ruxolitinib/INCB-18424 (ChemieTek, Indianapolis IN), JAK2 inhibitors, Tyrophostin AG490 and WP1066 (Sigma), and the ERK 1/2 inhibitor U0126 (EMD/Millipore, Danvers MA). When inhibitors required dimethyl sulfoxide (anhydrous, Life Technologies) for solubility then controls had dimethyl sulfoxide added at the same level as in the inhibitor solutions.

ELISA—ELISAs for LIF, myostatin, and OSM were from R&D Systems. ELISAs for TNFα and IL-6 were from eBioscience. All ELISAs were performed according to the protocols of the manufacturers.

Plasmids—To create a dominant negative STAT, we took the plasmid pMXs-Stat3-C (Addgene plasmid 13373) (15) and removed the C-terminal transactivation domain as described in Ref. 16. We then subcloned Stat3β into the EGFP-N1 plasmid to create our designation d.n.STAT3β-EGFPN1 (d.n.STAT3), which was sequenced (GeneWiz, Cambridge MA) to confirm the in-frame sequence of the fusion construct. The plasmids pPIAS1 and pPIAS3 were bought from Addgene (plasmids 15206 and 15207) (17). The STAT reporter plasmid STAT-luc (plasmid 8688) and the SMAD reporter plasmid SMAD-luc (plasmid 16495) were from Addgene (Cambridge, MA). The NF-κB and AP-1 reporters have been described previously (18). The C/EBP reporter was purchased from Signosis (Santa Clara, CA). The FOXO-luc reporter was obtained from the Greenberg laboratory and has been described previously (19). All plasmids were prepared using Qiagen Maxi or Mega endotoxin removal kits and stored at −20 °C in Tris-EDTA until used.

Cell Culture and Transfection—C26 adenocarcinoma cells obtained from the National Cancer Institute (Frederick, MD) were plated and maintained as described previously (8). C2C12 myoblasts were purchased from the ATCC and used within the first 15 passages. C2C12 cells were cultured in DMEM/10% FBS, passed at least every 2 days, and differentiated in DMEM/2% horse serum (HS) for 4 days. Transfections were done on C2C12 myoblasts using Effectene (Qiagen, Valencia, CA) for reporter assays and GeneCellin (Bulldog Bio, Portsmouth, NH) for transfections when myotube diameters were measured, with the following details. For Effectene, myoblasts were seeded in growth medium at $5 \times 10^5$ cells/well on a 6-well plate the night before transfection in growth medium. 12–16 h later, DNA (0.5 μg/well)-Effectene mixes were added to the wells in differentiation medium. For GeneCellin, cells were seeded in growth medium at $5 \times 10^5$ cells/well on a 6-well plate and allowed to attach overnight. The next day, cells were switched to differentiation medium and transfected with 2 μg of DNA/well for a 6-well plate using GeneCellin transfection reagent. The medium was refreshed 24 h later.

CM Collection—Conditioned medium for C26 cells was made using the method of Lokireddy et al. (10). C26 cells and C2C12 myoblasts were grown in 5% FBS or 10% FBS, respectively, at 37 °C in 5% CO₂. When the plates reached a confluency of >90%, the growth medium was removed, and the cells were washed twice with sterile PBS and three times with DMEM with no serum plus antibiotics and glutamine. It was found to be important that conditioned medium was taken from the cells in medium without serum. Fetal bovine serum contains myostatin (see below) and induces C26 cells to produce IL-6 at a level 50-fold higher than when it is not present. C26 cells were grown in DMEM plus antibiotics and glutamine with no serum for 24 h. After 24 h, the medium was collected and centrifuged in 50-ml Falcon tubes at 4500 rpm for 15 min at 4 °C. The supernatant was filtered through a 0.22-micron filter in a sterile environment. Aliquots of the filtered medium were stored at −80 °C for up to a year. Conditioned medium treatment was 33% CM in differentiation medium (2% HS in DMEM plus antibiotics and glutamine). Treatment for controls was 33% DMEM plus antibiotics and glutamine without serum.

Luciferase Reporter Assays—C2C12 myoblasts in growth serum were plated on a 24-well plate at a density of $5 \times 10^4$ cells/well and left overnight for attachment. Cells were then switched to differentiation medium and transfected with 0.5 μg of a luciferase reporter plasmid and 0.05 μg of EGFP/well. The differentiation medium was changed 24 h later, and was EGFP visualized for transfection efficiency. Cells were treated 4 days post-transfection, lysed with 200 μl of passive lysis buffer (Promega, Madison, WI), and luciferase activity was measured as detailed previously (20).

Immunoblotting—The antibodies for Western blots were anti-phospho-STAT3 (Tyr-705, catalog no. 9139), anti-STAT3 (catalog no. 9139), anti-phospho-STAT1 (Tyr-701, catalog no. 9172), anti-phospho-STAT5 (Tyr-694, catalog no. 4322), anti-STAT5 (catalog no. 9363), anti-pERK1/2 (Thr-202/Tyr-204, catalog no. 4370), anti-ERK1/2 (catalog no. 4695) (Cell Signaling, Danvers, MA), anti-myostatin (catalog no. AF788, R&D Systems), and anti-GAPDH (Sigma). Myotubes were lysed with 1× radioimmune precipitation assay buffer (Cell Signaling Technology) and 1× PMSF. The protein concentration of cell lysates was measured using the Bio-Rad DC assay (Bio-Rad). Equal amounts of protein from each sample were separated by electrophoresis, transferred to a membrane, and incubated with primary and secondary antibodies as detailed previously (21). Protein signals were visualized using indirect immunostaining with infrared fluorescence imaging using a LiCor Odyssey imager.

Myotube Diameter Studies—For phase and fluorescence micrographs of C2C12 myotubes, cultures were treated with differentiation medium supplemented with 33% DMEM (control) or 33% C26 CM for 48 h beginning at 3 d of differentiation. The myotubes were photographed and measured as detailed previously (20). When needed, differentiated myotubes were visualized with MF20, a sarcosomic myosin-specific antibody from Developmental Studies Hybridoma Bank (University of Iowa), followed by incubation with a secondary antibody conjugated to Alexa Fluor 488 (Life Technologies).

Gene Expression Studies—Total RNA was isolated from myotubes treated with LIF for 4, 8, or 24 h and from vehicle-treated (PBS) myotubes at each time point. Each of these six groups contained three independent samples. Total RNA was isolated using the miRNeasy mini kit (Qiagen), and quantity and quality were measured by NanoDrop spectroscopy and Agilent Bioanalyzer assay. The Boston University Microarray Resource Core Facility performed first-strand synthesis and hybridization to Affymetrix mouse 1.0 ST arrays. For
microarray studies, the RNA samples in each of the six groups were pooled. For quantitative real-time PCR, RNA samples were converted to cDNA with the Qiagen QuantiTect kit, followed by real-time quantitative PCR on an ABI 7300 thermal cycler using Fast Advanced Master Mix and TaqMan primer-probe sets purchased from Life Technologies. The probe sets were as follows: Mm00545913_s1, Socs3; Mm00504306_m1, Bcl-3; Mm00786711_s1, C/EBP/H9254; Mm01275601_g1, Bnip3; Mm00432307_m1, Casp4; Mm01197698_m1, Gusb; and Mm01545399_m1, Hprt.

**Animals**—Mice with C26 tumors were prepared as described previously (21). Eight-week-old male CDF1 mice purchased from Charles River Laboratories (Wilmington, MA) were used for all experiments. This study was carried out in strict accordance with the recommendations given in the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals. The protocol was approved by the Boston University Institutional Animal Care and Use Committee (protocol number 12-016).

Plasmids were prepared and injected into mouse tibialis anterior (TA) muscles 14 days after C26 tumor cell inoculations, as detailed previously (21). On day 25 post-inoculation, TA muscles were removed from control and C26 tumor-bearing mice and prepared for analysis. The myofiber cross-sectional area was measured as detailed previously (21). TA muscle fiber cross-sectional areas of plasmid-transfected fibers (green) were compared with fiber areas in the same fields that did not take up the plasmid (black) in both tumor-bearing and control mice. Laminin was stained with an antibody coupled to Texas Red-X to demarcate the muscle fiber membranes.

**Statistics**—For one variable analysis, an unpaired Student’s *t* test was used to determine significant difference. For multivariable analysis, a one-way analysis of variance with Tukey’s post test was used. Data are expressed as mean ± S.E., and statistical difference was defined at *p* < 0.05.

**Results**

**Characterization of a C26 CM Myotube Atrophy Model**—To focus on the factors that trigger muscle wasting in C26 mouse colon carcinoma, we used medium from C26 cells and treated cultured myotubes. First, we isolated C26 CM to verify that it produced atrophy, as measured by decreased myotube diameters of differentiated C2C12 myotubes (Fig. 1A). A 15% decrease in myotube diameter could be seen as early as 24 h after addition of the C26 CM, and there was a 20% decrease in diameter at 48 h (Fig. 1B).

**STAT-dependent Transcriptional Activation in C26 CM-treated Myotubes Is Mediated by LIF**—To test a role for several transcription factors as expediently as possible in CM-treated myotubes, we transfected a series of transcription factor-dependent reporter genes into myoblasts, differentiated the cultures to myotubes, and surveyed them for inducibility by C26 CM (Fig. 1C). Despite several transcription factors being implicated in different types of cancer cachexia in the literature, including AP-1 (2), C/EBP (22), FOXO (10, 13), NF-kB (10, 23), SMAD 2/3 (10), and STAT3 (8, 12), our survey of reporters showed that only the STAT reporter was activated by C26 CM (Fig. 1C). Although data for 4 h are shown, reporters dependent on the other transcription factors did not respond to CM at any time over a 24-h time period (data not shown). A time course showed
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The IL-6 concentration in C26 CM was 7 pg/ml, and this was not significantly different from the levels in C2C12 CM (Fig. 3A). The OSM concentration in C26 CM was ~350 pg/ml, but this was not different from levels in C2C12 CM. LIF was elevated significantly in C26 CM compared with undetectable levels in C2C12 CM (Fig. 3A). LIF was sufficient to induce atrophy when tested at the C26 CM level of 370 pg/ml (Fig. 3C). Moreover, the blocking antibody to LIF completely blocked the myotube atrophy induced by the C26 CM (Fig. 3D). Although not elevated in C26 CM, high levels of IL-6 (10 or 100 ng/ml) are sufficient to induce atrophy in cultured myotubes, as we and others have shown (12, 20). However, a blocking antibody to IL-6 did not reverse C26 CM-induced atrophy (Fig. 3E).

STAT1, STAT3, and STAT5—Because the STAT reporter was instrumental in leading our investigation into the signaling of C26 cachexia, we next studied the involvement of two major STATs. The activation of STAT is known to be due to specific phosphorylation (24), and so we investigated the phosphorylation of STAT3 and STAT1 by C26 CM and LIF. The time course for the appearance of pSTAT3 (Fig. 4A) and pSTAT1 (Fig. 4B) when C2C12 myotubes were treated with C26 CM or with LIF showed the following. There is a strong phosphorylation of STAT3 and a weaker but significant phosphorylation of STAT1 at the same time, peaking at 30 min after addition of either C26 CM or LIF. This precedes the peak for the time course of STAT reporter activity by C26 CM, as shown in Fig. 1.

We inhibited C26 CM-induced STAT activation and myotube atrophy using various methods. A blocking antibody to LIF inhibited C26 CM-induced STAT3 phosphorylation but not CM-induced STAT1 phosphorylation (Fig. 4C). We then used two naturally occurring protein inhibitors of active STAT1 and STAT3, PIAS1 and PIAS3 (17), respectively, to elucidate which STAT might be more responsible for STAT reporter activation in CM-treated myotubes. Overexpression of PIAS1 was unable to inhibit C26 CM-induced STAT reporter activation (Fig. 4D). On the other hand, PIAS3 overexpression inhibited ~50% of STAT reporter activation (Fig. 4D). We also used another genetic method to inhibit STAT3 by creating a dominant negative STAT3 (d.n.STAT3). We took a constitutive dimer-producing version of STAT3 (26) and removed the C-terminal transactivation domain, effectively creating the beta form (16). This new molecule forms dimers and binds to all STAT3 response elements but is not able to activate them. In addition, we added EGFp to the C terminus to visualize this molecule in cells. In C2C12 myotubes, our d.n.STAT3Cβ-EGFPN1 (d.n.STAT3) inhibited C26 CM and LIF-induced STAT reporter activation by 50% (Fig. 4E), similar to the inhibition by PIAS3. Because STAT reporter activation was not affected by STAT1 inhibition and was reversed by 50% with STAT3 inhibition, we studied another STAT and measured the expression of STAT5 and pSTAT5. STAT5 was phosphorylated to a similar extent as STAT1 and STAT3 when treated with C26 CM (Fig. 4F), although STAT5 is expressed at lower levels in C2C12 myotubes. In addition to the effects on STAT3 phosphorylation and STAT reporter activity, STAT3 was required for CM-induced myotube atrophy because d.n.STAT3 expression blocked CM-induced atrophy (Fig. 4G).
JAK2 Activates the STATs in C26 CM-induced Myotube Atrophy—The gp130 family of cytokines, including LIF, activate the STATs by JAK1/2 (24). Therefore, we tested whether the JAK1/2 inhibitor ruxolitinib (27) could block LIF and C26 CM-induced STAT reporter activation and myotube atrophy (Fig. 5). Ruxolitinib (3 μM) completely blocked the STAT reporter activation induced by LIF or by C26 CM (Fig. 5A) and reversed the phosphorylation of STAT3 by C26 CM (Fig. 5B). In addition, ruxolitinib blocked the decrease in myotube diameter induced by C26 CM (Fig. 5C) or LIF (Fig. 5D). Finally, two specific JAK2 inhibitors, AG490 and WP1066, produced complete inhibition of C26 CM STAT reporter activation (Fig. 5E). Therefore, it appears that JAK2 is the predominant STAT kinase active in this system.

ERK Signaling in C26 CM—The ERK pathway has also been implicated in C26 cachexia and is a known signaling arm of LIF (28). Although no reporter system for activated transcription factors in this pathway was available, we tested U0126, a known inhibitor of ERK activation by MEK (28). U0126 at 3 μM was able to inhibit myotube atrophy because of C26 CM (Fig. 6A) or because of LIF (Fig. 6B). The latter was used at the concentration (370 pg/ml) found in C26 CM. Phosphorylation of ERK1 and ERK2 occurred as early as 15 min after C26 CM addition (Fig. 6C). On the other hand, the STAT reporter induced by either LIF or C26 CM was not affected by ERK inhibition (Fig. 6D), and inhibition of JAK by ruxolitinib did not affect the phosphorylation of ERK1/2 by C26 CM (data not shown).

LIF and STAT3 in C26 Cancer-induced Muscle Wasting—We used the d.n.STAT3 plasmid to test whether STAT3 is required for atrophy in muscle fibers of C26 tumor-bearing mice (Fig. 7A). In non-tumor-bearing mice, expression of d.n.STAT3 resulted in 18% hypertrophy in myofiber cross-sectional area, results first reported by Bonetto et al. (12), who used a different STAT3 mutant plasmid (STAT3 Y705F). In C26 tumor mice, there was 24% fiber atrophy compared with control mice. However, C26 tumor mice showed a lack of atrophy in fibers expressing d.n.STAT3 (Fig. 7A), evidenced by the 30% increase in fiber size compared with fibers not expressing d.n.STAT3 and significantly higher than the 18% expected from d.n.STAT3 hypertrophy. We verified the overexpression of d.n.STAT3 by fluorescence microscopy (Fig. 7B) and also by Western blot analysis from plasmid-transduced muscle (Fig. 7C).

Levels of a number of cytokines in the serum from mice with and without C26 tumors were measured using ELISA. LIF (Fig. 7D) and IL-6 (Fig. 7E) increased with growth of the tumors. There was a significant increase in LIF compared with control mice by 19 days post-tumor inoculation (Fig. 7D), whereas IL-6 was only increased significantly at a later time (Fig. 7E). Myo-
statin was quite high in normal mouse serum, as shown previ-
ously (29), and was decreased at later times in C26 tumor-bear-
ing mice during cachexia (Fig. 7F). We did not detect serum
TNFα (data not shown), consistent with what was reported by
Strassmann et al. (14).

LIF can induce IL-6 secretion from muscle because, when
C26 CM was added to C2C12 myotubes, IL-6 was elevated (Fig.
7G). This C26 CM-induced IL-6 secretion was inhibited by an
antibody to LIF. However, the levels of IL-6 produced by myotubes
in response to C26 CM treatment are not high enough to partici-
pate in atrophy because an antibody to IL-6 had no effect on myo-
tube diameter reduction caused by C26 CM (Fig. 3E). Furthermore,
levels of IL-6 up to 2000 pg/ml are insufficient to stimulate
the STAT reporter or to cause myotube atrophy (Fig. 8).

Candidate LIF Target Genes in Myotubes—Quantitation of
increases in mRNAs in response to LIF treatment was per-
formed to identify potential LIF targets genes that could cause
the atrophy phenotype. Results from gene expression microar-
rays from RNA isolated at 4, 8, and 24 h after LIF treatment
served to guide the choice of qPCR candidates (see supplemen-
tary Tables S1 and S2 and GEO accession no. GSE68827). Pre-
viously identified STAT3 target genes found in other cell types
were up-regulated in myotubes treated with LIF (Socs3, Bcl-3, and
C/EBPβ) (Fig. 9). These genes were also up-regulated in
muscle from mice with C26 tumors (21). Socs3, Bcl-3, C/EBPβ,
and Bnip3 were up-regulated at 4 h after a single LIF treatment
and subsided at later times, whereas Casp4 was increased sig-
nificantly at 24 h after LIF treatment (Fig. 9).

Discussion
Experiments have been performed to identify the factors that
trigger skeletal muscle atrophy associated with cancer cachexia,
but treatment remains elusive. In this study, we used an in vitro
system consisting of the conditioned medium from C26 tumor
cells as a basis for a systematic study of the signaling pathways
in muscle. The model was found to simulate several charac-
teristics of cachexia observed in mice with C26 tumors (12, 13, 21)
when we evaluated C26 CM on differentiated C2C12 myotubes
(not shown), surrogates for skeletal muscle in cachectic mice.

From our survey of transcription factor reporters, including
NF-κB, C/EBP, FOXO, SMAD, AP-1, and STAT, we found that
C26 CM activates only the STAT reporter. This is in agreement
with others who have implicated STAT in the transcriptional
changes during C26 cancer cachexia (8, 12, 30), but it is the first
time that a STAT transcriptional assay has been used as evi-
dence. Subsequent to the finding that only STAT was activated
by C26 CM, we found, remarkably, that LIF is the cytokine
stimulating the JAK/STAT pathway in myotubes treated with

FIGURE 4. C26 CM and LIF effects on STAT activation. A, time course of STAT3 phosphorylation from 15 min to 4 h after CM treatment (left panel) and LIF (6
ng/ml) treatment (right panel). B, time course of STAT1 phosphorylation from 15 min to 4 h after CM treatment (left panel) and LIF (6 ng/ml) treatment (right
panel). C, LIF antibody (Ab) inhibits C26 CM-induced STAT3 phosphorylation but not STAT1 phosphorylation. D, C26 CM induction of the STAT reporter is not
inhibited by PIA1 but is attenuated by PIA3. E, d.n.STAT3 expression attenuates the C26 CM- and LIF-induced (370 pg/ml) STAT reporter. F, Western blot of
pSTAT5 in C26 CM-treated myotubes 15 min, 30 min, 1 h, and 2 h after C26 CM treatment. G, d.n.STAT3 expression reverses C26 CM-induced myotube atrophy.
*, p < 0.05 compared with control; †, p < 0.05 compared with CM.
medium from C26 cancer cells. The antibody to LIF was uniquely able to block both STAT-dependent transcriptional activation and myotube atrophy because of C26 CM treatment. Previously, LIF has been suggested as a candidate cachexia factor requiring further study (31). LIF is a known inhibitor of lipoprotein lipase, an enzyme that affects adipose tissue and whose inhibition results in the loss of body fat (32). Circulating LIF was found to be correlated with cachexia when expressed by melanoma (31), and when purified LIF is injected in normal mice, body fat is lost within 2 weeks (33). In our experiments, we show that LIF originates from the C26 tumor and has a direct atrophic effect on skeletal muscle. The requirement of LIF also explains the loss of adipose tissue in C26 cancer cachexia (7, 21).

The production of LIF appears to be widespread among tumors. In one study of 17 human cancer cell lines, 12 were found to secrete LIF (34). We measured LIF in medium from other cancer cell lines described previously as LIF secretors (35).

![Image](http://www.jbc.org/)

**FIGURE 5. JAK inhibitors block C26 CM- and LIF-induced STAT activation and atrophy.** A, ruxolitinib (3 μM) blocked STAT reporter induction by LIF (370 pg/ml) and C26 CM. B, Western blot showing that ruxolitinib blocks C26 CM-induced STAT3 phosphorylation. C, ruxolitinib reversed C26 CM-induced myotube atrophy. D, ruxolitinib reversed LIF-induced atrophy. E, C26 CM induction of STAT reporter is blocked by two JAK2 inhibitors, AG490 (5 μM) and WP1066 (10 μM). *, p < 0.05 compared with control; †, p < 0.05 compared with CM or LIF.

**FIGURE 6. ERK 1/2 in C26 CM and LIF-induced myotube atrophy.** A, the ERK 1/2 inhibitor U0126 (3 μM) blocks the atrophy produced by C26 CM. B, the ERK 1/2 inhibitor U0126 (3 μM) blocks the atrophy produced by LIF (370 pg/ml). C, time course of the appearance of pERK 1/2 induced by C26 CM. D, U0126 does not block STAT reporter induction by LIF or C26 CM. *, p < 0.05 compared with control.
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36) and found it to be elevated. These were A172, 5637, and Ehrlich cells (data not shown). In addition, conditioned medium from these three cancer cell lines produced atrophy in C2C12 myotubes, and the atrophy was blocked with a species-specific antibody to LIF (data not shown). This suggests that LIF is a cachectic factor in other tumor types besides C26.

LIF functions by activating at least two signaling pathways, STAT and ERK (28). We found that these signaling pathways are required for C26 CM and LIF-mediated myotube atrophy. The STAT pathway appears to have a component of STAT3 but not STAT1, which we discovered by differential inhibition of these two molecules. Additional evidence for STAT3 and not STAT1 is that a LIF-blocking antibody reversed the increased phosphorylation of STAT3 but not STAT1 in CM-treated myotubes. Using selective JAK inhibitors, we determined that JAK2 is the signaling pathway activating STAT in our system. However, because JAK2 inhibition with AG490 and WP1066 can abrogate STAT reporter activity but inhibitors of STAT3 are only 50% effective in inhibition, it appears that some other STAT species is also a part of the LIF-STAT reporter activation. We have evidence that STAT5 shows signs of participation because it is phosphorylated by C26 CM treatment. Therefore,
the STAT signaling pathway is activated by LIF in C26 CM, and interference with this pathway using a LIF antibody, JAK2 inhibitors, or STAT3 inhibition blocked atrophy in the C26 cell culture model of myotube wasting. In addition, STAT3 inhibition blocked the muscle wasting associated with C26-induced cancer cachexia.

ERK 1/2 is also required for both C26 CM and LIF-mediated myotube atrophy, but it does not appear to be part of the STAT response, and, therefore, we conclude that these two signaling pathways are required in parallel to produce cachexia with this tumor. In most cases, STAT and ERK are thought to work in parallel when induced by LIF (28). Previously, ERK inhibition was seen to attenuate cachexia in C26 tumor in mice, although the mechanism via LIF had not been determined at that time (11).

Treatment of myotubes with LIF confirms the elevation of several genes already shown to be targets of STAT3, especially Socs3 and Bcl-3 and including C/EBPδ (37). The finding of C/EBPδ is interesting because it has been linked to C26 cancer cachexia in a recent paper (30). Increased expression of Bnip3 and Casp4 is also suggestive of the activities that could produce atrophy of myotubes by LIF. Casp4, being delayed to 24 h from LIF treatment, appears to be induced secondarily to the primary STAT3 targets. It remains to be determined which of these genes contributes to the inhibition of atrophy when STAT3 is blocked.

The concentration of IL-6 in our C26 CM is quite low (≤10 pg/ml), but, in medium from myotubes treated with C26 CM, IL-6 increased to 300 pg/ml. However, this level of IL-6 was unable to contribute to C26 CM-induced atrophy. IL-6 can elicit atrophy in cell culture, but it requires a concentration of at least 10 ng/ml and above (Fig. 8A) (12, 20), which is several times more than found in sera from any reports on C26 tumors (6, 8, 9, 38). The STAT reporter was induced moderately by supraphysiological levels (10 and 100 ng/ml) of IL-6 but not at the pathophysiological level of 2 ng/ml (Fig. 8B). In the literature, it has been shown that the serum level of IL-6 does not correlate with cachexia in C26 cancer (39, 40). The sustained injection of IL-6 alone into mice was not sufficient for inducing cachexia in C26 cancer (39, 41) unless it is at supraphysiological levels (12, 42). The timing of the appearance of LIF and IL-6 in the sera from our cachectic mice suggests that an increase in LIF precedes that of IL-6. The effect of LIF on IL-6 production was shown by the blocking effect of anti-LIF on C26 CM-induced...
IL-6 production by myotubes. Villiger et al. (43) have also shown the ability of LIF to induce IL-6 in chondrocytes.

In a paper in which much work was described in muscle cell culture with C26 conditioned medium (10), myostatin has been shown to be secreted by C26 cells, as determined by ELISA and Western blots from the conditioned medium. In our work, myostatin is not measureable from C26 tumor cells by either ELISA or Western blot except when serum is present in the cell culture medium. Others have used myostatin to produce atrophy in C2C12 myotubes and, it required 3–10 μg/ml (25), 1000 times as much as reported in C26 CM if when it is found there (10). In contrast, in normal mouse sera, we find high levels of myostatin using the same ELISA that we used for the measurement of myostatin in the C26 CM, and these in vivo levels correspond to those reported previously (29). The levels of myostatin are then reduced significantly in the serum of mice with advanced cachexia (Fig. 7F). Therefore, in our case, the atrophy elicited by C26 cells in myotube cell culture and in skeletal muscle in vivo appears to develop without myostatin being a major factor.

Although FOXO is a transcription factor required for C26 tumor-induced muscle wasting (13), the lack of FOXO reporter activation in our C26 CM-treated myotubes indicates that an increase in FOXO activity does not play a primary role in the induction of atrophy but, rather, that the maintenance of active FOXO transcription is required for expression of the proteins that carry out atrophy. In fact, we found that overexpression of d.n.FOXO in myotubes treated with C26 CM blocks myotube atrophy (data not shown).

Cachexia is a major complication of cancer, but it is a complex pathology for which there are no known treatments. In this study, only STAT3-dependent transcription was activated of the six transcription factor families tested, and this led us to the identification of the gp130 cytokine LIF as the trigger of C26-induced wasting of cultured myotubes via the JAK2-STAT3 pathway. LIF is present in secretions from C26 cells at levels that produce myotube atrophy, and LIF is elevated in the serum from C26 tumor-bearing mice. LIF, JAK2, and STAT3 were all necessary for C26-induced atrophy, and LIF is elevated in the serum from C26 tumor-bearing mice.

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