Review

Atrophy and programmed cell death of skeletal muscle

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Striated skeletal is subject to nonlethal cycles of atrophy in response to a variety of physiological and pathological stimuli, including: starvation, disuse, denervation and inflammation. These cells can also undergo cell death in response to appropriate developmental signals or specific pathological insults. Most of the insights gained into the control of vertebrate skeletal muscle atrophy and death have resulted from experimental interventions rather than natural processes. In contrast, the intersegmental muscles (ISMs) of moths are giant cells that initiate sequential and distinct programs of atrophy and death at the end of metamorphosis as a normal component of development. This model has provided fundamental information about the control, biochemistry, molecular biology and anatomy of naturally occurring atrophy and death *in vivo*. The ISMs have provided a good complement to studies in vertebrates and may provide insights into clinically relevant disorders.

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Vertebrate Skeletal Muscle Development and Death

Striated skeletal muscle is the largest tissue in the body and accounts for about 40% of its total mass and 75% of its cellular mass. It also represents the body's primary amino acid reservoir, and as such, is subject to protracted periods of nonpathological atrophy and hypertrophy in response to starvation, activity and development. Skeletal muscle also undergoes cell death as a normal component of development or in response to pathological stimuli.

Skeletal muscles arise during embryogenesis from a pool of lineage restricted precursor stem cells known as myoblasts. Classical embryological studies have demonstrated that myoblasts typically arise in the somites in response to a variety of inductive and repressive signals emanating from the neural tube and floor plate/notochord, as well as the lateral ectoderm and mesoderm.^{1,2} Newer data suggest that myoblasts may also be derived from other tissues.³ Myoblasts migrate to the back or limb buds where they proliferate and assume one of three distinct fates. One subset upregulates a variety of muscle-specific basic helix–loop–helix transcription factors, including MyoD, myocyte enhancer factor-2 and myogenin, and then undergoes terminal differentiation to form multinucleated muscle fibers (Figure 1).^{1,4} *In vitro* studies suggest that these cells also express several survival proteins, including AKT, phosphoinositide-3 kinase and the cyclin-dependent protein kinase inhibitor p21.^{5,6} Expression of these proteins presumably contributes to the extreme apoptosis resistance displayed by myotubes and muscle fibers.

A second subset of myoblasts, termed satellite cells because of their location between the basal lamina and sarcolemma, exit from cell cycle but fail to undergo myogenesis (Figure 1).¹ These cells require the expression of the transcription factor Myf-5 and Pax-7 for differentiation^{1,7} and appear to express the antiapoptotic protein Bcl-2 as their primary survival factor.⁸ Satellite cells are lineage restricted stem cells that can be induced to proliferate in response to focal injury, such as follows extreme exercise.⁹ The progeny of satellite cells can repair damaged muscle, form new muscle fibers or replace themselves. In some diseases, such as Duchenne Muscular Dystrophy, the satellite pool becomes exhausted and the muscles lose the capacity for repair.^{10,11}

Like surplus precursor cells in other lineages, myoblasts that fail to differentiate or express appropriate survival proteins typically undergo programmed cell death by apoptosis (Figure 1).^{12,13} This 'default' to apoptosis may be triggered through TNF receptor TRAIL.¹⁴

Skeletal muscles are very plastic cells in terms of their mass and undergo atrophy in response to a variety of noxious stimuli, including: sarcopenia, denervation, disuse, sepsis, cancer, cachexia, or glucocorticoid treatment.¹⁵ While a variety of different signal transduction pathways control the activation of this process, the gross loss of muscle mass is ultimately mediated by two general mechanisms. The first is the ubiquitin–proteasome system (UPS).¹⁶ Inhibitors of the UPS greatly attenuate muscle protein loss following a variety of insults.¹⁷ Recently, this role was elegantly demonstrated in genetic studies that targeted the RING finger ubiquitin E3 ligases MuRF1 and Atrogin.^{18,19} Loss-of-function mutations in either of these genes is sufficient to reduce muscle atrophy in

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Figure 1 Muscle development and death in vertebrates. Myoblasts are muscle stem cells that migrate to their targets in the trunk and limb buds. At some point during maturation, the cells are forced to choose one of three distinct fates. Some cells become lineage-restricted stem cells known as satellite cells because of their location between the basement membrane and the sarcolemma. These cells express several transcription factors including Pax-7 and Myf-5, as well as the antiapoptotic Bcl-2 protein. A second population of cells fuses to form multinucleated muscle fibers. These cells express the transcription factors MyoD and myogenin and several survival proteins, including p21. A final group of cells fail to either differentiate or activate survival proteins, and then undergo programmed cell death via apoptosis. Muscle fibers can be induced to undergo atrophy in response to a wide variety of stimuli, including glucocorticoids, denervation or starvation. This process is nonlethal and muscle mass can be restored under some circumstances. There is still controversy regarding the apoptotic death of myonuclei within atrophic muscle fibers (denoted by the black nuclei). Muscle fibers can also be induced to die via necrosis, autophagy and apoptosis, depending on the stimulus and developmental programming of the cell

response to a variety of noxious stimuli. A third ubiquitin E3 ligase, NEDD-4, has been shown to facilitate muscle atrophy in response to denervation and unloading, but not from cachexia.²⁰

The second major mechanism responsible for muscle protein loss during atrophy is the autophagy/lysosome pathway.²¹ Autophagy is an ancient survival program that allows cells to literally consume themselves during times of extreme nutrient deprivation.²² During autophagy, double-membrane vesicles known as autophagosomes form around bulk cytoplasm and whole organelles such as mitochondria, and then fuse with lysosomes where the contents of the vesicles are degraded. While the autophagic- and UPS-dependent muscle atrophy programs are distinct, they can be coordinately regulated in cells via the FoxO3 transcription factor.²³

It has been reported that when skeletal muscle undergoes atrophy that a subset of myonuclei undergo apoptosis, even though the rest of the muscle fibers themselves remain intact.²⁴ These data have been used to support the hypothesis that muscle fibers carefully maintain their nuclear-to-cytoplasmic ratio even during atrophy.²⁵ One potential interpretation of these results is that the loss of myonuclei may functionally reduce the abundance of mRNAs encoding muscle proteins, which in turn helps shift the cells from anabolism to the catabolism that accompanies atrophy.¹⁶ This model has been challenged recently in an elegant series of experiments that monitored nuclei in the same muscle fibers, *in vivo*, over the course of up to 1 month following atrophic insults.²⁶ These authors demonstrated that atrophy occurs in the absence of demonstrable myonuclear loss. While they did confirm that atrophy is accompanied by a substantial increase

in apoptosis within the muscle tissue, they provided compelling data that the apoptotic nuclei were derived from mononucleated stromal or satellite cells, but not myonuclei from within the muscle fibers itself.

Although differentiated striated skeletal muscle is very apoptosis resistant, these cells can be induced to undergo apoptosis in response to either toxins such as staurosporin²⁷ or as part of a developmental program, such as the regression of the tadpole tail during amphibian metamorphosis (Figure 1).²⁸ However, necrosis is the normal mechanism of cell death in skeletal muscle that has been exposed to a highly toxic stimulus (Figure 1).

Intersegmental Muscles as a Model for Developmentally Regulated Atrophy and Death

Most of the studies reported above relied on experimental manipulation to induce muscle atrophy or death followed pathological stimuli, such as denervation, immobilization or exposure to toxins. These data provide insight into the kinds of processes that may occur during pathogenesis, but may not fully reflect the endogenous mechanisms that are activated during normal development and homeostasis. One animal model that does not rely on experimental intervention to induce either atrophy or death is the intersegmental muscle (ISMs) of moths. These giant muscle cells undergo a normal hormonally controlled atrophy that results in a 40% loss of muscle mass during the 3 days preceding adult emergence (eclosion).²⁹ More striking is the fact that the cells then die and disappear during a 30-hour period coincident with adult eclosion at the end of metamorphosis (described in detail below).^{30,31} In fact, the term 'programmed cell death' (PCD) was coined in by Richard Lockshin to describe the death of these cells.³⁰

The ISMs arise in the embryo and attach to each of nine of the segmental boundaries within the abdomen to create eight adjacent sheets of muscle. Each individual fiber within the ISMs is about 5 mm long and up to 1 mm in diameter (Figure 2a,c and d). Coordinated contraction of the ISMs allows the caterpillar to crawl, the pupa to defend itself, and the adult moth to extricate itself from the overlying pupal cuticle at the end of metamorphosis. The ISMs are not required for any adult-specific behaviors and subsequently undergo PCD (Figure 2a and b).

Shortly after pupation, the ISMs in the first two and last two abdominal segments initiate PCD and disappear during the next 30-plus hours. The remaining four segments of ISMs, which appear to be anatomically identical to the early dying cells, persist throughout the rest of pupal-adult development. It is unclear why some ISMs die at the beginning of metamorphosis and the remainder at the end, but differential expression of embryonic gap, pair-rule, and/or segment-polarity genes during embryogenesis may provide these groups with unique segmental identities.

The Physiology of Atrophy

Three days before adult eclosion the ISMs initiate a program of atrophy that results in a 40% loss of muscle mass and a 15–20% decline in fiber volume by the time of eclosion.^{29,30,32}

Figure 2 Morphology of ISM death in *Manduca*. The morphology of the ISMs at the light (a, b), scanning electron microscopic (c–e), and transmission electron microscopic (f) levels. The ISMs were examined in the abdomens of newly eclosed adults (a) and 30-hour old adults (b). Three sets of bilaterally symmetric ISMs span four abdominal segments (arrowheads) in the newly eclosed adult but they disappear during the subsequent 30 hours. Other abdominal muscle groups are spared and persist throughout adulthood. The ISMs are composed of large, well-defined muscle fibers (c, d) that rapidly lose mass as they die (e). This death is cell autonomous and the dying fibers are not phagocytosed (f). Ultrastructural analysis of the ISMs during death reveals pyknotic nuclei and numerous autophagic vesicles (arrows) (f). Scale approximately = 5 mm (a, b), 10 μ m (c), 4 μ m (d) and 40 μ m (e) and 2 μ m (f)

Atrophy is not accompanied by changes in muscle physiological parameters such as: resting potential, force/cross-sectional area, tetanic contracture, or sensitivity to intracellular calcium in skinned fiber preparations.³³ There is also no overt change in the stoichiometric levels of the major contractile proteins.^{34,35} These data support the hypothesis that atrophy involves a general increase in protein catabolism rather than the selective loss-specific contractile proteins as has been shown in mammals.³⁶ Supporting this hypothesis is the observed increase polyubiquitin mRNA expression and ubiquitin-dependent proteolysis during the period of atrophy (Figure 3).^{40,41}

Triggers for ISM Cell Death

All known examples of muscle PCD in insects are triggered by hormonal cues.⁴² In *Manduca*, there is a circadian decline in the titer of the insect molting hormone 20-hydroxyecdysone (20E) during the last several days of pupal–adult development that determines the timing of many of the terminal events in metamorphosis, including the onset of atrophy and the initiation of ISM PCD.²⁹ Maintaining an elevated level of 20E is sufficient to delay ISM death indefinitely, while a precocious

drop in the circulating 20E titer can greatly advance the time of death.

The Physiology of Death

ISM death is not just the natural conclusion of the on-going atrophy process, but instead represents a distinct developmental program that induces the destruction of the contractile apparatus.^{29,31,43} Coincident with eclosion, the resting potential declines at a rate of about 2.5 mV/h and the mass at a rate of $\sim 4\%/h$.³³ While the muscles are no longer contractile 15 h later, they still maintain a modest resting potential (~ -30 mV), suggesting that there has been no membrane rupture or necrosis. By 30 h post-eclosion, almost all the muscle mass is gone and the fibers are composed of whorls of internalized membranes and connective tissue.^{43,44}

In mammals, dying cells are almost always phagocytosed by either neighboring cells or circulating macrophages.⁴⁵ This is also the ultimate fate for most dying cells in insects, which tend to be consumed by circulating hemocytes.⁴⁶ Interestingly, dying ISMs are not phagocytosed and do not even attract circulating macrophages, suggesting that they do not produce the normal membrane-associated signals that are

Figure 3 Differential gene expression in the intersegmental muscles. The ISMs begin to atrophy on day 15 of pupal adult development and then initiate programmed cell death coincident with adult eclosion late on day 18. By 5 h post-eclosion the muscles have lost significant mass and physiological function.³³ Most genes are constitutively expressed, like the ubiquitin fusion 80 (Ubf-80) gene, which plays a role in ribosome biogenesis.³⁷ A small number of genes are induced or repressed with the commitment to die. Actin mRNA goes from being one of the most abundant transcripts within the ISMs prior to eclosion to all but disappearing in the dying cells.³⁸ Conversely, expression of the Small Cytoplasmic Leucine Rich Repeat Protein (SCLP) is almost undetectable in the muscles prior to day 18 and then is dramatically induced with the commitment to die.³⁹ Polyubiquitin is transiently induced on day 15, coincident with the onset of atrophy, and then is expressed at very high levels on day 18.⁴⁰ All of the death-associated changes in transcript abundance can be prevented with injection of 25 μ g of 20-hydroxyecdysone (20E) on day 17. D = day of pupal-adult development, hr = hours post-eclosion; 20E = 20-hydroxyecdysone

expressed by most dying cells (Figure 2e).^{43,46} Instead, the ISMs not only commit suicide, they also assume responsibility for the subsequent removal of the corpse. Interestingly, hemocytes do associate with the sarcolemma of some other dying muscles in *Manduca*, where they may function in remodeling the muscle to facilitate the *de novo* formation of new muscle groups during metamorphosis.⁴⁷

The Morphology of Atrophy and Death

The ISMs do not display any of the classic features of apoptosis, including: nuclear condensation, DNA ladders, membrane blebbing, caspase activation, or phagocytosis (Figure 2e).^{43,46,48,49} Instead, ISM death is accompanied by a dramatic increase in the number of both lysosomes and autophagic vesicles rich in cytoplasm and mitochondria (Figure 2f).^{43,50} In some systems, autophagy can serve to protect cells from nutrient deprivation or as a component of subsequent cell death.⁵¹ In the case of the ISMs, the autophagic process continues until the muscles become devoid of identifiable organelles or defined internal structure.³⁵ It is unclear if autophagy just serves to recycle the bulk of the cytoplasm and contractile apparatus or also plays an active role in death. The drug chloroquine, which can inhibit autophagy, inhibited the dissolution of the fibers but not muscle death, suggesting that autophagy may not be a triggering event.⁵² However, the answer to this question awaits the

application of more specific tools, such as the use of RNAi directed against essential autophagic proteins.⁵³

Molecular Mechanisms Controlling Atrophy and Death

The ability of the ISMs to die at the end of metamorphosis requires *de novo* gene expression, and this model provided the first examples of differential gene expression during PCD.^{54,55} Using both molecular and proteomic methods, several genes have been identified that are induced or repressed with the commitment of the ISMs to die (Figure 3, Table 1).

The first cell death-associated gene isolated from the ISMs encoded polyubiquitin.⁵⁶ *Manduca* has two alleles of polyubiquitin, one that encodes 9 head-to-tail repeats of the ubiquitin monomer and a second that encodes 18.⁶⁵ Translation and protein processing of these transcripts provides the cell with a mechanism to rapidly increase the free ubiquitin pool. The induction of polyubiquitin in condemned ISMs is tightly coupled to the cell death process since judicious injection of 20E can delay both cell death and the expression of this polyubiquitin mRNA.⁵⁶ In fact, ISM death is associated with a coordinated increase in all components of the ubiquitin–proteasome pathway, including: ubiquitin, 20S and 26S proteasome subunits, and E2 ubiquitin protein conjugases.^{41,59,66,57} The induction of the ubiquitin–proteasome pathway presumably serves to facilitate protein loss during PCD, especially since the cells are not phagocytosed.⁴⁶ Bayline *et al.*³² have shown that pharmacological inhibition of the proteasome is sufficient to block the initiation of ISM death. As an ubiquitin–proteasome-dependent step is also required for many apoptotic cell deaths,⁶⁷ the loss of cell death inhibitor(s) may be a key step in all PCDs.

Because adult *Manduca* only feed on nectar, the amino acid reserves liberated from the dying cells presumably serve as a valuable resource for the adult. Muscles are also lipid reservoirs with extensive internal membrane systems including the T tubule system and sarcoplasmic reticulum. One of the genes that is dramatically induced when the ISMs become committed to die is apolipoprotein III (ApoIII), a lipid transport protein.⁶⁴ Functional studies have not been performed with the ISMs, but it has been speculated that it helps recycle ISM lipids to the blood for utilization by other tissues. As with all the other cell death-associated transcripts in the ISMs, the expression of ApoIII is regulated by the 20E titer at the end of development.

Some of the genes isolated in the ISM screen encode novel proteins that may function as signal transduction proteins. For example, the newly described Acheron (Achn) protein is dramatically induced with the commitment of the ISMs to die.⁶² In humans and mouse, Achn is expressed in neurons, myoepithelial cells, skeletal muscle and cardiac muscle (C Brown, H Glenn, J Mueller and LM Schwartz, unpublished). Ectopic expression of Achn drives both myotube formation and apoptosis in mammalian myoblasts *in vitro* and zebrafish embryos *in vivo*, while blockade of Achn expression blocks both myogenesis and death (Z Wang, H Glenn, C Brown, J-X Liu, RO Karlstrom, and LM Schwartz, unpublished). Misregulation of Achn also appears to contribute to

Table 1 Differentially expressed genes from condemned manduca ISMs

Process	Gene	Expression	Reference
Proteolysis	Polyubiquitin	Induced	56
	14 kDa E2 ubiquitin conjugase	Induced	41
	18–56 (Sug1) 26S proteasome ATPase	Induced	57
	28.1 kDa subunit catalytic subunit of 20S proteasome	Repressed	58
	S6 (TBP7, MS73) 26S proteasome ATPase	Induced	59
	S6' (TBP1) 26S proteasome ATPase	Induced	58
	S7 (MSS1) 26S proteasome ATPase	Induced	58
	S10b (SUG2) 26S proteasome ATPase	Induced	58
Transcription	E75B	Repressed	60
Translation	eIF1A translation-initiation factor	Induced	60
	Oskar (maternal effect protein)	Repressed	61
Signal transduction	Acheron (putative RNA-binding protein)	Induced	62
	Small cytoplasmic repeat protein (SCLP)	Induced	
	G-coupled-receptor GPR85	Induced	61
	Calmodulin-dependent calcineurin A1 subunit	Induced	61
	Death-associated LIM-only protein (DALP) (insect ortholog of Hic-5)	Induced	63
Metabolism	Apolipoprotein III	Induced	64
	Low molecular wt lipoprotein PBMHPC-23	Induced	61
	Hydroxy acid oxidase 1	Induced	61
Contractile protein	Actin	Repressed	38
	Myosin heavy chain	Repressed	38
	Myosin light chain	Repressed	61
	Myosin regulatory Light chain isoforms 1 and 2	Repressed	61
	Calponin 1	Repressed	61
	Troponin I	Repressed	61

pathogenesis in human mammary ductal carcinomas and myoblast-derived rhabdomyosarcomas (R Shao, H Glenn, S Scully, J Mueller, and LM Schwartz, unpublished). These data suggest that Achn acts at an early point in differentiation and death in muscle and perhaps other tissues.

ISM death is also associated with the repression of the genes encoding actin and myosin heavy chain (Figure 3, Table 1).³⁸ Part of this control presumably reflects transcriptional regulation, since it is coordinated with the *de novo* expression of the cell death associated transcripts described above. The coordinated repression of structural genes and the induction of genes associated with cell death, allows the newly expressed transcripts to represent a greater percentage of the message pool and facilitate rapid muscle degradation.

In addition to transcriptional control, there may also be post-translational control of gene expression.⁶⁸ Analysis of cell-free extracts of *Manduca* ISMs has demonstrated that all mRNAs analyzed displayed a significant reduction in half-life on the day preceding ISM death. This regulation was dependent on sequences present in the 3' untranslated regions of the transcripts and could be transferred to ectopic mRNAs such as β -galactosidase. In the ISMs, actin mRNA stability and translatability may be regulated in part through the AU-rich elements (AREs) present in its 3' untranslated region.⁶⁸ AREs are key regulatory sites and recent studies have demonstrated that the binding of microRNAs to AREs can serve as a switch to both enhance and repress translatability of specific mRNAs.^{69–71} Thus, the regulation of gene expression in the ISMs may be regulated at multiple levels.

Concluding Remarks

Skeletal muscle atrophy and death are major contributors to human morbidity and mortality, and interventions that can block or retard these processes would be of obvious therapeutic value. The intersegmental muscles of moths have proven to be a powerful tool for examining the molecular mechanisms that mediate atrophy and death. In addition to providing the field with its name, the ISMs were the source for the first cloned cell death-associated transcripts and were one of the first models to demonstrate a role of the ubiquitin/proteasome pathway in muscle atrophy and death. Data from the ISMs also support the hypothesis that activation of programmed cell death is coupled to mRNA stability and raises the possibility that this process may be modulated by microRNAs, a largely unexplored area of regulation. Lastly, the ISMs constitute a powerful system for studying the role of autophagy in atrophy and cell death.

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