ANNUAL REPORT
Regional Research Project NC-131
1996

PROJECT TITLE: Molecular Mechanisms Regulating Skeletal Muscle Growth and Differentiation

PROJECT PERIOD: 10-1-95 to 9-30-96

COOPERATING AGENCIES AND PRINCIPAL LEADERS:

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*Denotes voting member of the Technical Committee

PROGRESS OF THE WORK AND PRINCIPAL ACCOMPLISHMENTS:

Objective 1: Characterize the signal transduction pathways that regulate skeletal muscle growth and differentiation.

Several stations reported studies on the function and expression of growth factors, growth factor receptors, and growth factor binding proteins during skeletal myogenesis. Investigators at the Arizona Station have shown that hepatocyte growth factor/scatter factor (HGF/SF) stimulates activation and
early division of adult satellite cells in culture. In addition to being stimulated by HGF/SF, cultured satellite cells also synthesize and secrete this factor, and thus, HGF/SF is a potentially important factor in satellite cell activation during growth.

The South Dakota Station reported the results of studies on avian muscle satellite cells. A comparison of the in vitro characteristics of clonal cultures of myogenic satellite cells derived from the pectoralis major and biceps femoris muscles of Cornish Rock broiler chickens demonstrated that cells derived from these two different muscles have different responsiveness to serum mitogens. In collaboration with the Ohio Station, researchers at the South Dakota Station have characterized satellite cells derived from chickens with the Low Score Normal (LSN) muscle weakness. In comparison to clones from control animals, the LSN clones demonstrated significantly lower percentage of attachment to the substrate, lower proliferation rates, and lower differentiation rates, suggesting that a defect in satellite cell physiology may contribute to the skeletal muscle weakness seen in the LSN line.

Studies at the Minnesota Station focused on the role of the insulin-like growth factor binding proteins (IGFBPs), particularly IGFBP-3, in regulating proliferation and differentiation of porcine muscle cells. Measurements of IGFBP expression in differentiating porcine muscle cell cultures demonstrated a drop in IGFBP-3 mRNA that roughly coincides with differentiation, and a 10-fold increase in IGFBP-3 mRNA levels after differentiation is complete. Treatment of cultures with IGF-I decreased production of BP-3, while treatment with TGF-ß1 increased BP-3 levels. Thus, IGFBP-3 may play a role in the differentiation process, and levels of IGFBP-3 are regulated by growth factors. The Minnesota Station also reported results of studies on the effects of Revalor-S, a combined trenbolone acetate and estradiol implant, on rate of gain, feed efficiency, hormone levels, and satellite cell proliferation in feedlot steers. Results demonstrated higher IGF-I concentrations and mitogenic activity in sera from treated animals, as well as a higher proliferative response of satellite cells isolated from muscle of the treated animals. These differences suggest a potential biological mechanism for the positive muscle growth effects of the implants.

Work reported by the Illinois Station was directed towards examination of the role of cations, particularly zinc, on interactions of the IGFs with their receptors and the IGFBPs. Zinc depresses IGF binding to the IGFBPs secreted by skeletal muscle cells in vitro, resulting in enhanced IGF binding to the cell surface receptor. Physiological levels of zinc (10-20 µM) depress IGF binding to the IGFBPs by 50%, while supraphysiological levels are needed to enhance receptor binding. Thus, the primary role of zinc in the IGFBP-IGF system appears to be to control binding to the BPs. Depressed growth of zinc deficient animals may be in part due to interactions with the IGF system.

The Washington Station reported the development of a defined myogenic and adipofibroblast co-culture system. Two methods were been developed for isolation of adipofibroblasts from sheep skeletal muscle and the cultures were characterized by their response to dexamethasone, insulin, IGF-I, growth hormone, and bFGF. Initial results of studies using co-cultures of satellite cells and 3T3-L1 preadipocytes indicated that growth of preadipocytes is enhanced by the satellite cells, and that preadipocytes induced an increase in IGFBP secretion by the satellite cells. Collaborative efforts with several other stations (Indiana, Illinois, US-MARC, Alabama) are in progress to characterize and utilize this co-culture system.

At the New York Station, animal studies were done to determine if addition of animal protein to the
diet of young, growing steers could increase amino acid availability and improve efficiency of protein utilization. Results demonstrated that addition of animal by-product protein sources to a corn-based, soybean-meal supplemented diet significantly increased N balance and efficiency of N used at all levels added, from 2.6-10.4%. Greater improvement was obtained in lighter (250 kg) than in heavier (425 kg) steers. Thus, use of undegradable intake proteins to balance essential amino acids at the site of adsorption improves efficiency of N use for growth.

Significant progress in studies designed to examine the role of the extracellular matrix in signal transduction pathways in developing muscle was reported by the Ohio Station. In collaboration with the South Dakota Station, studies were done to examine extracellular matrix expression in skeletal muscle of chickens with the Low Score Normal (LSN), muscle weakness in comparison to control animals. Transient elevation in LSN glycosaminoglycan concentration and in transcription of the proteoglycan decorin was observed at 20 days of embryonic development. Although decorin and LSN glycosaminoglycan levels return to control levels by six weeks posthatch, both sarcomere organization and collagen fibril arrangement were altered at six weeks in the LSN animals. Studies are in progress to further characterize these differences. Also in collaboration with the South Dakota Station, synthesis of proteoglycans was characterized in normal chicken satellite cells, demonstrating that these cells produce both decorin and heparan sulfate proteoglycans. Finally, the Ohio Station reported the characterization of the developmental pattern of proteoglycan expression in embryonic turkey pectoral muscle.

To examine the mechanisms by which growth factors stimulate protein synthesis in muscle cells, investigators at the Nebraska Station examined the effects of added compounds on the recruitment of free ribosomes to polysomes in C2C12 muscle cell cultures. Results demonstrated a decrease in polysomes in response to serum deprivation in both myoblasts and myotubes. Insulin, IGF-I, IGF-II, FGF, and PDGF all increased the polysome percentages. This method also provided more consistent results than the use of tritiated tyrosine uptake to measure protein synthesis.

Objective 2: Determine the nuclear mechanisms that control gene expression in skeletal muscle.

Investigators at the Alabama Station examined possible mechanisms for the increase in postnatal muscle growth that occur as a consequence of porcine somatotropin (pST) administration to bred gilts. Gilts were injected twice daily starting at gestation with 8.4 mg of pST. RNA was isolated from embryos at 16, 18, 20, 22, 24 and 28 days of gestation and RNA levels of the differentiation inhibitor gene Id and the myogenic regulatory genes Myf-5 and myogenin were analyzed. Treatment with pST appeared to shift the timing of expression of these key developmental genes in embryos, providing a possible explanation for increases in muscle cell number and muscularity postnatally. In addition, the Alabama Station reported the results of preliminary studies examining the relationship between the abundance and phosphorylation state of the Rb protein, a cell cycle regulator, and response to IGFs in myogenic cell cultures derived from 12-day chick embryos. Results indicated that Rb protein varies in abundance and phosphorylation state with time in culture, and that this relationship can be altered by IGFs.

The California Station reported the results of two studies on the organization of the chicken muscle myosin heavy chain genes. First, a YAC (yeast artificial chromosome) clone containing the fast myosin heavy chain locus was identified. PCR analysis confirmed that this clone contains a 650 kb
insert that encompasses the 3’-untranslated regions for at least 5 fast myosin heavy chain genes. Work is in progress to determine the order of the five genes. Second, a slow myosin genomic locus in the chicken was characterized. Results demonstrate that three myosin heavy chain genes are linked on a microchromosome. In the chicken, there are at least three genes that comprise the slow myosin locus, in contrast to mammals, where there are only two slow myosin genes.

Objective 3: Characterize muscle proteins and their functional domains involved in myofibrillar assembly and disassembly.

In studies on mechanisms of myofibrillar assembly, the California Station is examining assembly of the major myofibrillar protein myosin. Several approaches are being used to identify specific sites or domains on the myosin rod that are important in aggregation and assembly of myosin molecules into filaments, including use of antibodies to specific sites on the rod, characterization of aggregates formed by expressed domains, and characterization of filaments formed by peptides consisting of LMM domains attached to a globular domain. Studies on the assembly of homodimeric myosin molecules, using a model system to study exchange of LMM subunits following guanidinium denaturation, demonstrate that LMM subunits, like full length rods, preferentially renature as homodimers when different isoforms are denatured together. In addition, these investigators have constructed a baculovirus containing a full length adult myosin heavy chain and both light chains, to examine assembly of the molecule in an expression system. Results with this latter system suggest that the subunits are associating, but the myosin is not functional, and that there may be a chaperonin-like activity present in muscle cells that is needed for correct assembly of sarcomeric myosin molecules.

The Wisconsin Station reported results on the study of two myofibrillar proteins, troponin and titin. Troponin C (TnC), the Ca\(^{2+}\)-sensing subunit of troponin responsible for initiating contraction in striated muscle, can be extracted from myofibrils in low ionic strength, Ca\(^{2+}\)-free buffers. Fluorescein labeled TnC, in conjunction with high resolution digital fluorescence microscopy, was used to characterize TnC extraction and TnC binding to native thin filaments. Results showed that TnC extraction was not random, but occurred more readily in the non-overlap region, suggesting that binding of myosin to actin stabilizes TnC binding at low [Ca\(^{2+}\)] and providing new information about the structural organization of the thin filament. Analysis of the cDNA sequence of the giant myofibrillar protein titin demonstrated that several of the immunoglobulin domains of the molecule, from the region located adjacent to the Z-line, are missing key conserved residues in comparison to IgG domains elsewhere in the molecule. These unique, shortened domains may play an important role in the elasticity of the titin molecule. Investigators at the Wisconsin Station also described a new technique utilizing uranyl acetate as a primary fixative for electron microscopy of skeletal muscle. This new method appears to provide for retention of antigenicity after fixation, and thus, should be useful in future structural studies.

Studies related to myofibrillar organization and assembly were also reported by the Iowa Station. In studies on the function of ADP-ribosylation in muscle development, it was demonstrated, by use of antibodies to ADP-ribosylated arginine, that soluble desmin in myotube cultures of chick myogenic cells was ADP-ribosylated. In combination with earlier studies demonstrating that arginine ADP-ribosylation blocks assembly of desmin into intermediate filaments, this work suggests that
ADP-ribosylation could regulate intermediate filament assembly in muscle cells. Studies on the protein talin, which is found at actin attachment sites in cells and is thought to function in cytoskeletal attachment to the membrane in muscle, demonstrated that this protein can crosslink actin into either bundles or networks, depending on conditions. Finally, a cDNA sequence for the protein paranemin was isolated and sequenced. Paranemin is associated with desmin intermediate filaments in embryonic muscle. Analysis of the cDNA sequence demonstrated that this protein contains the rod domain characteristic of intermediate filament proteins, and thus paranemin is a novel intermediate filament protein, which appears to be involved in filament attachment and organization in muscle cells.

In studies on the molecular mechanisms regulating disassembly and degradation of myofibrils, the report from the Arizona Station described their work on the calpain protease system. These researchers have nearly completed mapping of six monoclonal antibodies specific for: (1) µ-calpain-2; (2) m-calpain-1; (3) calpastatin-2; and (4) the 28-kDa polypeptide common to both µ- and m-calpain-1. Based on the resolution to which they have been mapped thus far, the epitopes for these MAbs are in domains 3 and 4 of calpastatin; in domain 6 of the 28-kDa subunit; in domain 3or 4 of m-calpain; and in domains 2 and 3 of µ-calpain. Although these antibodies do not have any marked effects on calpain activity, they should provide useful structural probes. In addition, studies are in progress to isolate the protein form of skm-calpain. This calpain isoform was identified in 1989 as an mRNA that exists solely in skeletal muscle, but the protein form has not been identified or isolated. To identify the skm-calpain protein, the Arizona station has elicited several monoclonal and polyclonal antibodies to two peptide sequences predicted from the cDNA sequence. Characterization of these antibodies is in progress.

The Nebraska Station has developed capillary electrophoretic methods for the separation and quantitation of 3-methylhistidine and 4-hydroxyproline in muscle. The 3-methylhistidine protocol is capable of determining levels of this amino acid in as little as 1 mg of muscle tissue or 0.5 mg of myofibrils from cell cultures. In addition, a capillary electrophoretic method was developed for sensitive determination of 4- and 3-hydroxyproline in muscle and collagen tissues. In combination, these protocols provide a sensitive method for measurement of myofibrillar and connective tissue protein turnover in small samples, such as from biopsy or cell culture.

USEFULNESS OF FINDINGS:

The results summarized in this progress report represent fundamental, basic research of direct relevance to animal agriculture. The overall goal of this project is to increase the efficiency of lean meat production in domestic animals through the use of basic research into the biological mechanisms that regulate muscle growth and differentiation. Such basic research is absolutely essential for development of applied methods for improving meat production in animals. This overall goal fits squarely within national priorities for research in animal agriculture.

The committee continues to be highly productive, as evidenced by the number of publications resulting from this work. This report covers the first year of a five-year renewal of the NC-131 project. The approach and objectives of the project were substantially revised for this renewal, to emphasize the use of molecular techniques and approaches. The progress reported suggests that this approach has been successful. Significant progress was made in all areas, but particularly in understanding the molecular mechanisms involved in growth factor regulation of muscle differentiation, as described
under Objective 1. Collaboration, through the sharing of techniques, culture models, antibodies, molecular probes, and expertise in different areas, continues to be an important part of the project. Overall, the work summarized in this report provides a significant contribution to our understanding of the molecular mechanisms regulation muscle growth.

WORK PLANNED FOR NEXT YEAR:

Studies will continue as described in the project proposal. Some changes in project personnel and participation by specific stations have occurred since the project proposal was approved. Dr. Sandra G. Velleman has moved from the Connecticut Station to the Ohio Station. Thus, the Connecticut Station is no longer a participant, and the work outlined for the Connecticut Station will be done at the Ohio Station. There is no participating investigator at the Missouri Station, as Dr. David E. Gerrard has moved to the Indiana Station, where he will continue to participate in this project. The representative from the Michigan station, Dr. Werner G. Bergen, is now at Auburn University. The majority of key personnel remain with the project, and no changes in the approach or objectives are needed.

PUBLICATIONS FOR THE YEAR:

Refereed Journal Papers

Published Full-Length Articles (45)

California


Illinois


Iowa


Minnesota


**Nebraska**


**New York**


**Ohio**


**South Dakota**


**USDA - US-MARC, Clay Center**


Washington


Wisconsin


**Published Abstracts (40):**

**California**


**Illinois**


**Iowa**


**Minnesota**


**Nebraska**


**New York**


**Ohio**


**South Dakota**


**USDA - US-MARC, Clay Center**


**Washington**


**Wisconsin**


**Accepted Full-Length Articles (16):**

**California**


**Iowa**


**Illinois**


**Nebraska**


**New York**


**Ohio**


**South Dakota**


**Washington**


**Non-Refereed Publications**

*Proceedings, Book Chapters, etc.* (9):

**Arizona**


**Iowa**


**Nebraska**

Chu, Q., B.T. Evans and M.G. Zeece. 1996. Quantitative separation of 4-hydroxyproline from bovine skeletal muscle collagen by micellar electrokinetic capillary electrophoresis. Presented at Frederick Capillary Electrophoresis Conference, October 21-23, Frederick, MD.

**New York**


124-136.

Ohio

Velleman, S.G., C.S. Coy and R. Whitmoyer. 1996. The avian low score normal genetic muscle weakness alters skeletal muscle organization and transcript levels of the proteoglycan decorin. XVth FECTS Meeting, Munich, Germany.

South Dakota


Washington


Theses and Dissertations (3):

Iowa


APPROVED:

_________________________________________________
Dr. Ted W. Huiatt, Chair of Technical Committee, 1995-96 Date

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Dr. Robert Steele, Administrative Adviser Date