Changes in Expression of Proteolytic-Related Genes in Chick Myoblasts during Myogenesis

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Changes in expression of proteolytic-related genes in chick myoblasts during myogenesis were investigated. Expression of MyoD and myogenin, as an index of differentiation, was regulated in chick myoblasts during myogenesis. Expression of PAX7, as an index of myoblast proliferation, was decreased during myogenesis. Expression of ubiquitin, atrogin-1/MAFbx, proteasome C2 subunit, m-calpain large subunit, cathepsin B, and caspase-3 mRNA of chick myoblasts was also decreased during myogenesis. These results show that the expression of proteolytic-related genes of myoblasts was decreased during myogenesis, resulting in an increase in differentiation of chick myoblasts.

Key words: atrogin-1/MAFbx, myoblasts, myogenesis, differentiation, chicken


Introduction

Muscle cells undergo a distinct and well-characterized series of biochemical and morphological changes during the process of differentiation (myogenesis). The formation of skeletal muscle during embryonic development involves myoblast differentiation and fusion to multinucleated muscle fibers. Myoblast differentiation and fusion can be studied in myoblast cultures, employing primary myoblast cultures or myoblast cell lines (Wakelam, 1985). These events are correlated with each other and regulated by various intracellular mechanisms that mediate subsequent events, such as reorganization of cytoskeletal structure, synthesis of new protein, and destruction of old protein (Fulton et al., 1981). Since proteolysis may play a critical role in the cell cycle, intracellular proteases may regulate cell growth and differentiation. Differentiation of skeletal muscle involves withdrawal of undifferentiated myoblasts from the cell cycle, fusion into multinucleated myotubes, and coordinate induction of muscle-specific gene products (Florini et al., 1991).

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which could play an important role in muscle atrophy. Intracellular proteolysis is carried out by lysosomal and nonlysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpains, i.e., cysteine proteases in the cytosol, are the main agents of nonlysosomal Ca²⁺-dependent proteolysis, which occurs within the myofibril and can carry out the initial step in myofibrillar proteolysis (Goll et al., 1991, 1992). Cathepsins, i.e., the main agents of lysosomal degradation, have been well established as contributing to muscle protein breakdown (Hall-Angeras et al., 1991). Lysosomal proteases degrade sarcoplasmic proteins and release myofibrillar proteins (Lowell et al., 1986). Recently, caspase-3, an apoptotic protease, has been shown to be an initial trigger of accelerated muscle proteolysis in catabolic conditions (Du et al., 2004). The major proteolytic pathway involves an ubiquitin-proteasome system, which is ubiquitous throughout the body, is dependent on ATP, and degrades ubiquitin-conjugated proteins via the 26S proteasome (Glickman and Ciechanover, 2002). In skeletal muscle, the ubiquitin-proteasome system is also the main proteolytic pathway for overall proteolysis (Lecker et al., 1999). Studies with animals have consistently demonstrated that protein degradation by the ubiquitin-proteasome system is increased in muscle undergoing atrophy (Mitch and Goldberg, 1996; Dehoux et al., 2003; Dehoux et al., 2004; Lecker et al., 2004). Evidence suggests that atrogin-1/MAFbx, an E3 ubiquitin ligase, play a pivotal role in muscle atrophy (Gomes et al., 2001; Bodine et al., 2001). Atrogin-1/MAFbx is expressed only in skeletal muscle. Its expression is increased under catabolic conditions that result in muscle atrophy (Gomes et al., 2001; Bodine et al., 2001; Dehoux et al., 2003, Dehoux et al., 2004). However, the role of the expression of proteolytic-related genes in chick myoblasts during myogenesis has not been studied.
Materials and Methods

Cells were isolated from the thigh muscle of 13-day-old chick embryos (Nakashima et al., 2005). Briefly, the muscle tissue obtained from the embryos was digested with dispase (Invitrogen Life Technologies, Carlsbad, CA). The cell suspension was transferred to a 100-mm uncoated culture dish to allow fibroblast attachment. The cells were counted and plated onto gelatin-coated six-well plates (IWAKI SCITECH, Tokyo, Japan) at a density of 2.0×10^5 cells/well. The cells were cultured in M-199 medium containing 15% calf serum (Invitrogen Life Technologies, Carlsbad, CA) and 2.5% chicken embryo extract (growth medium). The cells were grown at 37°C in a 5% CO₂-enriched atmosphere of humidified air. The medium was replaced every other day for a 7 day incubation period.

Total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's directions. Complementary DNA was synthesized from of the total RNA using random hexamer primers (TaKaRa, Tokyo, Japan) and ReverTra Ace (TOYOBO, Tokyo, Japan). Real-time PCR primers were designed (software Primer3, http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi) for chicken MyoD, 5’-GGC TTT GGA GGA GAA GGA CT-3’-5’-CAG AGT GCT GCG TTT CAG AG-3’ (NM_204214); chicken myogenin, 5’-GGC TTT GGA GGA GAA GGA CT-3’-5’-CAG AGT GCT GCG TTT CAG AG-3’ (NM_204184); chicken Pax7, 5’-GCA TCA AAT TCG GGA AGA AA-3’-5’-CTC TTC AAA GGC AGG TCT GG-3’ (NM_205065); chicken ubiquitin, 5’-CGC ACC CTG TCT GAC TAC AA-3’-5’-GCC TTC ACG TTT TCA ATG GT-3’ (X02650); chicken atrogin-1/MAFbx, 5’-CCA ACA ACC CAG AGA CCT GT-3’-5’-GGA GCT TCA CAC GAA CAT GA-3’ (NM_00103956); chicken proteasome C2 subunit, 5’-AAC ACA CGC TGT TCT GGT TG-3’-5’-CTG CGT TGG TAT CTG GGT TT-3’ (AF207978); chicken m-calpain large subunit, 5’-ACA TCA TCG TGC CCT CTA CC-3’-5’-GAG ATC TCT GCA TCG CTT CC-3’ (D38026); chicken cathepsin B, 5’-CAA GCT CAA CAC CAC TGG AA-3’-5’-TCA AAG GTA TCC GGC AAA TC-3’ (U18083); chicken caspase-3, 5’-TGG CGA TGA AGG ACT CTT CT-3’-5’-CTG GTC CAC TGT CTT CGT CTT CA-3’ (AF083029); and chicken GAPDH, 5’-CCT TCT TGG CAA AGT CCA AG-3’-5’-CAT CTG CCC ATT TGA TGT TG-3’ (K01458). The levels of mRNA were measured by real-time PCR analysis using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) and the QuantiTect SYBR Green PCR system (Qiagen, Tokyo, Japan). The expression of GAPDH expression was measured as an internal control.

Data were analyzed by Student’s t-test. A p value of < 0.05 was considered statistically significant. Results were expressed as means±standard error (SE).

Results and Discussion

In this study, changes in expression of proteolytic-related genes in chick myoblasts during myogenesis were investigated as a first step for clarifying the mechanism for differentiation. The cultured muscle cell undergoes proliferation, followed by fusion to form postmitotic multinucleated myotubes. The formation of multinucleated myotubes was substantially observed by phase-contrast microscopy on days 1–7, as shown in Fig. 1 A–G. The cell cultures from chick embryo thigh muscle were prepared. After plating and incubating for 24–48 h in growth medium, the cultures consisted of cells which exhibit the morphology of myoblasts (Fig. 1 A and B). The myoblasts progressively exhibited the differentiated morphology characterized by fusion the cells to form myotubes on day 3–7 (Fig. 1 C, D, E and F, arrows). The cultures were fully formed myotubes on day 7.

Changes in expression of MyoD, myogenin, and PAX7

Fig. 1. Morphological changes of chick myoblasts from embryonic chick thigh muscle during myogenesis.

After plating, chick myoblasts cultured in growth medium (M-199 medium containing 15% calf serum and 2.5% chicken embryo extract) for 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F) and 7 (G) days. Arrows indicate chick myotubes.
mRNA in chick myoblasts during myogenesis were investigated. The mRNA levels of MyoD, myogenin, and PAX7 are shown in Fig. 2 patrs A, B, and C, respectively. MyoD mRNA expression (A) was significantly increased on day 3 (p<0.05), but on days 4–7, it was significantly decreased (p<0.01). Myogenin mRNA expression (B) was significantly increased on days 3, 4, 5, 6, and 7 (p<0.01). PAX7 mRNA expression (C) was significantly decreased during myogenesis (days 2–7, p<0.01). These data indicate that the chick myoblast provides a good model for the study of muscle cell differentiation. Skeletal muscle differentiation is characterized by the terminal withdrawal of myoblasts from the cell cycle, activation of muscle-specific gene expression, and cell fusion into multinucleated myotubes. The myogenic transcription regulatory factors MyoD and myogenin play essential roles in the differentiation of myogenic precursor cells to skeletal muscles (Zammit et al., 2004). MyoD is required for the determination of myogenic cell lineage and is expressed in the proliferating, undifferentiated myoblasts. The appearance of myogenin is associated with cell cycle withdrawal and the onset of terminal differentiation and fusion to multinucleated muscle fibers. PAX genes encode transcription factors that have important and highly conserved roles during development. In skeletal muscle, PAX7 genes have overlapping but nonredundant roles in the specification of embryonic progenitors and network with the myogenic transcription regulatory factors comprising MyoD and myogenin (Zammit et al., 2004; Halevy et al., 2004). PAX7 is expressed almost ubiquitously by quiescent satellite cells and is co-expressed with MyoD in their proliferating myoblast progeny (Zammit et al., 2004; Halevy et al., 2004). PAX7 is expressed by postnatal satellite cells or their progeny, but is down-regulated during myogenic differentiation (Zammit et al., 2004; Halevy et al., 2004).

Changes in the ubiquitin-proteasome proteolytic pathway during the myogenesis of chick myoblasts are shown in Fig. 3 A-C. The mRNA levels of ubiquitin (A), atrogin-1/MAFbx (B), and proteasome C2 subunit (C) were examined. Ubiquitin mRNA expression (A) was significantly decreased during differentiation (day 2, p<0.01, days 3–7 p<0.01). The mRNA expression of atrogin-1/MAFbx (B) was significantly decreased during myogenesis (days 2–7, p<0.01). Proteasome C2 subunit mRNA expression (C) was also significantly decreased during myogenesis (days 2–4, p<0.05, days 6–7, p<0.01). These results indicate that the suppression of atrogin-1/MAFbx expression plays an important role in the myogenesis of chick myoblasts. Changes in the expression of atrogin-1/MAFbx mRNA during myogenesis (differentiation) in skeletal muscle cells have not previously been reported. The present study demonstrates for the first time that expression of ubiquitin, atrogin-1/MAFbx and proteasome C2 subunit mRNA was decreased during the myogenesis of chick myoblasts. Changes in expression of ubiquitin-proteasome proteolytic-related genes regulate the morphological and biochemical events of myogenesis and may be key to the role of ubiquitin and the proteasome. Other researchers have also investigated aspects of the ubiquitin-proteasome proteolytic pathway in muscle development and differentiation. Ahn et al. (Ahn et al., 1991) reported that proteasome substrates (chymotrypsin- and trypsin-like peptidases) degrading ability of the 20S proteasome decreased during chick embryo development. Ebisui et al. (Ebisui et al., 1995) also reported that differentiated C2C12 myotubes contained a lower expression of proteasome subunits C2, C8, S4, and S7 mRNAs compared to that of myoblasts. Ueda et al. (Ueda et al., 1998) also reported that the expression of proteasome subunits of C2, C8, and S7, but not S4, was decreased during differentiation of L8 myoblasts. These data are consistent with our results. Our results show that the expression of
Results are expressed as ratios relative to the expression of GAPDH in 1 day incubation of chick myoblasts, whose expression level was taken to be equal to 1 and reflects the means ± SE (n = 5–6). Student’s t-test was performed, and the results were significantly different from that of day 1 (**p < 0.01, *p < 0.05).

Changes in the mRNA levels of calpain, cathepsin, and caspase during the myogenesis of chick myoblasts are shown in Fig. 3 D-F. The mRNA expression of m-calpain large subunit (D) was significantly decreased during myogenesis (days 2, 3, and 4–7, p < 0.05). The mRNA expression of cathepsin B (E) was significantly decreased during myogenesis (day 2, p < 0.05, days 3–7, p < 0.01). The mRNA expression of caspase-3 (F) was also significantly decreased on days 2–7 (p < 0.01). The present study demonstrates for the first time that expression of m-calpain large subunit, cathepsin B, and caspase-3 mRNA was decreased during the myogenesis of chick myoblasts.

It has been reported that calpain inhibitors prevent myoblast fusion and expression of creatine kinase activity (differentiation marker) (Kwak et al., 1993b; Ueda et al., 1998). In another report (Kwak et al., 1993a), m-calpain activity increased during the myogenesis of cultured chick...
muscle cells but µ-calpain was undetectable. Ebisui et al. (Ebisui et al., 1994) also investigated the role that calpains play in myogenesis, and there is an agreement that calpain plays an important role in the process. Our data also support these studies by showing that calpain participates with the ubiquitin and the proteasome in the process of myogenesis.

In the process of myogenesis, involvement of cathepsins B, L, H, and D has been reported (Kirschke et al., 1983; Colella et al., 1986; Bechet et al., 1991; Ebisui et al., 1994, 1995), though their physiological roles remain unclear. In fetal calf muscle, it appears that the low amount of cathepsin B mRNA probably accounts for the low concentrations of active cathepsin B (Bechet et al., 1991). We also observed that expression of cathepsin B mRNA was decreased during early growth in the chick skeletal muscle of hatched chicks (unpublished data). This is consistent with data obtained with C and L lines (Colella et al., 1986) and calf muscle cell cultures (Bechet et al., 1991) showing that cathepsin B mRNA was changed during myogenesis. Fernando et al. (Fernando et al., 2002) reported that caspase-3 is required for the differentiation of myoblasts into myofibers but apparently is sufficient for this differentiation. Because the mechanisms that control muscle protein degradation in birds have not been explored, the relative importance of alternative pathways involving ubiquitin conjugation, the calpain system, and lysosomal degradation are unknown. Although caspase-3 was identified previously in birds (Johnson and Bridgham, 2000), no assessment of enzyme activity or expression during myogenesis was reported. In the present study, we observed that the expression of caspase-3 mRNA, as well as calpain and cathepsin mRNA, was decreased during the myogenesis of chick myoblasts.

The degradation of muscle proteins constitutes an important regulatory mechanism in the process of muscle growth (Goll et al., 1992). Sacheck et al. (Sacheck et al., 2004) reported that suppression of atrogin-1/MAFbx expression was associated with a marked increase in muscle growth in C2C12 myotubes, and our results agree with those findings. We also showed previously that the expression of proteolytic-related genes was decreased during early skeletal muscle growth (myogenesis) after the hatching of chicks (unpublished data). Thus, an important component of muscle growth is the transcriptional suppression of proteolytic-related genes in the muscle growth (myogenesis) of chicks.

In conclusion, this study showed that the expression of proteolytic-related genes of myoblasts was decreased during myogenesis, resulting in an increase in differentiation in chick myoblasts. However, a limitation of our study is that results were derived only from cultured cells, which do not directly indicate the condition of skeletal muscle in ovo. Myogenesis in ovo will be investigated in future studies.

References


