Myogenic and nonmyogenic cells differentially express proteinases, Hsc/Hsp70, and BAG-1 during skeletal muscle regeneration

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Skeletal muscle is an adult, postmitotic tissue that retains the ability to repair and regenerate. Regardless of the type of injury, skeletal muscle regeneration is characterized by an intense remodeling of extracellular matrix (ECM) proteins and intracellular protein networks. Complete rebuilding of skeletal muscle during regeneration may thus require a tight regulation of proteinase expression.

Matrix metalloproteinases (MMPs), tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA) are important enzymes involved in the degradation and remodeling of ECM proteins (9, 11). Plasminogen activators (PAs) cleave plasminogen to plasmin. Plasmin then degrades several ECM components and extracellularly activates several pro-MMPs and pro-uPA, thus generating a self-maintaining feedback mechanism of pro-uPA and plasminogen activation (45, 46). Ca$^{2+}$-dependent (calpains) and -independent (lysosomal cathepsins and ubiquitin-proteasome) proteolytic pathways are major systems for muscle protein degradation. Ubiquitous calpains (calpains 1 and 2) have been shown to be involved in the limited proteolysis of myofibrillar proteins such as α-actinin and desmin (31, 54) but are also required for myoblast fusion during in vitro myogenesis (3, 4). Lysosomal cathepsins and ubiquitin-proteasome pathways, whose functions during muscle atrophy and catabolic states have been largely documented (30, 58, 62), are also involved in myoblast proliferation and differentiation (8, 18). Overall, these studies suggest that MMPs, PAs, and Ca$^{2+}$-dependent and -independent proteolytic pathways may contribute to the remodeling of skeletal muscle during regeneration.

The regulation of these proteolytic pathways is also important to consider. For example, molecular chaperones of the 70-kDa heat shock protein family, the 73-kDa constitutive heat shock cognate protein (Hsc70), and the stress-inducible 72-kDa heat shock protein (Hsp70) regulate the fate of aberrantly folded or damaged proteins (38). Interestingly, Hsc/Hsp70 function is regulated by the cochaperone Bel-2-associated gene product-1 (BAG-1), which acts as a coupling factor between Hsc/Hsp70 and proteasome (41). Furthermore, BAG-1 also exerts an inhibitory function on Hsc/Hsp70 chaperone activity (48, 59). Expression of...
Hsc/Hsp70 and BAG-1 may thus contribute to the regulation of proteasomal proteolysis during skeletal muscle regeneration.

Myogenic cells (degenerating myofibers, myoblasts, regenerating myofibers, and growing mature myofibers) and nonmyogenic cells (inflammatory cells) are involved in the different stages of skeletal muscle regeneration and may thus participate in the expression of proteinases (17, 20, 49). In the present study, an immunohistochemical analysis was first performed to assess the relative proportion of myogenic and non-myogenic cells over the course of muscle regeneration. In the light of these data, we then determined the impact of muscle regeneration on the expression of extracellular (MMP-2 and -9 and PAs) and intracellular (calpains 1 and 2, cathepsins B and L, and proteasome) proteinases. To gain further insight into the biological function of Hsc/Hsp70 and BAG-1 during muscle regeneration, their protein contents were also examined.

**MATERIALS AND METHODS**

**Animal care.** Animal procedures were approved by the Ministère de l’Agriculture et de la Forêt. This study was carried out on 44 male Sprague-Dawley rats (278 ± 2 g), as previously described (17). Briefly, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt), and a 0.75% solution of bupivacaine (600 μg/l) was injected into the tailis anterior (TA) muscle. The contralateral muscle was injected with a 0.9% saline solution. TA muscles were removed at 3, 5, 7, 10, 14, 21, and 35 days postinjection (n = 5–7/group). A first portion of TA muscle was embedded in cryopreservative (Cryomount, HistoLab), quickly frozen in isopentane, and stored in liquid N2 for subsequent histochemical and immunohistochemical analyses. A second portion was frozen in liquid N2 for subsequent biochemical analyses.

**Protein extraction.** Total proteins were extracted from powdered muscles, as previously described (22), and stored at −80°C. Concentrations were spectrophotometrically measured at 750 nm using the Bio-Rad Protein Assay.

**Immunohistochemical analyses.** Transverse muscle sections (10 μm) were cut in a cryostat microtome at −20°C and hydrated in buffer T (0.05 M Tris, 0.145 M NaCl, pH 7.5) for 5 min. Transverse sections were then blocked for 1 min at room temperature in buffer T-0.3% (vol/vol) BSA supplemented with 23% (vol/vol) rat serum. The following primary antibodies were used for immunohistochemical analyses: desmin [1:50 (vol/vol), DAKO, D33]; α-actin [1:50 (vol/vol), Sigma, AC-40]; and developmental/neonatal myosin heavy chain [MHCd/n; 1:20 (vol/vol), Novocstra, RNMy2/92D]. Primary antibodies were incubated at room temperature for 40 min. Rabbit anti-mouse IgG [1:20 (vol/vol), DAKO, P0161] conjugated to horseradish peroxidase was used for the detection of proteins (DAKO, DAB Tablet). Transverse muscle sections were then counterstained with hematoxylin. Desmin immunostaining was used to quantify the area characterized by the presence of myoblasts, desmin-negative mononucleated cells, and desmin-negative and desmin-positive myofibers. MHCd/n immunostaining was used to quantify the area characterized by the presence of regenerating myofibers. All analyses were performed with computerized image analysis systems (ImageTool 3.0 and NIH Image 1.61).

**Histochemical staining of acid phosphatase.** Transverse muscle sections (10 μm) were incubated in 57 mM sodium acetate, 35 mM sodium 5,5-dimethylbarbiturate, 1 mM 7-bromo-3-hydroxy-2-naphthoic-anisidine phosphate, 10% (vol/vol) dimethylformamide, 18 mM sodium nitrite, and 5 mM pararosanilin, 0.1 N HCl (pH 5) for 45 min at 37°C. Transverse muscle sections were then counterstained with hematoxylin.

**Enzyme activities.** MMP-2/9 enzyme activities (EC 3.4.24.24 and EC 3.4.25.35) were fluorometrically determined (λexc = 280 nm and λem = 670 nm) as previously described (47), with some modifications (5). PA activities (EC 3.4.21.73 and EC 3.4.21.68) were fluorometrically determined (λexc = 380 nm and λem = 460 nm) in the presence of benzoyloxy carbonyl-Gly-Gly-Arg-7-amido-4-methylcoumarin (Bachem, I-1140) as substrate (68). Proteins (10 μl) were added to 970 μl of 60 mM imidazole buffer (pH 7.4) containing 0.1% Triton X-100. The reaction was started by the addition of 200 μM substrate. Calpain 1/2 activities (EC 3.4.22.17) were fluorometrically measured (λexc = 380 nm and λem = 460 nm) using N-Succinyl-Leu-Tyr-7-amido-4-methylcoumarin (Bachem, I-1355) as substrate (55). Five microliters of protein extract were added to a buffer assay (60 mM imidazole, 2.5 mM β-mercaptoethanol, 2% dimethyl sulfoxide (vol/vol), 2% methanol (vol/vol), pH 7.3) supplemented or not with 5 mM CaCl2. The reaction was started by the addition of 250 μM of substrate. Calpain 1/2 activities represented the difference between samples to which CaCl2 was present and samples without CaCl2. Cathepsin B/L activities (EC 3.4.22.1 and EC 3.4.22.15) were fluorometrically measured (λexc = 380 nm and λem = 460 nm) with 10 μl of protein extract in 980 μl of 100 mM sodium acetate buffer (pH 9.0) (18). The assay was started by the addition of 10 mM benzoyloxy carbonyl-Phe-Arg-amido-4-methylcoumarin (Bachem, I-1160). Chymotrypsin-like enzyme activity of 20S proteasome (EC 3.4.25.1) was fluorometrically measured (λexc = 380 nm and λem = 460 nm) with 10 μl of protein extract in 980 μl of 60 mM imidazole buffer (pH 7.4) (18). The reaction was started by the addition of 100 μM fluorogenic peptide succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Bachem, I-1395).

**Immunoblotting.** Aliquots of protein (30 μg/lane for Hsc/Hsp70 and 60 μg/lane for BAG-1) were mixed with a loading buffer [62.5 mM Tris, pH 6.8, 5% glycerol (vol/vol), 1% SDS (vol/vol), and 2.5% β-mercaptoethanol (vol/vol)], boiled for 3 min, applied to a 12.5% (wt/vol) SDS-PAGE, and electrophoresed at 115 V for 4 h at 4°C. Separated proteins were electrotransferred for 1 h at 4°C onto nitrocellulose membranes. Membranes were then blocked in TBS-5% milk (wt/vol) solution at room temperature for 1 h. The following primary antibodies were used for immunoblotting: Hsc/Hsp70 (1:2,500 dilution NeoMarkers, W27) and BAG-1 (1:250 dilution, Santa Cruz Biotechnology, C16). Primary antibodies were incubated at room temperature for 2 h. Rabbit anti-mouse IgG [1:1,000 (vol/vol)] conjugated to horseradish peroxidase was used for chemiluminescent detection of proteins (ECL, Amersham). The films were scanned and quantified using NIH image 1.61.

**Statistical analyses.** All values are presented as means ± SE. One-way ANOVA was used to assess the effects of bupivacaine on desmin and MHCd/n expressions. Two-way ANOVA was used to evaluate the effects of bupivacaine as a function of time on MMP-2/9, PAs, calpains 1/2, cathepsins B/L, proteasome, Hsc/Hsp70, and BAG-1 protein levels. Post hoc comparisons were performed with Fisher’s protected least significance difference test. Individual means were com-
pared with a paired t-test. Differences were considered to be statistically significant at the 0.05 level of confidence.

RESULTS

Myogenic and nonmyogenic cells in regenerating TA muscles. The muscles analyzed in this study had already been used in a previous experiment, and data on the effects of bupivacaine over the complete course of skeletal muscle regeneration have been published (17). To assess the relative proportion of myogenic and nonmyogenic cells in these regenerating muscles, we performed desmin and α-actin immunostainings (Fig. 1). Desmin-negative mononucleated cells were largely represented at days 3–7 (Fig. 1B). Numerous desmin-positive mononucleated cells were also observed between days 3 and 10 and illustrated the presence of myoblasts (7). Intense desmin expression was particularly evident in myoblasts and newly formed myotubes in relation to myofibers (Fig. 1A). Interestingly, myoblasts were frequently observed at the periphery of disjointed fibers, which expressed desmin weakly or were desmin negative (Fig. 1, C and D). Furthermore, these rounded cells were characterized by low myonucleus content as illustrated by hematoxylin staining. Desmin-negative myofibers were thus considered as degenerating myofibers. Another interesting feature was the circular organization of myoblasts (Fig. 1, E and F), which disappeared after myoblast fusion (Fig. 1G). A different pattern of expression was observed with α-actin (compare Fig. 1, D and H). Although all

Fig. 1. Desmin and α-actin immunostainings in regenerating tibialis anterior (TA) muscles. Frozen muscle sections were immunostained for desmin (A–G) and α-actin (H) and counterstained with hematoxylin. Micrographs are representative of muscle cross sections observed on day 3 (A–F, H) and day 7 (G). The bundle organization of myofibers was preserved (A, B, and E). Serial muscle sections stained for desmin (D) and α-actin (H) indicate that desmin-negative fibers are α-actin positive. Bar, 50 μm.
muscle fibers expressed α-actin, its expression was low in myoblasts.

Desmin immunolabeling was used to quantify the areas occupied by myoblasts and desmin-negative mononucleated cells (Fig. 2, A and B). From 3 to 7 days, each cell population represented ~30% of the whole muscle cross-sectional area. These cell populations then rapidly declined and were not observed anymore after 2 wk of regeneration. The area of desmin-negative myofibers was also quantified (Fig. 2C). Desmin-negative myofibers, which represented ~30% on day 3, were no longer observed after 10 days of regeneration. Finally, the area of desmin-positive myofibers was progressively restored to represent >90% of muscle cross-sectional area by day 10 (Fig. 2D).

**MHCd/n expression in regenerating TA muscles.** To detect the presence of regenerating myofibers, MHCd/n immunostainings were also performed (15). MHCd/n expression was mainly observed in small bi/tri-nucleated muscle cells on day 3 (Fig. 3A), whereas only a minority of mononucleated muscle cells expressed MHCd/n. As the process of regeneration occurred (days 5–7), MHCd/n expression became inhomogeneous and was observed in larger myofibers with central myonuclei (Fig. 3, B and C). These growing myofibers then progressively lost MHCd/n immunoreactivity. Once quantified (Fig. 3D), our data indicated that MHCd/n expression progressively increased to peak on day 7 (P < 0.05) and was no longer detectable on day 14.

**MMP-2/9 and PA enzyme activities.** MMP-2/9 enzyme activities were significantly increased about two-fold on day 3 and remained elevated until day 14 (Fig. 4A). Activities then returned to control level by day 21. A similar profile was observed for PA enzyme activities (Fig. 4B). Activities remained significantly increased between days 3 and 14 and returned to control level at 21 days postinjection.

**Calpain 1/2, cathepsin B/L, and proteasome enzyme activities.** Ca²⁺-dependent proteolysis of calpains 1/2 was increased by about threefold between days 3 and 7 (Fig. 5A). Enzyme activities returned to control level on day 14. Cathepsins B/L and proteasome enzymes, markers of Ca²⁺-independent intracellular proteolysis, were also assayed. A dramatic 22-fold increase in cathepsins B/L enzyme activities was observed on day 3 (Fig. 5B). Activities were then markedly reduced to reach values nonsignificantly different from controls on day 14. Histochemical detection of acid phosphatase, a marker of lysosomal activity, stained myoblasts, inflammatory cells, and small regenerating myofibers (Fig. 5C). Proteasome enzyme activity displayed a different pattern (Fig. 5D). Activity peaked at 3 and 5

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**Fig. 2.** Quantification of myogenic and nonmyogenic cells in regenerating TA muscles. Desmin immunostaining was used to quantify the area occupied by myoblasts (A), desmin-negative mononucleated cells (B), desmin-negative myofibers (C), and desmin-positive myofibers (D). Myoblasts, desmin-negative mononucleated cells, and desmin-negative myofibers were not present on days 14, 21, and 10, respectively. Data are expressed as the percentage of whole muscle cross-sectional area. Values are means ± SE (n = 4–5/group). †P < 0.05, ††P < 0.01, and †††P < 0.001, significantly different from day 3.

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days of regeneration ($P < 0.01$) and then progressively returned to control level by day 21.

**Hsc/Hsp70 and BAG-1 protein levels.** The protein contents of the molecular chaperones Hsc/Hsp70 and BAG-1, which regulate the fate of aberrantly folded or damaged proteins toward proteasomal degradation, were also determined. Hsc/Hsp70 protein level remained statistically unchanged over the entire course of the regeneration process (Fig. 6A). In contrast, BAG-1 expression was modified during muscle regeneration. Two bands corresponding to molecular masses of $\sim 32$ kDa and $\sim 44$ kDa were detectable in contralateral rat skeletal muscles (Fig. 6A) and were identified as the small and large isoforms of BAG-1 [BAG-1S and BAG-1L, respectively (60); see also **DISCUSSION**]. BAG-1S protein level was significantly increased from 3 to 14 days postinjection (Fig. 6, A and B) and returned to control level by day 21. No BAG-1L isoform was detectable until 21 days of regeneration, whereas it was systematically present in contralateral muscles (Fig. 6A).

**DISCUSSION**

The aim of the present study was to determine the impact of muscle regeneration on the expression of extracellular and intracellular proteinases. Previous studies indicated that numerous cell populations (inflammatory cells, myoblasts, and myofibers) participated in skeletal muscle remodeling (17, 49, 50) and may thus contribute to the expression patterns of proteinases observed in the present study. To determine the relative proportion of these cells over the time course of regeneration, we first performed an immunohistochemical study based on the analysis of desmin, $\alpha$-actin, and MHC/d/n expressions. Next, we tentatively assigned the expression patterns of proteinases, Hsc/Hsp 70, and BAG-1 to the different cell populations.
Our immunohistochemical data clearly indicated the presence of desmin-negative fibers, desmin-negative mononucleated cells, myoblasts, and regenerating and growing mature myofibers during the first few days following bupivacaine injection (days 3–14). Desmin immunolabeling indicated the presence of desmin-negative myofibers. Such a negative staining was not observed when α-actin immunolabeling was performed with the same secondary antibody (compare Fig. 1, D and H), showing that our observations were not due to experimental drawbacks. They rather suggest that desmin proteolysis had occurred in these fibers (23), supporting the hypothesis that desmin-negative fibers were degenerating myofibers. These remnant myofibers, which represented 25–30% of muscle cross-sectional area on day 3, were not observed anymore on day 10. Desmin-negative mononucleated cells were considered as inflammatory cells. These cells have been shown to include a majority of macrophages (50). Concomitantly, myoblasts were also equally represented (30% on days 3–7). One intriguing feature was the circular organization of myoblasts, suggesting that the presence of basal lamina was required to initiate the reconstruction of myofibers (7). Furthermore, myoblasts strongly expressed desmin, suggesting that the formation of a desmin network may be required for the subsequent formation of myofibrils (10, 66). In agreement with this hypothesis, MHCd/n expression was temporally delayed and first observed in bi/tri-nucleated cells. Furthermore, MHCd/n immunoreactivity appeared punctuated, suggesting the formation of myofibril bundles (Fig. 3, A and C). The progressive disappearance of MHCd/n expression revealed the recovery of motor innervation and the replacement of MHCd/n by mature myosin isoforms (19, 52). Furthermore, the heterogeneity of the MHCd/n expression pattern could be indicative of differences in the reinnervation timing between muscle fibers. From day 14, growing mature myofibers were the main constituent of skeletal muscle (90–100%).

On the basis of the aforementioned observations, the twofold increase in MMP-2/9 and PA enzyme activities could be ascribed to inflammatory cells, myoblasts, and regenerating myofibers (days 3–7). MMP-2/9 and PAs, which are known to degrade ECM components such as fibrin and collagens (9, 11), may exert different functions during muscle regeneration. First, migration of inflammatory cells to the damaged area depends on fibrinolysis by uPA-dependent plasmin activity (39, 57). Similarly, in vitro myoblast migration also depends on MMP-2/9 activities (37) and PA system through interactions among uPA receptor, uPA regulator (plasminogen activator inhibitor-1), and vitronectin (12, 13, 21, 64). Second, MMPs and PAs may also participate in the proteolytic maturation of growth factors necessary for satellite cell activation and myo-
nóstains were visible in inflammatory and myogenic mononucleated cells (69). Ubiquitous calpain 2 has previously been shown to be required for myoblast fusion during muscle cell differentiation (3, 4). Particularly, calpains would be involved in the destabilization of membrane-cytoskeleton and membrane-ECM interactions (6). In the present study, the time course changes observed for myoblasts and regenerating and mature growing myofibers are consistent with this hypothesis.

Cathepsins are endopeptidases responsible for intracellular degradation of damaged proteins and organelles (14) and phagocytosis of extracellular debris. Alteration of cathepsin activities is a hallmark of a set of experimental and pathological situations associated with muscle wasting (16, 58, 62). Here, we report a new example of a condition under which cathepsin B/L enzyme activities are dramatically altered (22-fold increase) in skeletal muscle. A recent report (20) indicated that inflammatory cells expressed mainly lysosomal enzymes in response to local trauma. In agreement with that study, our histochemical analysis of lysosomal activity (acid phosphatase staining) showed that inflammatory cells were stained positively, whereas desmin-negative fibers did not react with the dye. Overall, these data suggest that inflammatory cells contributed strongly to the elimination of muscle cellular debris (days 3–7). However, acid phosphatase staining also indicated that myoblasts and small regenerating myofibers contributed to the synthesis of cathepsins B/L, supporting a role for these proteases in myoblast fusion and maturation (days 5–14) (8, 18, 26).

Targeted protein degradation by the ubiquitin-proteasome pathway is another dominant mechanism involved in myofibrillar protein degradation (2, 30). However, proteasomal proteolysis may also be involved in the control of myoblast proliferation through the selec-

blasts may be involved in myoblast fusion (13, 21, 57). Later (days 10–14), regenerating fibers and growing mature myofibers may constitute the main source of MMP-2/9 and PAs, suggesting that ECM remodeling may be required for muscle fiber enlargement. Furthermore, these enzymes may also be involved in the revascularization and reinnervation of skeletal muscle (27, 28, 35, 61a).

Ca$^{2+}$-dependent ubiquitous proteinases (calpains 1/2) are generally considered to be involved in the limited proteolysis of myofibrillar proteins such as α-actinin, desmin, nebulin, talin, titin, and vimentin (31, 54). Bupivacaine causes severe perturbation of calcium homeostasis (40, 56) associated with hypercontractions of muscle fibers and disruption of plasmalemma membranes (29, 53), which lead ultimately to rapid muscle fiber necrosis (27, 40). The increase in calpain 1/2 activities observed on day 3 is consistent with the aforementioned considerations. The presence of desmin-negative myofibers may thus reflect the proteolytic activity of calpains 1/2. The time course changes in calpain 1/2 activities, which closely paralleled those reported for myoblasts and inflammatory cells, suggest that both cell populations contributed to the expression of ubiquitous calpains during skeletal muscle regeneration. In agreement with our data, a previous study showed that ubiquitous calpain immu-

Fig. 7. Correlation between changes in proliferating cell nuclear antigen (PCNA) expression and proteasome enzyme activity. Values are means ± SE (n = 5–7/group). Solid line illustrates hypothesized relationship between PCNA protein level and proteasome activity following bupivacaine injection ($r = 0.991; P < 0.001$).
tive degradation of MyoD (1, 61, 63) and regulatory proteins of the cell cycle such as cyclins [25], for reviews see Refs. 24 and 67]. This latter hypothesis raised the possibility that a correlation existed between the change in proteasome activity and a marker of cell proliferation. We previously measured on these muscle samples the expression of proliferating cell nuclear antigen (PCNA) (17), a cofactor of DNA polymerase-δ synthesized in S-phase. Therefore, the mean PCNA protein level was plotted against the mean increase in proteasome activity (Fig. 7). A strong positive relationship between PCNA protein level and proteasome activity was observed ($r = 0.991; P < 0.001$). It should be noted that no correlation was obtained when PCNA protein level was plotted against the other enzyme activities. Although we cannot totally exclude that the increase in proteasome activity may be involved in the degradation of damaged myofibrils and in the immune response of inflammatory cells (24), this correlation suggests strongly that proteasome activity may be functionally linked to the regulation of myoblast proliferation.

The 73-kDa constitutive Hsc70 and the stress-inducible 72-kDa Hsp70 are required to regulate the fate of aberrantly folded or damaged proteins and thus have the folding capacity of Hsc/Hsp70 (34, 42). This is consistent with the huge demand on protein synthesis, which occurs during muscle regeneration. Time course changes in Hsp40 and Hsc70-interacting protein, which are known to regulate Hsc/Hsp70 activity (36, 43), would be necessary to further elucidate the functions of BAG-1S during muscle regeneration. The expression pattern of BAG-1L was more restricted, such that BAG-1L expression was not detectable before day 21 of regeneration. A different function may thus emerge from the expression of BAG-1L and could be related to the late maturation of muscle fibers. Many other cellular functions could potentially be attributed to BAG-1, such as protection from cell death as well as the regulation of growth factor receptor activity (51, 65). More studies are clearly needed to elucidate the complex physiological functions of BAG-1 isoforms during muscle regeneration.

In summary, the present study points to several new important events of skeletal muscle regeneration. Our report documents profound adaptations in proteolytic metabolism and BAG-1 expression, which can be partly explained by the changes in the relative proportions of myogenic and nonmyogenic cells observed during muscle regeneration.

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