Hyaluronic acid, HAS1, and HAS2 are significantly upregulated during muscle hypertrophy

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Calve S, Isaac J, Gumucio JP, Mendias CL. Hyaluronic acid, HAS1, and HAS2 are significantly upregulated during muscle hypertrophy. Am J Physiol Cell Physiol 303: C577–C588, 2012. First published July 11, 2012; doi:10.1152/ajpcell.00057.2012.—Hyaluronic acid (HA) is a component of the extracellular matrix (ECM) in most vertebrate tissues and is thought to play a significant role during development, wound healing, and regeneration. In vitro studies have shown that HA enhances muscle progenitor cell recruitment and inhibits premature myotube fusion, implicating a role for this glycosaminoglycan in functional repair. However, the spatiotemporal distribution of HA during muscle growth and repair was unknown. We hypothesized that inducing hypertrophy via synergist ablation would increase the expression of HA and the HA synthases (HAS1–HAS3). We found that HA and HAS1–HAS3 were significantly upregulated within the plantaris muscle in response to Achilles tenectomy. HA concentration significantly increased 2.8-fold after 2 days but decreased towards levels comparable to age-matched controls by 14 days. Using immunohistochemistry, we found the colocalization of HAS1–HAS3 with macrophages, blood vessel epithelia, and fibroblasts varied in response to time and/or tenectomy. At the level of gene expression, only HAS1 and HAS2 significantly increased with respect to both time and tenectomy. The profiles of additional genes that influence ECM composition during muscle repair, tenascin-C, type I collagen, the HA-degrading hyaluronidases (Hyal) and matrix metalloproteinases (MMP) were also investigated. Hyal1 and Hyal2 were highly expressed in skeletal muscle but did not change after tenectomy; however, indicators of hypertrophy, MMP-2 and MMP-14, were significantly upregulated from 2 to 14 days. These results indicate that HA levels dynamically change in response to a hypertrophic stimulus and various cells may participate in this mechanism of skeletal muscle adaptation.

SATELLITE CELLS ARE MUSCLE progenitor cells that play a central role in regenerating diseased or injured skeletal muscle tissue. Engraftment of muscle progenitor cells as a therapy to increase the regeneration of damaged muscle tissue has shown some success in animal models, but the technique has been hindered by limited survival and migration of the implanted cells (8). A complex set of intrinsic and extrinsic factors regulate satellite cell activity (51). Studies on the regulation of satellite cell activity have predominantly focused on signaling molecules and cytokines, and little is known about the role that the extracellular matrix (ECM) plays in controlling satellite cell function. As the ECM within damaged muscle undergoes remodeling during the repair process, it is likely signals from the ECM also significantly contribute to the ability of transplanted satellite cells to functionally restore diseased tissue (10, 51).

Mature myofibers and associated satellite cells are enclosed within a mesh-like basal lamina predominantly composed of type IV collagen and laminin (49). Force generated by the myofibers is transmitted through the basal lamina to the surrounding connective tissues via a network of type I and III collagen fibrils (30). Capillaries, nerves, and other supporting cells reside between the myofibers within the interstitial space filled with hyaluronic acid (HA), fibronectin, and assorted proteoglycans (24, 42). When muscle is damaged as a result of direct trauma or contraction-induced injuries, there is a transient upregulation of key ECMs that are expressed only at low levels in mature muscle such as tenascin-C (TN-C) and heparan sulfate proteoglycans (12, 36). TN-C is a hexameric glycoprotein that, in homeostatic tissues, is typically restricted to areas of high loading, such as tendons and myotendinous junctions and is hypothesized to add mechanical stability (27). However, TN-C is upregulated within injured skeletal muscle and thought to decrease cellular adhesion, promote migration and inhibit premature fusion (10, 36). While the fibrillar collagens are critical for structural support, their dysregulation during repair for muscle can lead to fibrosis and scar formation, as seen in muscular dystrophy (50).

Instrumental in maintaining the fine balance between fibrosis and repair are the matrix metalloproteinases (MMPs), enzymes that facilitate the remodeling and homeostasis of connective tissues by breaking down components of the ECM. MMP-2, -9, and -14 are the three main MMPs expressed in skeletal muscle and are regulated by two of the tissue inhibitors of metalloproteases, TIMP1 and TIMP2 (11). Disruption of ECM synthesis or MMP activity has been shown to dramatically affect functional skeletal muscle repair (21, 40, 41, 50), highlighting the important role that ECM composition has in controlling cellular behavior.

HA may also play a role during muscle repair since it has been shown to upregulate myoblast migration and reversibly suppress differentiation in vitro (10, 20, 31). In vivo, the content and distribution of HA in skeletal muscle have been characterized for different mammalian systems (4, 32, 42, 44). While exercise did not induce an immediate increase in intramuscular HA in humans (44), the content within rat skeletal muscle was found to increase in response to 1 wk of immobilization (42), indicating that the dynamics of HA distribution during tissue remodeling remains unclear.

HA is a large, linear glycosaminoglycan found within the ECM of most vertebrate tissues and is highly expressed during development, wound healing, and regeneration (10, 55). Due to the combination of a net anionic charge with a stiffened random coil structure, HA has a large hydrodynamic volume...
that facilitates the influx of water, regulating tissue hydration (18). In mammals, HA polymerization is carried out by three plasma membrane-bound hyaluronic acid synthases, HAS1, HAS2, and HAS3 (55). These transmembrane enzymes assemble HA on the cytosolic side via the alternate addition of the sugars N-acetyl-D-glucosamine and D-glucuronic acid and subsequently extrude the polysaccharide through the plasma membrane (55). HAS isoforms vary in enzymatic activity; HAS1 and HAS2 synthesize chains of molecular masses up to 4 MDa, while HAS3 generates chains shorter than 300 kDa and is more active than HAS1 and HAS2 (23). Degradation of HA is primarily carried out by the hyaluronidases, six of which have been identified in the mouse genome, Hyal1–Hyal5 and PH-20 (16). Hyal1 and Hyal2 are the main mammalian hyaluronidases and are expressed by the majority of soft tissues investigated (17). Studies (7, 14, 35, 56) have shown both HAS and hyaluronidase enzymes to be differentially expressed in developing, homeostatic and diseased tissues, but their distribution in skeletal muscle remains poorly characterized.

HA has been used as a therapeutic tool to enhance repair in damaged tissues since it is a biocompatible hydrogel and is indispensable during embryonic development (2, 45). When incorporated into engineered scaffolds designed to replace damaged tissues of the musculoskeletal system, HA has been shown to support tissue ingrowth and incorporation into the host (2, 46). However, the biological action of HA in these systems has not been well described. Future implementation of HA-based scaffold design for skeletal muscle regeneration can only be enhanced once the underlying mechanisms controlling HA synthesis and the biological response have been characterized during native repair in vivo. We hypothesized that the induction of muscle hypertrophy in response to a strong mechanical growth stimulus would transiently increase the expression of HA and HAS1–HAS3 within murine plantaris muscles. To investigate how HA deposition is regulated during skeletal muscle remodeling, we utilized a synergist ablation model of mechanical load-induced growth.

Hypertrophic growth in response to synergist ablation is primarily due to the fusion of myogenic precursors with existing muscle, resulting in the increase of myofiber diameter (37). Before progenitor cells can successfully fuse with intact myofibers, the tightly woven basal lamina needs to be broken down. To gain insight into how the integrity of the ECM is regulated at the enzymatic level during hypertrophy, we determined the expression pattern of Hyal1–Hyal4 and MMP-2, -9, and -14, and two of the MMP inhibitors, TIMP1 and TIMP2. In addition, we investigated the temporal response of TN-C and type I collagen, matrix components that should be differentially regulated during muscle repair.

### MATERIALS AND METHODS

**Animals.** Adult male C57BL/6 mice were housed in specific pathogen-free conditions and fed standard laboratory chow and water ad libitum. All experiments were approved by the University of Michigan Committee on the Use and Care of Animals. To induce hypertrophic growth in the plantaris muscle, mice were anesthetized with isoflurane and bilateral Achilles tenectomies were carefully performed as previously described (3). After tenectomy, mice were allowed to recover, and at either 2, 7, or 14 days after the surgery, plantaris muscles were harvested from both limbs. Age-matched mice that did not undergo surgery were used as controls. Four mice were utilized for each group (control and tenectomy) at each time point for a total of 24 animals. Immediately after removal, the right plantaris muscle from each mouse was embedded in Tissue-Tek OCT (Sakura, Torrance, CA), frozen in isopentane cooled in liquid N2, and stored at −80°C until processed for immunohistochemistry. The left muscles were weighed and finely minced with scissors, and half was placed in T-PER tissue protein extraction solution + 1% halofate protein inhibitor (ThermoScientific, Rockland, IL) to measure HA content and half into QIAzol lysis reagent (Qiagen, Valencia, CA) for mRNA isolation. To compare the expression of HAS enzymes in skeletal muscle to tissue with known HAS expression, samples from muscle, tendon, testes, liver, lung, heart, and skin were isolated from a control mouse after plantaris muscle harvest and were placed in QIAzol for mRNA isolation.

**Immunohistochemistry.** OCT-imbedded specimens were sectioned at 10 μm on a cryostat, fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 5 min, permeabilized in 0.1% Triton X-100 (Sigma) in PBS for 5 min, and blocked with 10% donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min. Sections were incubated with primary detection reagents (Table 1) diluted in 0.2% BSA (Sigma) in PBS for 1 h and then with the appropriate secondary detection reagents diluted 1:300 in 0.2% BSA in PBS for 30 min at room temperature [Alexa Fluor (AF) 488 anti-rat (Invitrogen, Grand Island, NY), AF546 anti-rabbit, AF546 streptavidin, AF647 anti-goat, and AF647 anti-rabbit; all raised in donkey and DAPI to label nuclei]. Antibody specificity was determined by incubating sections with both primary and secondary reagents, only primary reagents, or only secondary reagents that were then imaged using the same parameters to assess for the presence of any background signal. In addition, HAS1, HAS2, and HAS3 specificity was confirmed by staining cryosections of mouse lung tissue, which has been reported to express all three HAS enzymes (14) (data not shown). Slides were mounted with Gel-Mount (Electron Microscopy Sciences) and imaged using a Zeiss Axioplan 200M outfitted with the ApoTome system (Carl Zeiss, Thornwood, NY).

The colocalization of HAS and specific cell types was quantified using ImageJ (National Institutes of Health, Bethesda, MD) on ≥ 3 images for each HAS-cell combination (average = 225 cells/image). For CD31 positive vessels, colocalization was only counted if the HAS label distinctly overlaid the vessel lumen.

**HA quantification.** Minced plantaris muscles in T-PER were homogenized for 10 s immediately after harvest with a Qiagen TissueRuptor. Total protein content was determined using the BC-X protein assay (G-Biosciences, St. Louis MO). Samples were diluted to a final concentration of 150 μg protein/ml, and HA content was determined using a competitive ELISA per the manufacturer’s instructions (Echelon Biosciences, Salt Lake City, UT).

**RNA isolation and quantitative PCR.** Minced plantaris muscles in QIAzol were homogenized for 10 s immediately after harvest and RNA was isolated using a miRNA mini kit (Qiagen). Total RNA

<table>
<thead>
<tr>
<th>Table 1. Primary detection reagents used for immunohistochemistry</th>
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<tbody>
<tr>
<td>Probe</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>HAS1</td>
</tr>
<tr>
<td>HAS2</td>
</tr>
<tr>
<td>HAS3</td>
</tr>
<tr>
<td>HABP-biotinylated</td>
</tr>
<tr>
<td>Tenascin-C</td>
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<tr>
<td>Laminin α-2</td>
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<tr>
<td>F4/80</td>
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<tr>
<td>CD31</td>
</tr>
<tr>
<td>FSP-1 (S100A4)</td>
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<td>m-Cadherin</td>
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HAS, hyaluronic acid synthase; HABP, hyaluronic binding protein.
was quantified using a NanoDrop (ThermoScientific), RNA from each sample was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For each quantitative PCR reaction, cDNA generated from 3 ng of RNA was combined with primers for genes of interest (Table 2) and SsoFast EvaGreen reagents (Bio-Rad) in a CFX96 real-time thermal cycler (Bio-Rad) using a program of 3 min at 95°C to activate the enzyme, followed by 40 cycles with 10 s denaturing at 95°C and 30 s annealing at 55°C. The presence of single amplicons was validated by performing a melt-curve analysis. Relative copy number and PCR efficiencies were calculated using the linear regression of efficiency method (47, 48), which is based on a four-parameter sigmoidal model that captures the dynamics of amplification efficiency for each individual reaction (34).

For endpoint PCR, cDNA was amplified using GoTaq Green reagents (Promega, Madison, WI) using a program of 2 min at 95°C for initial denaturing, 30 s at 95°C to denature then 1 min at 55°C to anneal and 2 min at 72°C to extend for 35 cycles and a final extension for 5 min at 72°C. Reactions were then ran out on a 2% agarose gel and visualized using a FluorChem SP imaging system (Alpha Innotech, Santa Clara, CA).

**Table 2. Primers used for quantitative PCR**

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
<th>Size, bp</th>
<th>Ref Seq</th>
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<td>HAS3</td>
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<td>Hyl2</td>
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<td>Hyl3L</td>
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<td>Hyl4L</td>
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<td>CCATCAAATCGGATAGCG</td>
<td>151</td>
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Statistical analysis. One-way ANOVA, followed by Bonferroni’s post hoc analysis (α = 0.05), was used to determine the influence of time on HAS-cell type colocalization. Two-way ANOVA, followed by Bonferroni’s post hoc analysis (α = 0.05), was used to assess the effects time and tenectomy on HA content and gene expression within individual primer sets using Prism 5.0 (GraphPad Software, La Jolla, CA).

**RESULTS**

To investigate how the ECM of the murine plantaris muscle remodels in response to compensatory overload, a tenectomy of the Achilles tendon was performed. This tenectomy completely unloaded the gastrocnemius and soleus muscles, leaving the much smaller plantaris muscle as the only remaining ankle plantarflexor. Plantaris muscles harvested 2, 7, and 14 days post-tenectomy were compared to control muscle (Fig. 1). Hyaluronic acid (HA) and tenascin-C (TN-C) were upregulated during compensatory hypertrophy in the mouse plantaris muscle. A and A’: in cross-sections of control muscles, HA (indirectly labeled with hyaluronic acid binding protein, red) was expressed at low levels and TN-C (green) was restricted to the aponeurosis (a). B and B’: 2 days after Achilles tenectomy, the epimysium became enriched with HA (arrowheads), and both HA and TN-C appeared to infiltrate the basal lamina surrounding individual myofibers (white = laminin). C and C’: HA and TN-C were ubiquitous within the interstitial space after 7 days. D and D’: by 14 days, HA and TN-C expression had decreased within the muscle body, whereas the epimysium was still strongly labeled for HA compared with the control (A’). Blue = DAPI; bar = 200 μm; ×10.

**Fig. 1.** Hyaluronic acid (HA) and tenasin-C (TN-C) are upregulated during compensatory hypertrophy in the mouse plantaris muscle. A and A’: in cross-sections of control muscles, HA (indirectly labeled with hyaluronic acid binding protein, red) was expressed at low levels and TN-C (green) was restricted to the aponeurosis (a). B and B’: 2 days after Achilles tendonectomy, the epimysium became enriched with HA (arrowheads), and both HA and TN-C appeared to infiltrate the basal lamina surrounding individual myofibers (white = laminin). C and C’: HA and TN-C were ubiquitous within the interstitial space after 7 days. D and D’: by 14 days, HA and TN-C expression had decreased within the muscle body, whereas the epimysium was still strongly labeled for HA compared with the control (A’). Blue = DAPI; bar = 200 μm; ×10.
days after Achilles tenectomy showed a 44 ± 15, 57 ± 16, and 82 ± 41% increase (respectively) in muscle wet weight over age-matched controls, consistent with previous reports (26). The distribution of HA in plantaris muscles was assessed using hyaluronic acid binding protein (HABP) as an indirect probe. In cross-sections of control muscles, individual myofibers were surrounded by a basal lamina rich in laminin (Fig. 1A, white). HABP (red) identified low levels of HA in the interstitial space between myofibers and around the whole muscle within the epimysium (Fig. 1, A and A', arrowheads). TN-C (green) was restricted to the aponeurosis (Fig. 1, A and A'). After 2 days of overload, identification of HA via HABP staining was significantly enhanced within both the epimysium (arrowheads) and interstitial space (Fig. 1, B and B'). TN-C was also upregulated within the interstitial space and by 7 days was found surrounding the majority of myofibers throughout the cross-section (Fig. 1, C and C'). After 14 days of compensatory overload, the distribution of HABP and TN-C was more restricted, although both were still highly expressed within the plantaris muscle compared with controls (Fig. 1, D and D').

To confirm our immunohistochemical observations that HA was upregulated in response to Achilles tenectomy, the HA content of plantaris muscles was quantified using a competitive ELISA (Fig. 2). In plantaris muscles overloaded for 2 days, the HA content was 2.8-fold higher than the age-matched controls (53.7 ± 19.9 ng HA/µg protein; Fig. 2). After 7 days, HA content decreased but was still significantly higher than controls (39.1 ± 3.4 vs. 16.7 ± 2.5 ng HA/µg protein). By 14 days, HA content was not different between control and overloaded muscles (34.8 ± 11.3 vs. 23.0 ± 4.6 ng HA/µg protein). Two-way ANOVA indicated that both tenectomy and time significantly influenced HA content of plantaris muscles, and Bonferroni’s post hoc analysis revealed that the amount of HA in overloaded muscles after 14 days was significantly less than at 2 and 7 days. Together, our immunohistochemical and biochemical data show that HA deposition is significantly enhanced in skeletal muscle after both 2 and 7 days of compensatory overload and is downregulated towards control levels after 2 wk.

To determine which cells have the potential to contribute to the synthesis of HA, we utilized antibodies raised against HAS1, HAS2, and HAS3. Cross-sections of 2-day-overloaded plantaris muscles were stained for HAS1–HAS3 (red) and cell-specific markers (green): anti-F4/80 for macrophages (19), anti-CD31 for capillary endothelia (13), anti-FSP-1 for activated fibroblasts (54), and anti-m-cadherin for activated muscle satellite cells (15). F4/80+ macrophages were predominantly found in the epimysium (Fig. 3, A–C, right of dotted line) after 2 days and colocalized with HAS1 and HAS2 staining. Cells associated with the periphery of CD31+ blood vessels occasionally stained for HAS1 (Fig. 3D, arrowhead). Notably, HAS3 colocalized with the endothelium of many blood vessels (Fig. 3F, arrowhead). FSP-1 expression overlapped with the three isoforms and was most pronounced with HAS1 (Fig. 3, G–I). M-cadherin+ satellite cells were also colabeled with all three synthases (Fig. 3, J–L).

Quantification of cell-specific marker colocalization with HAS1–HAS3 revealed the dynamic expression of these iso-
HAS isoforms are differentially colocalized in response to hypertrophic overload. A–C: percentage of cells labeled with HAS1 and HAS2 significantly increased at all time points, whereas HAS3 only modestly increased at 2 days. D–F: colocalization of F4/80+ macrophages with HAS1–HAS3 significantly increased in response to hypertrophy with the maximum achieved at 7 days. G–I: CD31+ cells predominantly colocalized with HAS3. J–L: FSP-1+ cells showed little change in HAS2 and HAS3 overlap, while HAS1 colocalization was significantly enhanced in response to tenectomy (G). M–O: muscle satellite cells showed no variation in HAS isoform codistribution. One-way ANOVA was performed on the colocalization of HAS isoforms by distinct cell populations and the following combinations were significantly affected by tenectomy: A–H and J (\( P < 0.05 \)). Bonferroni’s post hoc test was utilized to assess significant differences in HAS with respect to time (\( * P < 0.05 \)). Values are presented as means \( \pm \) SD. M–O: codistribution was determined by analyzing \( n \geq 9 \times 20 \) images with ImageJ; A–L: \( n \geq 3 \times 20 \) images.
forms within the muscle interstitium in response to tenectomy (Fig. 4). Overall, we found that the total number of cells that colocalized with HAS1 and HAS2 significantly increased at all time periods investigated, with a maximal 9.7- and 4.8-fold increase over control expression at 7 days (Fig. 4, A and B). In contrast, HAS3 expression only modestly increased after 2 days of compensatory overload and declined to control values thereafter (Fig. 4C). In the control mice, there were very few macrophages in each image (n = 0–1) and none colocalized with the HAS (Fig. 4, D and F). After tenectomy, all three synthases highly colocalized with the invading macrophages and HAS1 and HAS3 significantly increased between 2 and 7 days (Fig. 4, D–F). CD31⁺ epithelia showed a slight, but significant enhancement of HAS1 and HAS2 colocalization with respect to time; however, at all time points HAS3 was the predominant isoform that colocalized with the blood vessel epithelium (Fig. 4, G–I). HAS1 colabeling of FSP-1⁺ cells significantly increased 2 days after tenectomy, whereas the distribution of all three isoforms highly overlapped with cadherin⁺ satellite cells at all time points (Fig. 4, J–O).

During our immunohistochemistry investigation, we observed that HAS2 colocalization with F4/80⁺ macrophages in muscle epimysium and adjacent connective tissue varied with respect to time. Cross-sections from 2, 7, and 14 days post-tenectomy were stained for HAS2 (red) and F4/80 (green). Macrophages in the epimysium that newly formed connective tissue were elongated and did not significantly colocalize with HAS2 expressing cells (Fig. 5B). After 7 and 14 days, macrophages in the epimysium and newly formed connective tissue were elongated and did not significantly colocalize with HAS2 expressing cells (Fig. 5, C and D). When quantified, we found that the colocalization of HAS2 and F4/80 in the epimysial cells significantly decreased between 2 and 14 days, in contrast to what was observed in the muscle belly proper (Figs. 5E and 4, D–F). These data suggest that multiple cell types have the ability to contribute to HA synthesis during hypertrophy via the differential expression of HAS1–HAS3.

Primer specific for HAS1–HAS3 and the four hyaluronidases, Hyal1–Hyal4, were used to assess how HA deposition is regulated at the molecular level in overloaded plantaris muscle. By carrying out end-point PCR on tissues from control adult mice, we confirmed primer specificity of the three isoforms using lung (HAS1–HAS3), heart (HAS2), skin (HAS1–HAS3), and liver (absence of HAS2) (14, 35, 53). To extend the profiles for heart, liver, and skeletal muscle, as well as establish the previously unknown patterns in tendon and testes, we determined the expression of HAS1–HAS3 and Hyal1–Hyal4 in these soft tissues. We found that the mRNA of all three synthases was expressed by skeletal muscle, tendon, and heart (Fig. 6). HAS1 and HAS3 transcripts were present in both the liver and the testes (the former at very low levels), but HAS2 could not be detected in either tissue after 35 cycles. Using the testes as a positive control (7), we validated our primers against Hyal1, Hyal2, Hyal3, and Hyal4. Hyal1 and Hyal2 were present in all tissues investigated (Fig. 6), consistent with previous studies (17, 52). While Hyal3 was detected in various murine soft tissues and Hyal4 in human skeletal muscle (17, 52), we were unable to find significant expression of either transcript outside of the testes or skin (Fig. 6). The functions of Hyal5 and PH-20 are not well characterized and were not investigated in this study.

Next, the influence of compensatory overload on the expression of matrix remodeling genes was determined using quantitative PCR. We attempted to normalize target gene expression to the typical housekeeping genes GAPDH, β-actin, β-2-microglobulin, and 18S rRNA; however, expression was not...
constant between overloaded and control animals across the time points investigated in our study (Fig. 7). Therefore, we used the linear regression of efficiency method to quantify relative copy number for each target gene normalized to input RNA quantity (34, 47, 48). HAS1 and HAS2 were significantly upregulated as a result of Achilles tenectomy, whereas HAS3 remained relatively unchanged (Fig. 8, A–C). Hyal1 was significantly increased after 2 days overload, but Hyal2, Hyal3, and Hyal4 showed no difference (Fig. 8, D and E, and data not shown). MMP2 expression was initially suppressed at 2 days and then significantly increased over controls by 14 days (Fig. 9A). In contrast, MMP9 was upregulated at 2 days and suppressed at 7 and 14 days (Fig. 9B). Similar to MMP2, MMP14 expression was enhanced at 7 and 14 days (Fig. 9C). The MMP regulators TIMP1 and TIMP2 displayed differential behaviors (Fig. 9, D and E). TIMP1 was dramatically upregulated in response to tenectomy and then decreased from 2–14 days, whereas TIMP2 was initially downregulated then significantly increased by 14 days. Finally, we found that TN-C was significantly increased in overloaded tissues (Fig. 10A). Col1a2, which encodes for one of the chains of the type I collagen triple helix that contributes to overall tissue stability, was initially unchanged but then significantly increased with time (Fig. 10B).

**DISCUSSION**

We hypothesized that compensatory overloading of the murine plantaris muscle would transiently upregulate the expression of HAS and HAS1–HAS3. After 2 days, we found that HA content and HAS1–HAS3 expression significantly increased in response to Achilles tenectomy, although HAS3 upregulation was attenuated when compared with the other two isoforms. Multiple cell types, including macrophages, blood vessel epithelia, activated fibroblasts, and myogenic satellite cells, appeared to have the ability to contribute to HA synthesis. Between 7 and 14 days, HA concentration decreased towards levels comparable to age-matched controls; however, HAS1 and HAS2 remained upregulated and these two enzymes were still significantly expressed within the muscle body.

When the murine soleus and gastrocnemius muscles are inactivated by Achilles tenectomy, the mass of the plantaris muscle eventually doubles to compensate for the loss of the synergists (26). The sudden increase in loading has been described to induce two distinct phases: 1) an immediate inflammatory reaction, and 2) a slower compensatory growth of myofibers in response to increased mechanical demands (3). The acute inflammatory response is highly active the first 5 days posttenectomy, which is critical not only for the clearing of debris but also to facilitate the rebuilding of damaged muscle (3, 5, 43). Then, myogenic cells activated by inflammation begin to proliferate by 7 days and induce hypertrophy by both fusing with existing myofibers and replacing damaged ones, inducing an increase in contractile protein machinery synthesis and overall muscle mass that gradually reaches a steady state after one month (3, 37, 57).

The dramatic 40% increase in plantaris muscle wet weight we observed after 2 days of overload is most likely due to the combination of an increase in HA deposition and the influx of water and inflammatory cells (Figs. 1 and 2). Previous studies (19, 26) have revealed that synergist ablation induces both significant edema and a decrease in overall protein concentration during the first 5 days posttenectomy. HA is thought to play a significant role in the induction of edema owing to the ability of the large hydrophilic chains to draw in water and may facilitate the immune response by enhancing diffusion of paracrine signals and clearing of tissue debris (22, 26). TN-C was also upregulated throughout the muscle interstitium during the initial response to overload (Figs. 1 and 10). Due to its hypothesized role as a mechanical stabilizer in homeostatic connective tissues (27), TN-C upregulation may help maintain the structural integrity of muscle during the early response to synergist ablation by counteracting the swelling induced by HA.

By 7 days posttenectomy, the plantaris continued to increase in mass; however, based on previous results (26), this was probably a result of an increase in muscle protein synthesis and subsequent myofiber hypertrophy. These data correlate well with our observations that HA content, and reported contribution of edematous swelling to tissue weight, decreased compared with 2 days (Figs. 1 and 2) (19). Nevertheless, HA and TN-C were still upregulated with respect to the control muscles and may act to create a permissive environment that recruits a large number of myogenic progenitors by enhancing migration and proliferation, while inhibiting premature fusion. We (10) previously showed that both HA and TN-C significantly enhanced migration and inhibited differentiation compared with ECM that are representative of the matrix of homeostatic muscle (i.e., laminin, Matrigel, and type I collagen). Additionally, TN-C promotes myoblast proliferation (10), indicating the
deposition of these two matrixes during repair can act to support the recruitment and replication of myoblast progenitors, increasing the overall number of satellite cells available for fusion with preexisting myofibers to enhance hypertrophy. Notably, the effect of HA on differentiation was shown to be reversible in vitro (20, 31). We hypothesize that once a critical cellular density is reached, myoblasts are able to overcome the spatial and biochemical barriers to differentiation that HA presents through direct cell-cell contact and/or the upregulation of HA degrading molecules.

**Differential distribution of HAS in hypertrophic skeletal muscle.** After tenectomy, HAS1 mRNA was significantly upregulated and strongly colocalized with both F4/80 and FSP-1+ cells (Fig. 3), indicating that activated fibroblasts may contribute to HA deposition during hypertrophy primarily through the upregulation of HAS1 since FSP-1 costaining with HAS2 and HAS3 was invariant during this study (Fig. 4). HAS2 mRNA and protein both appeared to be constitutively expressed at higher levels in control tissues relative to HAS1 (Figs. 4 and 6). Two days after Achilles tenectomy, the number of HAS2+ cells increased 3.5-fold within the muscle interstitium (Fig. 4B). However, the increase in mRNA at this time point was not significant, suggesting that HAS2 is regulated at the posttranscriptional level. Indeed, native antisense HAS2 is expressed and negatively regulates HA synthesis in mammalian kidney cells and may prevent HAS2 translation in homeostatic muscles (39). Alternatively, the prominent phase of HAS2 expression occurred earlier than the first time point we investigated. By 2 days posttenectomy, there was already a pronounced number of HAS2+ cells and previous studies found the peak upregulation of HAS enzymes in response to trauma to occur within the first 2–6 h (14, 35).

Of the four cell types investigated in this study, HAS2 was predominantly expressed by F4/80+ macrophages in the epimysium after 2 days of overload (Figs. 3 and 5). However, it is clear that other cell types not investigated in this study also utilize HAS2 for the synthesis of HA. There were a large number of unidentified rounded HAS2+ cells within the interstitial space after 2 days that, based on their early expression pattern, may correspond with other components of the immune response. We were also unable to identify the types of cells responsible for the discrete patches of HAS2 at 7 and 14 days, and we posit that these regions may represent remodeling of nervous tissues, as HA has been shown support neural cell migration (6).

While HAS3 expression was not significantly influenced by tenectomy, the pronounced coexpression in blood vessel endothelia suggests that this synthase may play a role in blood vessel homeostasis (Figs. 3 and 4). In addition, all three HAS enzymes colocalized with m-cadherin+ satellite cells, revealing that myogenic precursors may directly contribute to the deposition of the transitional matrix that prevents their premature differentiation.

**Hyaluronidase gene expression remains relatively unchanged during hypertrophy.** We found Hyal1 and Hyal2 to be the dominant hyaluronidases expressed in murine plantaris muscles (Fig. 6). Two days posttenectomy, there was a 2.2-fold induction of Hyal1, which may play a critical role in inflammation by generating HA fragments that can stimulate the inflammatory response in many cell types (28). Notably, HA...
oligomers were shown to activate macrophages during murine lung inflammation (38) and may have facilitated the recruitment of F4/80$^+$ macrophages we observed after 2 days (Fig. 5).

We expected to see a large upregulation of Hyal1 and Hyal2 at 7 and 14 days to account for the decrease in HA during the hypertrophic stage. Surprisingly, we only detected a slight increase in Hyal1 at 14 days and no change in Hyal2–Hyal4 at any time (Fig. 6 and data not shown). Since Hyal1 and Hyal2 were constitutively expressed in control muscles, the regulation of enzymatic activity may be at the protein level. Chondroitin, dermatan, and heparan sulfate proteoglycans can significantly inhibit hyaluronidase activity (1). During muscle repair, the heparan sulfate proteoglycans perlecan, syndecan-3, and glypican are transiently increased (12) and have the potential to interfere with hyaluronidase activity during the early stages of compensatory hypertrophy, indirectly inhibiting the premature degradation of HA. Alternatively, biomolecule-degrading oxygen free radicals or receptors responsible for HA endocytosis (e.g., HARE, CD44, and RHAMM) may also be significantly upregulated in response to hypertrophic overload to facilitate the clearing of excess HA (7).

Differential expression of MMPs. The expression pattern of MMP-2 suggests that it plays a role during hypertrophic growth (Fig. 8). MMP-2 is constitutively expressed in skeletal muscle and is necessary for the homeostasis of connective tissues (29). In response to cardiotoxin injury, MMP-2 was upregulated in murine skeletal muscle, with maximum activation correspond-
collagens, is an activator of pro-MMP-2 (25). TIMP-2, a primary inhibitor of many MMPs, can also facilitate the cleavage of pro-MMP-2 into the active form by binding with, and positively regulating, MMP-14 activity (25). MMP-14 has also been implicated in regulating muscle fiber diameter during cardiotoxin induced injury (41), potentially an indirect result of the role it plays in mediating the behavior of MMP-2.

These data indicate that, after synergist ablation, MMP-9 is upregulated in the plantaris muscle, likely to help break down damaged basal laminar components as part of the inflammatory response, whereas MMP-2 and -14 and TIMP2 act in concert to control basal laminar deposition so that it does not interfere with hypertrophy. It should be noted that we only investigated gene expression and that the activities of the MMPs and TIMPs

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Fig. 9. MMPs and their regulators TIMP1 and TIMP2 are differentially expressed in response to hypertrophic overload. A: MMP2 expression was initially suppressed at 2 days and then increased by 14 days with a 5.8-fold upregulation compared with controls. B: in contrast, MMP9 showed the greatest upregulation at 2 days and decreased to control values at 7 and 14 days. C: similar to MMP2, MMP14 expression was enhanced 1.2- to 11.3-fold between 2–14 days. D and E: 2 MMP regulators, TIMP1 and TIMP2 displayed reverse trends in expression where TIMP1 was greatly upregulated at 2 days and significantly decreased from 98- to 41-fold upregulation and TIMP2 was more modestly expressed, increasing from baseline values to 2.6-fold over controls by 14 days. F: two-way ANOVA was performed on overloaded and control muscles within each primer set to determine the effect of time and tenectomy on gene expression. Bonferroni’s post hoc test was utilized to assess significant differences in relative copy number between control and tenectomy and time after tenectomy (*P < 0.05). Data from overloaded plantaris were normalized to age-matched controls and, for each time point after tenectomy, control values were plotted on the left and overloaded on the right (black bars). Values are presented as means ± SD; n ≥ 3 mice.

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Fig. 10. TN-C and Col1a2 are differentially transcribed in response to hypertrophic overload. A and B: TN-C expression was significantly enhanced in response to tenectomy whereas Col1a2 was initially unchanged, then increasing to 17.8-fold by 14 days. C: Two-way ANOVA was performed on overloaded and control muscles within each primer set to determine the effect of time and tenectomy on gene expression. Bonferroni’s post hoc test was utilized to assess significant differences in relative copy number between control and tenectomy and time after tenectomy (*P < 0.05). Data from overloaded plantaris were normalized to age-matched controls and, for each time point after tenectomy, control values were plotted on the left and overloaded on the right (black bars). 18S, 18S RNA; Values are presented as means ± SD; n ≥ 3 mice.
can be regulated posttranscriptionally or posttranslationally. MMPs are secreted in a latent form that needs to be cleaved by cofactors to become activated (11). Future studies utilizing zymography to assess enzymatic activity will be necessary to resolve the spatiotemporal distribution of MMP activity in response to compensatory overload.

**Macrophages.** Macrophages play a dual role during muscle regeneration, having both proinflammatory and anti-inflammatory roles. Proinflammatory F4/80+/MMP-9+ macrophages help with the clearing of muscle debris during the initial stages of inflammation, which then differentiate into anti-inflammatory F4/80− cells that facilitate muscle repair and growth (5). Consistent with previous observations (9), during the inflammatory stage we found a large number of F4/80+/MMP-9+ cells embedded within the epimysium which then migrated into the muscle during hypertrophy (Figs. 3, 4, and 5). These cells were predominantly spherical and strongly colocalized with HAS2 expression, indicating they may have contributed to the enrichment of HA in the epimysium during migration.

By 7–14 days, at which time the inflammatory response is attenuated and hypertrophy dominates, the F4/80+/MMP-9− macrophages within the epimysium and connective tissue adopted a more elongated mesenchymal morphology and few cells expressed HAS2, whereas those in the muscle belly still colocalized with all three isoforms (Figs. 4 and 5). Interestingly, when myogenic precursors were cultured with proinflammatory macrophages in vitro, muscle differentiation was inhibited (5). In contrast, anti-inflammatory macrophages stimulated differentiation (5), suggesting that one way macrophages can regulate muscle repair during inflammation is by secreting HA to inhibit premature myoblast fusion. Additionally, the proinflammatory macrophages have been reported to be responsible, directly or indirectly, for the transient upregulation of MMP-9 (29, 33). Potentially, macrophages downregulate MMP-9 during the switch to the anti-inflammatory phenotype, which is critical since constitutively expressed MMP-9 is associated with fibrotic pathologies such as muscular dystrophy (33).

**Summary.** In context with our previous findings that HA enhances the recruitment, and prevents the premature fusion, of muscle progenitors in vitro, the transient upregulation of HA, HAS1, and HAS2 in response to mechanical load-induced hypertrophy may play an important role in the functional repair of mammalian skeletal muscle in vivo. Satellite cells, key players in muscle regeneration, colocalized with all three HAS isoforms, further supporting the notion that HA plays an important role during myogenesis. Additional studies into the pathways that regulate HAS expression in skeletal muscle, along with the design of engineered scaffolds that incorporate HA, will help promote the development of therapies that enhance muscle regeneration following massive tissue injury or disease.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

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Calve S, Isaac J, Gumucio JP, Mendias CL. Hyaluronic acid, HAS1, and HAS2 are significantly upregulated during muscle hypertrophy. Am J Physiol Cell Physiol 303: C577–C588, 2012. First published July 11, 2012; doi:10.1152/ajpcell.00057.2012 (http://ajpcell.physiology.org/content/303/5/C577).—In RESULTS, Figs. 8 and 9 were presented incorrectly. The corrected Figs. 8 and 9 appear below. In addition, in the section entitled “Differential expression of MMPs,” the first sentence should read as “The expression pattern of MMP-2 suggests that it plays a role during hypertrophic growth (Fig. 9).”

Fig. 8. HAS1–HAS3 and the hyaluronidases are differentially expressed in response to hypertrophic overload. A and B: HAS1 and HAS2 were significantly upregulated in response to tenectomy with HAS1 displaying an additional effect of time. C–E: HAS3 and Hyal2 in overloaded muscles did not vary from controls at any time point, whereas Hyal1 was slightly, but significantly, increased in response to tenectomy. F: two-way ANOVA was performed on overloaded and control muscles within each primer set to determine the effect of time and tenectomy on gene expression. Bonferroni’s post hoc test was utilized to assess significant differences in relative copy number between control and tenectomy and time after tenectomy (*P < 0.05). Data from overloaded plantaris were normalized to age-matched controls and, for each time point after tenectomy, control values were plotted on the left and overloaded on the right (black bars). Values are presented as means ± SD; n = 3 mice.
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