M-cadherin transcription in satellite cells from normal and denervated muscle

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Submitted 2 September 2003; accepted in final form 11 November 2003

Maier, Annette, and Antje Bornemann. M-cadherin transcription in satellite cells from normal and denervated muscle. Am J Physiol Cell Physiol 286: C708–C712, 2004; 10.1152/ajpcell.00369.2003.—Satellite cells (SC) in adult muscle are quiescent in the G0 phase of the cell cycle. In the present study we determined whether SC after denervation upregulate M-cadherin, an adhesion molecule that is upregulated with differentiation and fusion. We also monitored primary cultures of SC from denervated muscle for expression of the transcription factors of the MyoD family. Hindlimb muscles of rats were denervated under anesthesia, and rats were killed after 2–28 days. The SC of the denervated limbs were pooled and either assessed for M-cadherin mRNA by using real-time RT-PCR or cultured in vitro. The cultures were processed for RT-PCR or immunofluorescence for expression of the transcription factors of the MyoD family. Hindlimb muscles of M-cadherin knockout mice were denervated under anesthesia, mice were killed after 2–28 days, and cells were stained for β-galactosidase activity by X-gal histochemistry. In vitro, primary SC cultures from rat muscle denervated for 2–28 days expressed transcripts of myf5, MyoD, myogenin, and MRF4 as SC from normal innervated muscle. In vivo, M-cadherin transcription was not upregulated in SC from denervated rat muscle when compared with normal muscle. Moreover, β-galactosidase activity was not detected in denervated mouse muscle. The finding that SC do not upregulate M-cadherin after denervation supports the notion that they remain in the G0 phase of the cell cycle in vivo. However, the cells retain the capacity to pass through the proliferative and differentiative program when robustly stimulated to do so in vitro.

SATELLITE CELLS (SC) are mononuclear muscle cells lying dormant in the G0 phase of the cell cycle in normal adult muscle. They are localized between the plasma membrane and the basement membrane of myofibers. They are considered to be reserve cells that contribute to new myofiber formation and to postnatal myofiber growth (reviewed in Ref. 9). Growth factors that are able to recruit SC into the mitotic and differentiative program are released from crushed muscle (3). Muscle necrosis occurs in diseases such as muscular dystrophies, and it can be experimentally generated by transient devascularization, injecting toxic substances, or grafting. Denervated muscle also has been suggested to be a model for SC activation. Some studies using repeated injections of tritiated thymidine or bromodeoxyuridine (BrdU) into mice or rats yielded an increase in autoradiographically labeled SC nuclei in the first few days or weeks (13, 20, 23). However, other investigators reported that repeated injections or continuous application of BrdU yielded negative results: no increased SC proliferation took place compared with controls in a study on rat extensor digitorum longus (EDL) and soleus muscles (26) and in a study on mouse tibialis anterior (TA) or EDL muscles (31). Moreover, studies applying one injection only before the animals were killed never found labeled SC after denervation (14, 28). Another approach used immunolabeling with an antibody against the proliferating cell nuclear antigen (PCNA). SC from isolated cultured myofibers from muscle denervated 1–32 wk before culturing did not express PCNA (17). It had been shown previously that the two approaches of incorporation of tritiated thymidine and monitoring of proliferating cells with anti-PCNA both yielded similar conclusions regarding the kinetics of SC proliferation (34).

M-cadherin is a calcium-dependent adhesion molecule of the cadherin family (reviewed in Ref. 32). In normal adult muscle, the protein is localized to the SC-myofiber interface (4). The protein continues to be expressed after denervation, and it is also present in the SC of regenerating muscle (14). The use of M-cadherin protein as a marker renders the distinction of quiescent SC from SC of denervated or regenerating myofibers difficult, if not impossible, at the single-cell level. However, M-cadherin is differentially expressed at the transcript level in different conditions. Its mRNA was below the detection level in an in situ hybridization study of SC from normal adult mouse muscle, but it was detected in regenerating muscle (21). Moreover, the promoter of M-cadherin was activated in regenerating muscles in a transgene mouse model in which the LacZ gene was fused to the start codon in exon 1 (12). An in vitro study of a myoblast cell line determined that M-cadherin mRNA was upregulated with the myoblasts’ differentiation and fusion (8). In the present study we monitored the transcription of M-cadherin after denervation because this appears to be a suitable marker to determine whether a SC is in the differentiative compartment.

The exit of SC from the quiescent state can also be monitored by studying the expression of MyoD and myogenin of the MyoD family, since these molecules are expressed after activation in a precisely regulated manner (5, 16, 33). In vivo studies in which a SC marker was used to distinguish these cells from myonuclei did not detect MyoD+ and/or myogenin+ SC after denervation (19), or the percentage of labeled cells was low when compared with myonuclei (13). In the present study we determined whether SC after denervation express the transcription factors of the MyoD family when isolated from muscle and placed in culture. This treatment is a stronger stimulus on SC when compared with culturing isolated myofibers because the cells lose contact with the myofiber, which renders them more susceptible to the effect of growth factors (3).

Collectively, our results demonstrate that, in vivo, M-cadherin transcription is not upregulated after denervation by either method employed in this study. This corroborates the
finding that most, if not all, SC continue to remain in the G0 phase of the cell cycle after denervation in vivo, since they continue to express myf5 (19), which is expressed by quiescent SC (16). However, SC retain the capacity to pass through the proliferative-differentiative program in vitro when robustly stimulated to do so in primary cultures.

**MATERIALS AND METHODS**

**Animal experiments.** All animal experiments were performed in adherence to the standards of German law for the care and use of laboratory animals. The experiments have been approved by the Regierungspräsidium Tübingen (file no. 35/9185.81-2). Male Wistar rats (Charles River, Kissing, Germany) (8 wk old, 250 g body wt) were used. All experiments were performed under Ketanest (ketamine hydrochloride, 100 mg/kg; Essex Pharma, Munich, Germany) and Rompun (xylazine hydrochloride, 10 mg/kg; Bayer, Leverkusen, Germany) anesthesia. Both substances were injected intraperitoneally. To prevent xerophthalmia during anesthesia, both eyes were covered with Oculotect gel (retinolpalmitate; CIB Vision, Novartis, Germany). The right sciatic nerve was cut at midthigh, and a 10-mm-long segment was resected. The animals were killed after 2, 7, or 28 days.

We complemented the study on rat muscle with a knockout mouse model of M-cadherin to circumvent potential problems created by species differences and, also, to study M-cadherin at the single-cell level. In this transgene model, a neomycin-resistant cassette including the LacZ gene was fused to the start codon in exon 1. Homologous level. In this transgene model, a neomycin-resistant cassette including species differences and, also, to study M-cadherin at the single-cell level was resected. The animals were killed after 2, 7, or 28 days. The right sciatic nerve was cut at mighth, and a 10-mm-long segment was resected. The animals were killed after 2, 7, or 28 days. The concentration of anesthesia was only 10 mg/kg Ketanest and 1 mg/kg Rompun.

*TaqMan RT-PCR gene expression analysis.* For isolation of the RNA from SC, we employed the Mini Isolation kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Complementary DNA (cDNA) synthesis was performed with 1 μg of RNA at 37°C for 30 min by random hexamer priming, using a hexaamideptide mix (Promega, Mannheim, Germany) and reverse transcriptase (Life Technologies, Karlsruhe, Germany). Real-time PCR was performed in a total of 50 μl per capillary by using the SYBR Green PCR Master Mix at a 1× concentration (Applied Biosystems, Weiterstadt, Germany) and a primer pair at a concentration of 50 pmol/μl. The following primers were designed, using Primer Express software (Applied Biosystems), yielding the indicated fragment base pair (bp) lengths: M-cadherin: forward primer 5′-ctg gac tct cgg cac gag tga-3′, reverse primer 5′-atg ctc cca gtt ggt tga-3′, yielding a fragment of 160 bp; β-actin: forward primer 5′-cgg cct ccc cta cca gtt ggt tgc atc g-3′, reverse primer 5′-atc gtc cca gtt ggt tgc atc g-3′, yielding a fragment of 158 bp. Each primer contained at least one intron on genomic DNA. The fragment length of each sample was checked by agarose gel electrophoresis. The specificity of the primer pairs was confirmed by sequence analysis. DNA amplification was achieved by an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s. Subsequently, a dissociation protocol was created for each fragment to confirm specificity for the fragment. The dissociation temperature was 87.7°C for M-cadherin and 85.7°C for β-actin. As a negative control, the template DNA was replaced with water. TaqMan PCR was carried out on an ABI Prism 5700 sequence detector. A relative quantification was based on the relative standard method (user bulletin no. 2, ABI Prism 7700 sequence detection system; Applied Biosystems). For this method, a standard curve was created for the M-cadherin fragment and for the β-actin fragment by using cultured differentiating satellite cells (SC) as standard, because the M-cadherin gene is highly upregulated in differentiating SC (8). The standard curves were generated by plotting the threshold cycle vs. the known dilution of the cDNA. From each standard curve the slope was calculated, as well as the x value (amount from the unknown probe). The target amount (M-cadherin) was then divided by the endogenous reference (β-actin) to obtain a normalized target value. The calibrator in our experiments was innervated SC and means a 1× sample. Each of the normalized target values was divided by the calibrator normalized target value to generate the relative expression levels. The cutoff used for difference calls was a twofold change in expression (either increase or decrease) according to the manufacturer’s instructions. The samples for constructing the standard curves were run in duplicates, and the samples for measuring were run in quadruplicate.

*Isolation of SC.* All hindlimb muscles of five innervated control animals, or the muscles from the denervated hindlimb of five animals, were resected for each preparation. The pieces were rinsed several times with PBS and minced into small pieces. The cells were digested with Pronase (Sigma, Deisenhofen, Germany) at a concentration of 1.4 mg/ml PBS in a shaking bath for 1 h at 37°C. After digestion, the SC were separated from the tissue by spinning for 1 min at 750 g. The supernatant that contained the SC was precipitated by spinning for 5 min at 1,500 g. The pellet was resuspended in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL, Karlsruhe, Germany) to which 20% fetal calf serum (FCS) was added. For a second digestion step, the tissue was incubated in trypsin (0.25% in PBS, pH 7.8) at 37°C for 25 min. Thereafter, the tissue was vigorously titurated with a 10-ml pipette to dissolve the residual SC in the tissue. The cells were spun down for 1 min at 750 g. The supernatant was then spun down for 5 min at 1,500 g, and the pellet was resolved in 10 ml of DMEM to which 20% FCS was added. The cells were passed through a nylon filter (70 μm) to remove cell debris and processed for culturing. The cell cultures were fed 2% FCS for 3 days to stimulate proliferation and 2% horse serum for 2 days to stimulate differentiation. The cultures were then fixed in acetone and immunolabeled.

**Immunofluorescence.** Polyclonal anti-M-cadherin (19) in combination with monoclonal anti-myogenin was used for double-labeling experiments. This was followed by incubation in a goat anti-mouse antibody conjugated with Alexa Green (MobiTec, Göttingen, Germany) in conjunction with a Cy3-conjugated donkey anti-rabbit antibody (Dianova). The dilutions used were 1:50 (anti-M-cadherin), 1:100 (anti-myogenin), and 1:350 (fluorescent dye-conjugated antibodies). 4,6-Diamidino-2-phenylindole (Boehringer, Mannheim, Germany) for nuclear counterstaining was added for 5 min after the secondary antibody at a concentration of 1 μg/ml. Observations were made with the Olympus BX60 microscope equipped for epifluorescence.

![Fig. 1. Calculation of the arbitrary copy number of the M-cadherin was done according to the relative standard curve method. Serial dilutions of the standard [cultured differentiating satellite cells (SC)] were used to construct the target (M-cadherin) and the internal control (β-actin) standard curves to obtain a normalized target value. The mean threshold cycle (Ct) values are plotted against the arbitrary copy number of the 2 transcripts. The slopes (m) of the curves are indicated.](http://ajpcell.physiology.org/)
**RESULTS**

**M-cadherin transcription in normal innervated and denervated muscle.** The amount of M-cadherin transcript at the single-cell level in normal innervated muscle and in denervated muscle is too low in abundance to permit detection by in situ hybridization (21). A method to circumvent the scarcity of transcript is to use pooled SC from the hindlimbs of several animals. Most (97%) rat hindlimb SC are in G0 at the end of the isolation procedure (1). We monitored the first 28 days after denervation. During this period, the fiber diameter is severely reduced in rat to reach 10% of control values (29).

We determined whether the transcription of M-cadherin was upregulated in denervated muscle when compared with normal innervated controls. First, we assessed whether the relative amount of M-cadherin mRNA was increased in SC from rat muscle after denervation. Second, we determined whether the gene locus of M-cadherin was activated in the knockout mice.

Real-time PCR of pooled SC from normal innervated rat hindlimb muscles revealed a baseline transcription level (Figs. 1 and 2). We tested whether M-cadherin mRNA was upregulated in vivo after nerve lesion. The amount of M-cadherin RNA was not significantly altered when pooled SC from muscle denervated for 2, 7, or 28 days were compared with SC from normal innervated muscle (Fig. 2). Because differentiation and fusion are accompanied by an upregulation of M-cadherin in myoblast development (8) and regeneration (21), this finding suggests that the SC did not fuse with the myofiber.

LacZ staining was detected in growing muscle at P13 (Fig. 3) but not in soleus or TA muscles from normal adult innervated M-cadherin knockout mice (not shown). Likewise, no LacZ staining was present in muscles from knockout mice denervated for 2 days to 4 wk (Fig. 3). This finding indicates that the M-cadherin gene locus was not activated.

**DISCUSSION**

Denervation of skeletal muscle affects not only the myofibers but also the SC. The regenerative potential of SC isolated from denervated muscles appears to be impaired. After necrotization, new fibers are formed but do not fully mature (2, 10, 24). The molecular basis for this is only beginning to emerge. In a previous study we demonstrated that SC are less easily recruited into the mitotic cycle after denervation: SC did not express PCNA in cultures of isolated myofibers from muscle denervated 1–32 wk before culture (17). We failed to observe increases in M-cadherin transcription in SC isolated from muscles that were denervated for up to 28 days. A recent work (6) on rat TA muscle demonstrated that a 2-mo denervation...
period resulted in an increase of the number of M-cadherin+ SC per myofiber when compared with normal innervated animals. Depending on the age of the rats, a two- to threefold or six- to sevenfold increase was found (6). It cannot be excluded that after long-term denervation, M-cadherin is differentially regulated when compared with the time periods examined in the present study.

Denervation leads to a reduction of the number of SC. It decreases over time to reach up to less than one-fifth of normal (7, 25, 30). It has been suggested that SC fuse with the myofiber to account for the loss (26). Fusion of SC with myofibers is accompanied by the upregulation of M-cadherin transcription. This has been demonstrated in vivo with the use of muscle stretching and electrical stimulation as a model for SC activation (11). Moreover, the promoter of M-cadherin was activated as shown by X-gal staining in the mouse M-cadherin null mutant when regeneration was induced by necrotization (12). The promoter is also activated in postnatal muscle growth (Fig. 3), which is also accompanied by SC fusion with myofibers (22). After denervation, we did not find the promoter of M-cadherin to be activated (Fig. 3), and we did not detect an upregulation of M-cadherin transcription (Fig. 2). We interpret this to mean that fusion of SC is not likely to occur over the time period studied. Electron microscopy of SC in growing denervated (27) and adult denervated muscle (18) demonstrated basement membrane encroachments of SC, suggesting that SC detach from the parent myofiber to account for the loss (18, 28).

Fig. 4. RT-PCR analysis of the muscle transcription factors of the MyoD family. SC cultures were derived from either normal innervated muscle or muscle denervated 2–28 days before culturing. In all conditions, SC expressed myf5, MyoD, and myogenin in proliferating medium (left) and also MRF4 in differentiating medium (right).
In the present study we detected M-cadherin immunolabeling at the single-cell level in SC cultures of low density, which prevented the cells from fusing with one another (Fig. 5). Whereas M-cadherin is usually associated with myoblast fusion (15), this finding might be interpreted to mean that the molecule is part of the differentiative program of a myoblast, even when fusion does not occur. If this assumption is true, then the failure of SC to upregulate M-cadherin in vivo after denervation suggests that SC do not go through the differentiative program. This is in keeping with previous in vivo results demonstrating that SC retain mY5 expression after denervation to indicate their quiescent state (19) and hardly, if at all, upregulate MyoD, myogenin, or MRF4 (13, 19).

The SC of primary cultures were able to undergo the proliferative and differentiative program of the cell cycle regarding transcription of the factors of the MyoD family as in normal muscle (Fig. 4). This finding suggests that SC retain the capacity to go through the proliferative-differentiative program, as far as the expression of these factors is concerned, when robustly stimulated to do so. This might be interpreted to mean that the impaired maturation of aneurally regenerating myofibers cannot be explained by defective SC, at least not by defective SC alone. It might be speculated that the maturation of muscle fibers requires action potentials, contractile activity, and possibly even unknown neurotrophic substances, all of which are lacking during aneural regeneration.

ACKNOWLEDGMENTS

M-cadherin knockout mice were generously provided by Angela Hollnagel and Hans-Henning Arnold. We thank Thomas Bock, Tübingen, for advice on the real-time PCR in the beginning of the study.

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