Embryonic mesenchymal cells share the potential for smooth muscle differentiation: myogenesis is controlled by the cell’s shape

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SUMMARY

Undifferentiated embryonic mesenchymal cells are round/cuboidal in shape. During development, visceral myogenesis is shortly preceded by mesenchymal cell elongation. To determine the role of the cell’s shape on smooth muscle development, undifferentiated embryonic mesenchymal cells from intestine (abundant visceral muscle), lung (some visceral muscle) or kidney (no visceral muscle) were plated under conditions that maintained cell rounding or promoted elongation. Regardless of their fate in vivo, all the cells differentiated into smooth muscle upon elongation as indicated by the expression of smooth muscle-specific proteins and the development of membrane potentials of −60 mV and voltage-dependent Ca²⁺ currents, characteristic of excitable cells. Smooth muscle differentiation occurred within 24 hours and was independent of cell proliferation. Regardless of their fate in vivo, all the round cells remained negative for smooth muscle markers, had membrane potentials of −30 mV and showed no voltage-activated current. These cells, however, differentiated into smooth muscle upon elongation. The role of the cell’s shape in controlling smooth muscle differentiation was not overcome by treatment with retinoic acid, TGF-β₁, PDGF BB or epithelial-conditioned medium (all modulators of smooth muscle differentiation). These studies suggest that the mesenchymal cell shape plays a main role in visceral myogenesis.

Key words: Smooth muscle, Differentiation, Myogenesis, Cell shape, Mouse

INTRODUCTION

Smooth muscle (SM) is a significant portion of many organs including those of the gastrointestinal, urogenital and respiratory tracts, and the vascular system. With the exception of the aorta, the appearance of visceral SM precedes that of the vascular musculature (Mitchell et al., 1990; Woodcock-Mitchell et al., 1993; Miano et al., 1994; McHugh, 1995). Visceral SM originates by differentiation of local mesenchymal cells that in mid-gestation begin to express and accumulate SM-specific proteins (Ruzicka and Schwartz, 1988; Sawtell and Lessard, 1989; Kedinger et al., 1990; Roman and McDonald, 1992; Miano et al., 1994, 1996; McHugh, 1995; Li et al., 1996).

Although our understanding of SM biology has advanced significantly over the years, the mechanisms controlling SM differentiation still remain unknown. It is currently believed that the process of SM myogenesis results from interactions between mesenchymal cell precursors and epithelial and/or endothelial cells (Cunha et al., 1992; Duluc et al., 1994; Baskin et al., 1996; Hirschi et al., 1998). As in any process of heterotypic cell induction, the epithelial and/or endothelial cells could produce morphogens that by acting on the surrounding mesenchyme will trigger SM differentiation. Factors shown to modulate SM differentiation are transforming growth factor β1 (TGF-β₁) (Desmouliere et al., 1993; Orlani et al., 1994; Hirschi et al., 1998), platelet-derived growth factor-BB (PDGF-BB) (Holycross et al., 1992; Hirschi et al., 1998) and retinoic acid (RA) (Blank et al., 1995).

Interestingly, the appearance of visceral musculature is preceded by a change in the shape of the SM cell precursors, which gradually turn from round to elongated. This change in cell shape is followed by the synthesis of SM-specific proteins a few hours later (Theiler, 1989; Roman and McDonald, 1992; Y. Yang and L. Schuger, unpublished observations). Our recent studies suggested a possible correlation between mesenchymal cell shape and SM differentiation. These studies showed that embryonic lungs exposed to antibodies to laminin α₁ chain have decreased levels of SM α-actin and desmin and show round instead of elongated peribronchial mesenchymal cells (Schuger et al., 1997).

Here we determined the role of the cell shape in SM differentiation by plating undifferentiated mesenchymal cells on microsurfaces that stimulated cell rounding or cell elongation. We found that the round cells remained undifferentiated and expressed high levels of α-fetoprotein (produced by fetal but not by mature cells), whereas the elongated cells differentiated into SM. In all the mesenchymal cells studied, induction or prevention of SM differentiation was consistently determined by the cell shape and involved activation of mitogen activated protein kinase (MAPK) 38. The cell shape was the main factor in controlling SM
MATERIALS AND METHODS

Cell culture assemblies

Multi-perforated polycarbonate membranes with holes of 10 and 20 μm nominal diameter (Osmotics, Livermore, CA) were coated on one side with Cell-Tak cell and tissue adhesive (Collaborative Biomedical Products, Bedford, MA), left at room temperature for 10 minutes until semi-dry and attached to the bottom of 6-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ).

Cells

CD-1 strain mice (Charles River) were mated and the day of finding a vaginal plug was designated as day 0 of embryonic development. Mesenchymal cells were isolated by differential plating (Schuger et al., 1993), from embryonic intestine and lung on day 11 and from kidney on day 12 of gestation, prior to the onset of SM differentiation. Absence of SM-specific proteins was confirmed by immunohistochemistry (not shown) and western blot analysis (Fig. 1) that showed no SM-specific proteins earlier than day 12 in intestine and lung and no SM-specific proteins in the kidney up to day 15 of gestation, the latest gestational age studied. The cells were isolated directly on the dishes containing the multi-perforated membranes. To assess cell viability and metabolic state of the round and elongated cells, one well of each was labeled for three hours with [35S]methionine every 24 hours. The radiolabeled methionine was incorporated each day was then determined for a total period of 4 days.

Antibodies and immunoblot analysis

The following antibodies were used for detection of SM-specific proteins: a mouse monoclonal antibody to SM α-actin (Boehringer Mannheim Biochemica, Indianapolis, IN) at a concentration of 0.25 μg/ml, a mouse monoclonal antibody to desmin (Dako, Carpinteria, CA) at a concentration of 1.125 μg/ml, a mouse monoclonal antibody to calponin (Sigma, St Louis, MO) at a concentration of 3.8 μg/ml, rabbit polyclonal antibodies to SM-22 (a gift from Dr Rodrigo Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ) at a concentration of 0.2 μg/ml, and rabbit polyclonal antibodies to SM-myosin (Biomedical Technologies, Stoughton, MA) at a concentration of 10 μg/ml. A rat monoclonal antibody against laminin β1 chain was purchased from Chemicon (Temecula, CA) and at a concentration of 1.2 μg/ml and a polyclonal antibody against α-fetoprotein (Dako) was used at a concentration of 0.45 μg/ml. Mouse monoclonal antibodies against MAPKs 42 and 38 were obtained from Zymed Laboratories Inc., (San Francisco, CA) and a mouse monoclonal antibody against Rac 1 and another against phosphotyrosine were purchased from Upstate Biotechnology (Lake Placid, NY). All of them were used at a concentration of 1 μg/ml.

Cell cultures were lysed by boiling for 10 minutes in reducing sodium-dodecyl-sulfate (SDS) sample buffer. 30 μg of sample were resolved in a 12% acrylamide gel. The samples were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and blocked with 5% non-fat dry milk in TBS-T (20 mM Tris base, 137 mM NaCl, 0.05% Tween-20, pH 7.6, all from Bio-Rad). The membranes were then blotted for 1 hour to antibodies to either SM α-actin (0.25 μg/ml), desmin (1.125 μg/ml), SM22 (0.2 μg/ml) or myosin (1:150 dilution). This was followed by 1 hour of incubation with a 1:3000 dilution of the appropriate secondary antibody. The bands were detected by chemiluminescence using a commercial kit (Amersham Life Science, Arlington Heights, IL) according the manufacturer's instructions. In some studies the membranes were probed with antibodies to phosphotyrosine, stripped and reprobed with antibodies against signal transduction proteins.

Immunohistochemistry

Cell cultures were fixed for 5 minutes in absolute ethanol. The slides were then treated with 5 μg/ml of monoclonal antibody to desmin for 45 minutes at room temperature. The sections were washed in PBS and exposed to a 1:50 dilution of goat anti-mouse IgG (Sigma) for 30 minutes at room temperature and staining was completed using a commercial peroxidase-anti-peroxidase kit (Vector), following the manufacturer’s instructions. To identify epithelial cells, the cultures were immunostained with anti-cytokeratin antibodies using a commercial peroxidase-anti-peroxidase kit (Vector), following the manufacturer’s instructions.

RT-PCR

The following primers were used for PCR. SM α-actin: 5′ forward primer, 5′-ttcctggagaagactgtgagc-3′ and 3′ reverse primer, 5′-gtagcctcgctgactcgttg-3′; desmin: 5′ forward primer, 5′-gtagcctcgctgactcgttg-3′ and 3′ reverse primer, 5′-gtagcctcgctgactcgttg-3′; α-fetoprotein: 5′ forward primer, 5′-gtagcctcgctgactcgttg-3′ and 3′ reverse primer, 5′-gtagcctcgctgactcgttg-3′; for SM-myosin: 5′ forward primer, 5′-gtagcctcgctgactcgttg-3′ and 3′ reverse primer, 5′-gtagcctcgctgactcgttg-3′; for SM-22: 5′ forward primer, 5′-gtagcctcgctgactcgttg-3′ and 3′ reverse primer, 5′-gtagcctcgctgactcgttg-3′; and for SM-myosin: 5′ forward primer, 5′-gtagcctcgctgactcgttg-3′ and 3′ reverse primer, 5′-gtagcctcgctgactcgttg-3′. The RT-PCR were performed with the GeneAmp® RNA PCR kit (Perkin Elmer, Foster City, CA) following the manufacturer’s instructions. 25 cycles were run for all amplifications besides SM-α actin and myosin, which were run for 35 cycles. Message for all the SM-specific proteins, except SM-α actin and SM myosin, was detected in the round cells if the number of cycles was increased above 30.

Patch-clamp recordings

Cellular electrical properties were recorded using established protocols (Przywara et al., 1991). Culture dishes with attached cells were mounted on the stage of an inverted microscope (Nikon diaphot...
with Hoffman Contrast Modulation optics). The bathing solution contained (in mM) 120 NaCl, 4.7 KCl, 1 MgCl₂, 2.5 CaCl₂, 10 Hepes buffer, 10 glucose and 20 tetraethylammonium-Cl (TEA), adjusted to pH 7.3 with NaOH. Electrodes of 0.8-3 MΩ resistance contained (in mM) 100 CsCl, 5 MgCl₂, 10 EGTA, 40 Hepes buffer, adjusted to pH 7.3 with CsOH. The whole-cell variation of the patch-clamp technique, in current clamp mode, was used to record membrane potential immediately after rupture of the cell patch. The amplifier was then switched to voltage clamp mode and Ca²⁺ current evoked by a 200 ms second step depolarization from a holding potential of -70 mV to 0 mV.

The signal from the patch clamp amplifier (EPC&, List-electronic) was digitally stored and analyzed with the pCLAMP software (Axon instruments). Data was filtered (8-pole Bessel, Frequency Devices) at 3 kHz and digitized at 25-80 kHz. Capacitative and linear leak currents were subtracted on-line using the P/n protocol, with n chosen such that the largest subpulse would not be expected to evoke voltage-dependent currents.

**Treatments**

Mesenchymal cells isolated from mouse embryonic lung on day 11 of gestation and from lung and intestine on day 12 of gestation, were cultured for 24 hours under conditions that promote either cell rounding or cell elongation, and concomitantly treated with RA (0.1 μM; kindly provided by Dr James Varami), TGF-β1 (1 ng/ml; R&D Systems, Minneapolis, MN), PDGF-BB (20 ng/ml; kindly provided by Dr Heong-Reh Kim) and epithelial-conditioned medium. For the latter studies, epithelial cell monocultures were generated and cultured for 24 hours as previously described (Schuger et al., 1993). The culture medium was then removed and immediately used to generate round and elongated mesenchymal cell monocultures. The controls were generated using either fresh medium or medium obtained from 24 hour-old mesenchymal cultures (round and elongated).

**RESULTS**

Embryonic mesenchymal cells attached to 10 μm diameter microsurfaces conserved their original round shape and diameter (undifferentiated mesenchymal cell diameter in vivo ranges from 8-12 μm). Embryonic mesenchymal cells attached to 20 μm diameter surfaces became elongated, with a shape similar to their in vivo counterparts (elongated SM cell precursors and fetal SM cells have a diameter of 20-30 x 2-5 μm). These results were observed regardless of whether the cells were isolated from intestine (abundant visceral muscle), lung (some visceral muscle) or kidney (no visceral muscle) (Fig. 2). Metabolic radiolabeling studies performed every 24 hours for a period of 4 days indicated that round and elongated cells were metabolically active. After 48 hours, the round and elongated mesenchymal cells incorporated radiolabeled methionine at the rates of 20,500±500 and 33,000±700 cpm/μg protein, respectively (round:elongated ratio = 0.61). In the next 2 days, methionine incorporation by elongated cells decreased to 22,000±500 cpm/μg protein (round:elongated ratio = 0.93).

Within 24 hours in culture, the elongated cells differentiated into SM, whereas the round cells remained undifferentiated as long as they conserved their shape. SM differentiation was demonstrated by the expression of SM-myosin, SM-α actin, SM-γ actin, calponin, SM-22 and desmin (Fig. 3A-C) and by the development of membrane potentials of -60 mV and voltage-dependent Ca²⁺ currents, characteristic of excitatory cells (Fig. 3D). The round cells remained negative for SM markers, regardless of the organ of origin, had membrane potentials of -30 mV or less and showed no voltage-activated current. The last two are characteristic of non-excitable cells. These results were consistently observed in all the cells regardless of the organ of origin (Fig. 3). The synthesis of ubiquitously expressed proteins such as laminin β1 (Fig. 3A) and γ1 (not shown) chains was not affected by cell shape. Notice that round cells had more α-fetoprotein than elongated cells (Fig. 3A).

Round cells were able to differentiate into SM when transferred to culture conditions that facilitated cell elongation, (Fig. 4A). Elongated cells, however, did not lose their SM differentiation markers when forced to re-adopt a round configuration (Fig. 4A).

To eliminate the potential effect of cell proliferation on the differentiation process, the round and elongated cells were cultured under conditions that prevented cell proliferation (tenfold reduction in serum concentration; Yang et al., 1998). Upon elongation, the growth-arrested mesenchymal cells differentiated into SM in an identical manner to their proliferating counterparts, indicating that the process was independent of cell proliferation (Fig. 4B).

Western blotting with antibodies against phosphotyrosine, followed by reprobing with various signal transduction proteins, showed that cell elongation resulted in increased synthesis and phosphorylation of MAPK 38, which preceded the synthesis of SM-specific proteins (Fig. 5). The synthesis of MAPK 42 and Rac 1 remained unchanged during the whole process and neither of them showed phosphorylation (Fig. 5). The synthesis of FAK was not determined by specific antibodies; however, no phosphorylated proteins were observed around its molecular position (160 kDa).
Exposure of round and elongated cells to TGF-β1, PDGF-BB, RA and epithelial-conditioned medium showed no major changes in the expression of SM-specific proteins as controlled by the cell’s shape (Fig. 6). However, TGF-β1 had a mild stimulatory effect on SM-specific protein synthesis, whereas PDGF-BB had a mild inhibitory effect on SM differentiation.

**DISCUSSION**

Here we have determined the role of cell shape in controlling SM myogenesis. The rationale behind these studies was that visceral SM development is shortly preceded by a change in precursor cell shape from round to elongated. Furthermore, we recently observed that embryonic lung explants exposed to a monoclonal antibody against laminin α1 chain showed abnormalities in peribronchial mesenchymal cell configuration and had less SM α actin and desmin (Schuger et al., 1997).

In the current study, undifferentiated mesenchymal cells were isolated from three embryonic organs committed to produce either abundant, intermediate or no visceral SM. To control cell shape we used culture dishes with multi-perforated polycarbonate membranes attached to their bottom. Since polycarbonate does not support cell attachment, by selecting reduced cell adhesion.
membranes with 10 µm and with 20 µm diameter perforations we created culture microsurfaces that allowed either cell rounding or cell elongation. This tissue culture approach resulted in cells that conserved the shape and diameter of undifferentiated mesenchymal cells, or became elongated as the SM cell precursors during the onset of SM formation.

Within 24 hours in culture, the elongated cells differentiated into SM, whereas the round cells remained undifferentiated as long as they conserved their original shape. These cells, however, were able to differentiate into SM when transferred to culture conditions that facilitated cell elongation. In comparison, the SM phenotype was not reversed when elongated cells were forced to re-adopt a round configuration.

We initially considered the possibility that the round cells may not differentiate due to the potential stress of limiting their attachment surface. This possibility, however, was ruled out by observing that the round cells incorporated radioactive methionine and synthesized non-SM proteins such as laminin β1 and γ1 chains in similar amount as elongated cells and even synthesized more α-fetoprotein than their elongated counterparts.

Since identical responses were observed in all the cells, regardless of the organ of origin, these studies provided two important clues about the process of SM myogenesis. First, that the potential for SM differentiation is shared by most embryonic mesenchymal cells, whether committed or not to form SM. Second, that cell shape plays a prime role in controlling myogenesis, to such an extent that in vitro manipulation of the cell’s shape can overcome its natural differentiation pathway. The critical role of cell elongation as a triggering factor for myogenesis was demonstrated by the response of kidney mesenchymal cells. Although originating from an organ with no visceral SM, these cells consistently differentiated into SM upon elongation. In contrast, the role of cell rounding in preventing myogenesis was indicated by the response of cells isolated from a highly muscular organ such as intestine. According to the developmental stage at the time of isolation, the same intestinal mesenchymal cells should have undergone SM differentiation within 24 hours. However, in culture these cells did not differentiate as long as they conserved their original round shape.

Membranes with 10 µm-diameter perforations did not allow space for cell growth. However, membranes with 20 µm diameter perforations did allow some cell proliferation, due to overlaps between holes, as seen in Fig. 2. Hence, the two types of membranes used in these studies differed, although slightly, in their potential for promoting cell proliferation. Then, as part of these studies, we tested the effect of cell proliferation on SM

**Fig. 5.** Immunoblots of round and elongated mesenchymal cells using antibodies against phosphotyrosine, MAPK 38, MAPK 42 and Rac 1. (A) Membrane blotted with anti-phosphotyrosine and reprobed with anti-MAPK 38. Notice the increment in synthesis and phosphorylation of MAPK 38 in elongated cells. (B) No phosphorylation and no alteration in synthesis of MAPK 42 and Rac 1 occurred upon cell elongation. (C) Shows the concomitant synthesis of SM α-actin. hs, hours in culture.

**Fig. 6.** Mesenchymal cells isolated from mouse embryonic lung on day 11 of gestation were cultured for 24 hours under conditions that promote either cell rounding or cell elongation and treated with RA (0.1 µM), TGF-β1 (1 ng/ml), PDGF-BB (20 ng/ml) and epithelial-conditioned medium. None of the treatments stimulated differentiation of the round cells, although TGF-β1 had a mild stimulatory effect on SM-specific protein synthesis whereas PDGF-BB had the opposite effect.
differentiation. We observed, however, that when cell growth was arrested by a tenfold reduction in fetal bovine serum concentration, we obtained the same results as those seen with the usual serum levels, demonstrating that the proliferative status of the cells does not affect the control over differentiation exerted by the cell’s shape.

The possibility that what induces or prevents SM differentiation is not the cell shape but the amount of surface area available to the cells cannot be excluded. Similarly, the potential differences in diffusion when cells are grown in small vs larger wells and how this may affect SM differentiation are not clear. It is more likely, however, that the mechanical tensions generated by cell spreading/elongation, and their absence in the round cells, may play a role in determining how the cell’s shape controls SM differentiation. Ongoing studies in our laboratory seem to support this mechanism.

In recent years it has been shown that changes in cell shape activate signaling pathways involving mainly FAK, MAPK 42 and Rac 1 (Wang et al., 1993; Schiaepfer et al., 1994; Zhu and Assoian, 1995; Price et al., 1998; Kheradmand et al., 1998). Interestingly, none of these proteins was activated during the process of cell elongation. We found, however, that cell elongation activated MAPK 38 and that its activation preceded the synthesis of SM-specific proteins. Our studies therefore suggested that MAPK 38, but none of the other signaling molecules previously associated to cell shape regulation, is likely to play a role during SM differentiation. In previous studies cell rounding and elongation were mainly induced by detaching and attaching cells, whereas here the two different cell shapes were established by controlling cell spreading. It is likely that different signaling proteins are activated during the initial phase of cell attachment and the following process of cell spreading.

The tight anatomical relationships between visceral SM and epithelium, and vascular SM and endothelium led to the prevailing idea that inductive and/or permissive heterotypic cell interactions may be required for SM myogenesis (Cunha et al., 1992; Li et al., 1996). Studies using tissue recombinants, in which a fragment of epithelium is assoicated to a fragment of mesenchyme, seem to support this view. SM differentiation has been shown in recombinants of embryonic uterus (Cunha et al., 1992), intestine (Duluc et al., 1994) and urinary bladder (Baskin et al., 1996). In such cases the differentiation of mesenchymal cells into SM has been attributed to epithelial signals which, in the form of diffusible factors/gradient, induce differentiation of the surrounding mesenchyme. Among the factors known to participate in epithelial-mesenchymal interaction, TGF-β1 and RA have been shown to stimulate (Orlani et al., 1994; Blank et al., 1995; Hirschi et al., 1998; Serini et al., 1998), whereas PDGF BB has been reported to inhibit SM differentiation (Holycross et al., 1992; Hirschi et al., 1998). Our studies showed that none of these effectors stimulated round cells to differentiate or prevented elongated cells from differentiating. TGF-β1, however, stimulated the synthesis of SM-specific proteins on elongated cells, whereas PDGF-BB inhibited it. This is the characteristic response of SM cells to the two cytokines (Orlani et al., 1994; Hirschi et al., 1998; Serini et al., 1998; Holycross et al., 1992) and it was observed by us in cultures of adult intestinal SM cells, whether round or elongated (Y. Yang and L. Schuger, unpublished observations).

The cell shape has been shown to control important biological functions, including the activation of transcription factor NF-kappa B (Rosette and Karin, 1995), the expression of interleukin-1 and collagenase-1 by synovial fibroblasts (Kheradmand et al., 1998) and even the switching between cell growth or apoptosis (Chen et al., 1997). However, no previous studies have shown a causative link between cell shape and cell lineage determination.

Although our findings challenge the importance of epithelial-derived biochemical signals in the induction of visceral myogenesis, they do not imply that the epithelium is not required for visceral myogenesis. On the contrary, our studies suggest that the epithelium may be instrumental in the process of visceral myogenesis since its presence is likely to affect the shape of surrounding mesenchymal cells. Developing epithelia may cause mesenchymal cell elongation by generating new basement membrane on what surrounding mesenchymal cells attach, spread and elongate (Yang et al., 1998). In addition, developing epithelial tubes, such as bronchi and intestine, may induce mesenchymal cell elongation by their gradual increment in caliber, whereby causing the surrounding mesenchymal cells to stretch. Our ongoing studies seem to support this mechanism.

In summary, our findings provide a new insight on the process of visceral myogenesis by demonstrating the potential of embryonic mesenchymal cells to differentiate into SM and its control by the cell’s shape. We propose that during development, mesenchymal cell elongation initiates visceral myogenesis, whereas cell rounding prevents ectopic SM formation.

REFERENCES


