Communication

In Vitro System for Differentiating Pluripotent Neural Crest Cells into Smooth Muscle Cells*

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The change in vascular smooth muscle cells (SMC) from a differentiated to a dedifferentiated state is the critical phenotypic response that promotes occlusive arteriosclerotic disease. Despite its importance, research into molecular mechanisms regulating smooth muscle differentiation has been hindered by the lack of an in vitro cell differentiation system. We identified culture conditions that promote efficient differentiation of Monc-1 pluripotent neural crest cells into SMC. Exclusive Monc-1 to SMC differentiation was indicated by cellular morphology and time-dependent induction of the SMC markers smooth muscle α-actin, smooth muscle myosin heavy chain, calponin, SM22a, and APEG-1. The activity of the SM22a promoter was low in Monc-1 cells. Differentiation of these cells into SMC caused a 20–30-fold increase in the activity of the wild-type SM22a promoter and that of a hybrid promoter containing three copies of the CARG element. By gel mobility shift analysis, we identified new DNA-protein complexes in nuclear extracts prepared from differentiated Monc-1 cells. One of the new complexes contained serum response factor. This Monc-1 to SMC model should facilitate the identification of nodal regulators of smooth muscle development and differentiation.

Arteriosclerosis and its complications (heart attack and stroke) are the major causes of death in developed and developing countries (1), and the change in vascular smooth muscle cells (SMC) from a differentiated to a dedifferentiated state is the critical phenotypic response that promotes occlusive arteriosclerotic disease (1–3). Despite its importance, we know little about the genes that regulate SMC differentiation (2). This is in contrast with our more thorough understanding of transcription factors involved in the differentiation of skeletal muscle cells (4–7). A major reason for the rapid progress in our understanding of skeletal as opposed to smooth muscle biology is the availability of an in vitro system for studying differentiation of skeletal muscle cells. The ability to induce myogenic differentiation by the use of demethylating agents and serum starvation in 10T1/2 cells and C2C12 cells, respectively, has been critical to the study of skeletal muscle development and differentiation (8, 9). Indeed, the establishment of immortalized myogenic cell lines may have been the single most important development in the field of skeletal myogenesis (10). Until the neural crest cell to SMC differentiation system described here, a way of rapidly and uniformly inducing precursor cells to differentiate into SMC had been lacking.

Pluripotent neural crest cells can differentiate into neurons, glia, chondrocytes, melanocytes, and SMC (11–13). Arterial SMC of the chic ascending and thoracic aorta are of a neural crest origin (14–16), and various members of the transforming growth factor-β superfamily can instructively promote differentiation of primary cultured neural crest cells into neuronal cells or SMC (12). Unfortunately, our ability to work with neural crest cells in primary culture has been limited by the difficulty of obtaining quantities sufficient for biochemical and genetic analysis. This problem was solved recently by the generation of an immortalized neural crest cell line, Monc-1, by retroviral transfection of mouse neural crest cells with the v-myc gene (17, 18).

We hypothesized that Monc-1 cells could be used to develop an in vitro SMC differentiation system. We describe in this report the culture conditions under which Monc-1 cells can be differentiated efficiently into SMC. Exclusive Monc-1 to SMC differentiation was indicated by cellular appearance and induction of the SMC markers smooth muscle α-actin, smooth muscle myosin heavy chain, calponin, SM22a, and APEG-1. Also, a 20–30-fold increase in the activity of the SMC-specific promoter SM22a coincided with the formation of new DNA-protein complexes during differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The Monc-1 cell line was kindly provided by David Anderson (Pasadena, CA). Monc-1 cells were cultured in the undifferentiated state on fibronectin-coated plates in an L-15 CO2-based medium supplemented with chick embryo extract, hereafter referred to as complete medium, as described by Stemple and Anderson (11). Differentiation down the neuronal and glial pathways was performed on plates coated sequentially with poly-b-lysine (0.5 mg/ml) and fibronectin (0.25 mg/ml) in complete medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) plus 5 μM forskolin (Sigma) as described (17). SMC differentiation was induced by application of M199 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 μM HEPE (pH 7.4), hereafter referred to as SMC differentiation medium (SMDM).

RNA Extraction, Northern Analysis, and Reverse Transcription Po-
lymase Chain Reaction (PCR)—Total RNA from cultured cells was prepared by guanidinium isothiocyanate extraction and centrifugation through cesium chloride (19). RNA from mouse aorta was prepared by the RNAzol B method (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions. Total RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose filters, which were hybridized with the appropriate, randomly primed, 32P-labeled probe (19, 20). The smooth muscle α-actin was provided by J. Lessard (Cincinnati, OH); the angiotsin type II receptor (AT2) cDNA was provided by A. D. Strosberg (Paris, France). The calponin cDNA was isolated from a mouse aortic cDNA library.

mRNAs for the smooth muscle myosin heavy chain isoforms SM1 and SM2 were detected by reverse transcription PCR with primers designed from the mouse SM1 and SM2 cDNAs (GenBank accession numbers D85923 and D85924, respectively). The forward primer 5′-AGGAA-CACCAAGGTCAAGCA-3′ and the reverse primer 5′-GGGACTGTAC-TACAGGTTAG-3′ were used to amplify a 324-base pair SM1 fragment and a 363-base pair (alternatively spliced) SM2 fragment. To control for efficiency of reverse transcription, an aliquot of template cDNA was analyzed by PCR with a forward primer (5′-TGAAAGCTCGGTGTGAA-3′) and a reverse primer (5′-CATCTAGGCGCATGGGG-TCCACACC-3′) designed from the mouse glyceraldehyde-3-phosphate dehydrogenase cDNA sequence.

Immunocytochemistry and Western Analysis—Monc-1 cells were grown on glass slides coated with fibronectin or fibronectin plus d-lysine in the appropriate medium (see “Cell Culture and Reagents”). Immunostaining for smooth muscle α-actin, calponin, glial fibrillary acidic protein, and peripherin was performed as described (12, 21). Proteins from undifferentiated and differentiated Monc-1 cells and mouse aortas were prepared according to standard procedures (19) with minor modifications. Proteins were resolved on 5% sodium dodecyl sulfate-polyacrylamide gels (22), transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell), and incubated with a rabbit anti-smooth muscle myosin heavy chain antibody (23) (kindly provided by U. Groschel-Stewart of London, UK) diluted 1:5000, followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 1:4000. Membranes were processed with an enhanced chemiluminescence reagent (Pierce) and exposed to film.

Transfection and Luciferase Assays—A 1.4-kilobase fragment of the SM2α promoter was obtained by PCR with mouse genomic DNA and the following primers: forward 5′-CAGTGCTGGAACCCAAGGC-3′ and reverse 5′-GGGCTGGGGCCAGACGG-3′. The promoter fragment was subcloned into the pGL2-Basic vector (Promega, Madison, WI). Generation of the multimerized CarG and CarG mutant constructs in the pGL2-Basic vector will be described elsewhere.2 Monc-1 cells were transfected transiently by electroporation as described (24). Cell extracts were prepared 48–72 h after transfection, and luciferase and β-galactosidase assays were performed as described (25, 26). Each construct was transfected at least six times. Data for each construct are presented as the mean ± S.E.

Electrophoretic Mobility Shift Analysis—Nuclear extracts were prepared according to the method of Ritzenthaler et al. (27) with minor modifications. Electrophoretic mobility shift analysis was performed as described (28, 29). In brief, double-stranded oligonucleotide probes synthesized according to the sequence of the SM2α CarG element 5′-TCGAGACTTGTTGCTTTTCGCCAACATGAGCCTGTGTGGAGTG-3′ were radiolabeled as described (21). The reaction mixture was incubated at room temperature for 20 min and analyzed by 5% native polyacrylamide gel electrophoresis in 0.25 × TBE buffer (22 mM Tris base, 22 mM boric acid, and 0.5 mM EDTA). A 250-fold excess of specific or nonspecific oligonucleotide was used for competition experiments. For supershift experiments, 1 μl of antibody to serum response factor (sc-335X, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-YY1 antibody (sc-281X, Santa Cruz) was incubated with nuclear extracts and probes.

RESULTS AND DISCUSSION

Identification of Culture Conditions That Promote Monc-1 to SMC Differentiation—Monc-1 cells on fibronectin-coated plates can be maintained in the undifferentiated state in an L-15 CO2-based medium supplemented with chick embryo extract (11, 17). After confirming that Monc-1 cells incubated in this complete medium expressed the low-affinity nerve growth factor receptor (data not shown), a marker for undifferentiated neural crest cells (11, 12, 17), we looked for culture conditions that would allow us to differentiate the Monc-1 cells down the smooth muscle lineage.

Although several media had little effect on the Monc-1 phenotype, culturing the cells in M199 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mM HEPES (pH 7.4) induced a dramatic morphologic change. We studied two different lots of fetal bovine serum and observed no difference in their ability to induce Monc-1 cell differentiation.

Within 24 h of placement in this SMDM, the cells began to assume a flat, fusiform appearance and the size of the cytoplasm increased. By 4 days of culture in SMDM nearly 100% of the cells had assumed this form (Fig. 1A, bottom). In comparison with undifferentiated Monc-1 cells (Fig. 1A, top), cells cultured in SMDM grew much more slowly. At confluence the differentiated cells took on the “hill and valley” appearance characteristic of differentiated SMC (not shown). Immunostaining with antibodies specific to smooth muscle α-actin (Fig. 1B) and calponin (Fig. 1C), two well established markers of the smooth muscle lineage (2), revealed robust expression of these genes in Monc-1 cells cultured in SMDM for 4 days but not in controls. As expected, differentiation of Monc-1 cells into glial and neuronal cells (17, 18) was accompanied by immunoreactivity to glial fibrillary protein (Fig. 1D) and peripherin (not shown). In contrast, SMC differentiated from Monc-1 cells did not stain for glial acidic fibrillary protein or peripherin (not shown).

Time-dependent Induction of SMC Markers in Response to Monc-1 Cell Differentiation—We also measured expression of the mRNAs for these and other smooth muscle markers over the course of Monc-1 cell differentiation in SMDM (at 2, 5, and

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8 days). RNA from mouse aorta was used as a positive control for differentiated SMC. As a negative control, Monc-1 cells were induced to differentiate down the neuronal and glial pathways (17, 18) in a parallel experiment. Smooth muscle α-actin and calponin mRNA expression increased as early as 2 days after placement in SMDM (Fig. 2). Neither message was detected after differentiation down the neuronal pathway.

Another well studied marker of SMC is the SM22α gene (2, 28, 30–32), which is expressed exclusively in vascular and visceral SMC in adult animals (28). As with the smooth muscle α-actin and calponin mRNAs, expression of the SM22α mRNA increased after incubation in SMDM (Fig. 2). APEG-1, a nuclear protein preferentially expressed in vascular SMC, was cloned recently by our laboratory (33). Because its expression is high in arterial SMC in vivo and undetectable (by passage 3) in dedifferentiated arterial SMC in culture (33), APEG-1 represents a sensitive marker for differentiated SMC. Expression of APEG-1 mRNA also increased during Monc-1 cell culture in SMDM (Fig. 2), indicating that these cells share properties of differentiated vascular SMC. The last marker we tested in this experiment, the AT2, is expressed in the vasculature and many other tissues during embryogenesis (34). Although AT2 expression is down-regulated in the adult vasculature, it continues in adult neuronal cells (34). As Fig. 2 shows, AT2 mRNA was expressed in undifferentiated Monc-1 cells (day 0). Although AT2 receptor mRNA was still visible after 2 days of incubation in SMDM, it disappeared after 5 and 8 days of incubation. AT2 mRNA expression increased as neuronal/glial cell differentiation progressed, as reported previously (34). Expression of these five genes in the Monc-1 cell to SMC differentiation system was similar to that in the aorta, where SMC are highly differentiated.

Induction of Smooth Muscle Myosin Heavy Chain Expression in Response to Monc-1 Cell Differentiation—To determine whether smooth muscle myosin heavy chain, a specific marker of differentiated SMC, is expressed in Monc-1 cells, we performed reverse transcription PCR with a pair of primers that amplify 324- and 363-base pair fragments of the SM1 and SM2 isoforms, respectively. The SM1 and SM2 DNA fragments were both amplified from reverse transcribed mouse aorta RNA (Fig. 3A). SM1 was amplified from differentiated but not undifferentiated Monc-1 cell RNA. We then used primers for glyceraldehyde-3-phosphate dehydrogenase to amplify a specific band from all RNA samples. SM1 and SM2 were both detected in samples prepared from mouse aortas by high resolution Western analysis with an antibody to smooth muscle myosin heavy chain (23) (Fig. 3B). Although undifferentiated Monc-1 cells expressed only non-muscle myosin heavy chain (Fig. 3B, asterisk), differentiated Monc-1 cells expressed SM1. Taken together, these data indicate the presence of SM1 in SMC differentiated from Monc-1 cells.

Induction of SM22α Promoter Activity by Monc-1 to SMC Differentiation—The molecular mechanisms regulating expression of the SM22α gene are very well characterized. For example, we know that a cis-acting element, the CARG box (CC ATG G), is critical for expression of SM22α in vascular SMC, in vitro and in vivo (28, 30, 32). To see whether the same cis-acting element is critical to induction of SM22α in Monc-1 cells differentiated down the smooth muscle lineage, we performed transient transfection assays with the SM22α promoter.

First we generated a luciferase reporter construct, −1.4 kilobase SM22α, containing 1.4 kilobases of the SM22α promoter. Monc-1 cells were transfected by electroporation and cultured in complete medium or SMDM, and luciferase activity was measured in cell lysates 72 h after transfection. The −1.4 kilobase SM22α promoter was minimally active in undifferentiated Monc-1 cells (Fig. 4A). After differentiation into SMC, however, promoter activity increased by 20–30-fold. We then generated two more reporter constructs to see whether the CARG element was critical to regulation of SM22α promoter activity after Monc-1 cell to SMC differentiation in vitro. The first, CARG (3X) (Fig. 4A), contained three SM22α CARG elements upstream of the luciferase reporter gene, and the second, mt CARG (3X) (Fig. 4A), contained three CARG elements in which the core sequence had been modified from 5’ CCACATATGG 3’ to 5’ CCACACATGG 3’. This mutated sequence cannot function as an enhancer in SMC. Like the activity of the native SM22α promoter, the activity of the multimerized CARG reporter construct increased dramatically (Fig. 4A) after Monc-1 cell to SMC differentiation, again by about 20-fold. The mutated CARG construct was not active after neural crest cell to SMC differentiation.

Induction of Serum Response Factor DNA Binding Activity by Monc-1 to SMC Differentiation—We hypothesized that specific trans-acting factors may be induced after Monc-1 differentiation that would bind to the CARG element and thereby regulate SM22α expression. We tested this hypothesis in electrophoretic mobility shift assays with the CARG (3X) oligonucleotide used as a probe. Five specific DNA-protein complexes (Fig. 4B) were revealed. Complexes 1 and 4 appeared in nuclear extracts from differentiated Monc-1 cells but not in those from undifferentiated cells. Complex 2 was present under both conditions but appeared to intensify after differentiation. Antibody supershift experiments showed that complex 4 (shifted to 5) contains a protein antigenically identical or related to serum...
SM22α and other genes (28). Together our studies of the SM22α promoter suggest that the genetic program normally instituted in SMC in vivo is recapitulated during Monc-1 cell to SMC differentiation in vitro.

Because the excessive proliferation of vascular SMC is critical to the development of occlusive vascular diseases such as heart attack and stroke, the identification of nodal regulators of SMC differentiation is essential to the design of strategies for treating vascular disease. These nodal genes have been difficult to identify because there has been no system for producing differentiated SMC in vitro. We believe that the Monc-1 cell to SMC differentiation system described here represents the equivalent of the 10T1/2 cell system used to study regulators of skeletal muscle differentiation in vitro (2). Further study in the Monc-1 cell to SMC system may permit identification of analogous regulators of vascular SMC differentiation.

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