Isolation and Culture of Arterial Smooth Muscle Cells From Human Placenta

Courtney E. Leik, Amy Willey, Martin F. Graham, Scott W. Walsh

Abstract—A simple and economical technique was developed to isolate and culture human arterial smooth muscle cells from chorionic plate vessels. Placentas from healthy women were collected at the time of term delivery. Chorionic plate arteries were identified, excised, and cut into small pieces. An explant technique was used to grow cultures of placental arterial smooth muscle (PASM) cells. Small pieces of vessel with lumens down were placed in 100-mm culture plates and grown in Dulbecco modified eagle medium and 10% fetal bovine serum. Cells appeared from explants within 1 week and grew to confluence in approximately 4 weeks. At confluence, PASM cell cultures had a uniform cell morphology that was characterized by elongated cells in parallel rows, typical of smooth muscle cells. Smooth muscle cell phenotype was evaluated by morphology and by immunoblotting and immunofluorescence of smooth muscle myofilament proteins. All PASM cell cultures expressed α-smooth muscle actin, β-tropomyosin, and h-caldesmon. Expression was similar to that of human aortic smooth muscle cells, but not to endothelial cells or fibroblasts. PASM cells stained uniformly for α-smooth muscle actin and lacked staining for a fibroblast-specific antigen. PASM cells were evaluated for their response to inflammatory mediators, tumor necrosis factor-α, and interleukin-1β by measurement of interleukin-8 production. Cells cultured for 18 hours showed a progressive increase in interleukin-8 production with time. Treatment with inflammatory mediators increased interleukin-8 production by 3-fold as compared with media control. This technique provides a simple method to obtain normal human arterial smooth muscle cells for in vitro studies of physiology and pathophysiology. (Hypertension. 2004;43:837-840.)

Key Words: vascular smooth muscle • interleukins • tissue culture

The availability of normal human arterial smooth muscle cells for experimental studies is limited. Primary cultures of vascular smooth muscle cells can be difficult to obtain and grow, and commercially available cells are expensive. Vascular tissue can be obtained from various organs during organ transplant procedures, but access to this tissue is limited. Furthermore, the age of the donor is often advanced and health problems or death of the donor may have altered the phenotype of the cells. There is always a question as to whether donor cells are truly normal. Consequently, many investigators use arterial smooth muscle cells obtained from animal vessels. A readily available source of normal human arterial smooth muscle cells for in vitro studies would be useful.

Recently, we demonstrated increased vascular smooth muscle expression of interleukin-8 (IL-8), a potent chemotactic agent for neutrophils, coincident with neutrophil transendothelial migration into the systemic vasculature of women with preeclampsia, a hypertensive disorder of pregnancy. Increased vascular smooth muscle expression of IL-8 indicated inflammation and dysfunction of vascular smooth muscle in preeclampsia, but the cause of this dysfunction is unknown.

The purpose of this study was to develop a simple and economical technique for the isolation and culture of human arterial smooth muscle cells and to characterize the phenotype of these cells. Such cells would be of value for in vitro experiments to explore mechanisms for vascular smooth muscle cell dysfunction. This has been performed for endothelial cells, which are commonly used for cardiovascular studies, by isolating endothelial cells from human umbilical veins. We applied a similar rationale and developed a simple and economical technique to isolate and culture arterial smooth muscle cells from placental chorionic plate arteries. Smooth muscle cell phenotype was verified by surveying expression of smooth muscle cell myofilamentous proteins and functionality of the cells by responsiveness to inflammatory mediators.

Methods
Placental Arterial Smooth Muscle Cell Isolation and Culture

Placentas were collected at term deliveries from normal pregnant women at MCV Hospitals of Virginia Commonwealth University Medical Center. Informed consent was obtained before delivery.
This study was approved by the Virginia Commonwealth University Office of Research Subjects Protection. Procedures were in accordance with institutional guidelines.

Under sterile conditions, chorionic plate artery specimens were identified and excised from the chorionic plate. The vessels were rinsed 3 times in Hank’s balanced salt solution containing 2× strength antibiotics (200 U/mL of penicillin, 200 μg/mL streptomycin; Gibco, Invitrogen Corp, Grand Island, NY) and 50 U/mL Nystatin (Sigma, St. Louis, Mo). Vessels were cut longitudinally and dissected into small pieces of tissue (3 to 5 mm). Explants with vessel lumens down were placed in 100-mm culture plates (approximately 20 per plate). Five mL of Dulbecco modified eagle medium (Gibco, Invitrogen Corp) supplemented with 10% fetal bovine serum (Gibco, Invitrogen Corp) and 1× strength antibiotics/antimycotic was carefully added to the culture plates so as not to disturb adhered explants. Culture plates were placed in a 37°C incubator (5% CO₂).

Media were removed and replaced with fresh media twice per week. Culture growth and cell morphology were examined daily using an inverted light microscope. Cells started growing from explants within 1 week. At 2 weeks, the volume of media per 100-mm culture plate was increased to 10 mL because of increased numbers of cells.

Cell cultures became confluent in approximately 4 weeks. At confluence, cells were placed in Dulbecco modified eagle medium without serum for 24 hours. This was performed to eliminate any contaminating cells, such as fibroblasts and endothelial cells, because these cells do not survive without serum. After 24 hours, placental arterial smooth muscle (PASM) cells were placed in Dulbecco modified eagle medium with 10% fetal bovine serum for trypsinization, passage, or freezing, according to standard protocols. PASM cells were used between passages 2 and 7.

### Verification of Vascular Smooth Muscle Cell Phenotype

#### Western Blot

Vascular smooth muscle cell phenotype for all PASM cultures was verified by immunoblotting for smooth muscle myofilament proteins: α-smooth muscle actin, β-tropomyosin, and h-caldesmon. Human aortic smooth muscle (HASM) cells (Cambrex, East Rutherford, NJ), adult dermal fibroblasts (Cambrex), and human umbilical vein endothelial cells (HUVEC) were evaluated for comparison. HUVECs were isolated from umbilical veins as previously described⁵ and verified as endothelial cells by cobblestone morphology and positive staining for von Willebrand factor.

Fibroblasts and HASM cells were cultured according to the supplier’s instructions, and HUVECs were cultured according to standard protocols for endothelial cells. PASM cells were seeded and grown to confluence in 100-mm culture plates with 10 mL media 199 (M199) and 10% fetal bovine serum. PASM cells were harvested 6 days after the last addition of fresh media and serum. At confluence, media were removed and exchanged with M199 without serum for 24 hours. Cells were harvested and lysed with Laemmli buffer (250 μL). Cell lysates were diluted with sample buffer (0.5 mol/L Tris-HCl, 0.1% glycerol, 10% SDS, 0.05% 2-mercaptoethanol) to equalize protein concentrations and loaded on a 9% polyacrylamide gel for electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane. After overnight blocking with a 5% solution of dry milk in Tris-buffered saline, the membrane was incubated for 1 hour with mouse monoclonal anti-human primary antibodies (1:1000; Sigma-Aldrich, St. Louis, Mo) directed at α-smooth muscle actin (IgG2a), β-tropomyosin (IgG), and h-caldesmon (IgG), with β-actin (IgG) used as a control for loading.

Blots were then incubated for 1 hour with secondary antibody, a goat monoclonal anti-mouse IgG antibody conjugated to horseradish peroxidase (1:10,000; Roche Molecular Biochemicals, Indianapolis, Ind). Western lightning chemiluminescence reagent plus enhanced luminol (PerkinElmer Life Sciences, Boston, Mass) was used to develop horseradish peroxidase using Kodak film (Hyperfilm MP; Amersham Pharmacia, Piscataway, NJ).

#### Immunofluorescence

PASM cells and fibroblasts were seeded on sterile coverslips in a 24-well plate at 5000 cells per coverslip and grown in M199 and 10% fetal bovine serum for 1 to 2 days. Cells were rinsed with PBS and treated with 500 μL/well permeabilization buffer (50 mmol/L 2-n-morphoelnyl ethane sulfonic acid, 2.5 mmol/L EGTA, 5 mmol/L MgCl₂) with 0.5% Triton and 1% formaldehyde, pH 7, for 3 minutes. Permeabilization buffer was aspirated and cells were fixed with 500 μL well of 3.7% formaldehyde and 0.01% gluteraldehyde in PBS for 20 minutes. Cells were washed with PBS and then incubated at room temperature for 30 minutes in PBS with 1% bovine serum albumin (BSA) and mouse anti-human antibody for α-smooth muscle actin (1:100; Sigma) or for 60 minutes with mouse anti-human antibody for a fibroblast-specific antigen (CD90/Thy-1, 1:10; Oncogene Research Products, San Diego, Calif). To visualize the proteins, cells were treated for 30 minutes with tetramethylrhodamine isothiocyanate conjugate goat anti-mouse IgG (Fab-specific) antibody (1:40; Sigma), which resulted in a red fluorescence. To identify the cells, they were double-stained with fluorescein isothiocyanate-phalloidin (1 mg/mL) (1:40; Sigma), which stained filamentous actin (f-actin) green. Cells were rinsed and mounted on glass slides. Negative controls were performed in parallel, except that cells were treated with a solution of 1% BSA in PBS without primary antibody.

#### Response to Inflammatory Mediators

To determine responsiveness to inflammatory mediators, PASM cells were cultured overnight in M199 media in 24-well culture plates and media were collected at 2, 4, 6, and 18 hours to be analyzed for IL-8 production. To demonstrate that PASM cells could be stimulated, cells were treated with tumor necrosis factor-α (TNF-α) (1 ng/mL) or IL-1β (1 ng/mL) for 18 hours, and media were analyzed for IL-8 production. To compare PASM cell function to that of commercially available vascular smooth muscle cells, treatments also were performed using HASM cells. HASM cells, however, were grown in T-75 flasks because of difficulty with growing HASM cells. Cell media were analyzed for IL-8 by enzyme-linked immunosorbent assay using commercially available reagents (R & D Systems, Minneapolis, Minn). Continuous data were analyzed by 1-way ANOVA with Newman-Keuls post hoc test using Prism software (GraphPad Software, San Diego, Calif).

### Results

Cells were observed growing from chorionic plate vessel explants within 1 week (Figure 1a). Primary cultures of PASM cells grew to confluence in approximately 4 weeks (Figure 1b). At confluence, PASM cell cultures had uniform cell morphology that was characterized by elongated cells in ridges, which is characteristic of smooth muscle cell cultures. PASM cell morphology was retained after several passages.
All PASM cell cultures expressed smooth muscle myofilament proteins: α-smooth muscle actin (MW: 42 kDa), β-tropomyosin (MW: 36 and 39 kDa), and h-caldesmon (MW: 150 kDa) (Figure 2). PASM cultures showed similar protein expression to that of HASM cells. HUVECs and fibroblasts did not express α-smooth muscle actin or h-caldesmon.

PASM cell cultures showed uniform immunostaining for α-smooth muscle actin (Figure 3a). PASM cell cultures did not stain for a fibroblast-specific protein (Figure 3c), which strongly stained fibroblast cultures (Figure 3e). Panels b, d, and f of Figure 3 show staining for f-actin to identify cells in panels a, c, and e, respectively. Negative controls for α-smooth muscle actin and fibroblast-specific protein had no staining (data not shown).

Figure 2. Western blot for smooth muscle myofilament proteins. All PASM cell cultures expressed smooth muscle myofilament proteins. PASM protein expression was similar to that of HASM cell cultures. HUVECs and fibroblasts expressed tropomyosin, but not h-caldesmon or α-smooth muscle actin.

Figure 3. Immunofluorescence for α-smooth muscle actin and fibroblast surface antigen (CD90/Thy-1). PASM cells showed uniform filamentous expression of α-smooth muscle actin (a). PASM cell staining for a fibroblast-specific surface antigen was similar to background (c). Fibroblasts were run as a positive control for the fibroblast-specific surface antigen. Fibroblasts strongly expressed the fibroblast-specific surface antigen (e). Cells were identified by costaining for f-actin (b, d, and f). Magnification is 400×. Scale bar window that can be seen in the lower right corner (e) is equal to 50 μm.

Figure 4. Time course for the spontaneous production of IL-8 by PASM cells cultured in M199 for 2, 4, 6, and 18 hours. Concentrations of IL-8 increased progressively during 18 hours of incubation (n=5). Data represent mean ±SE.

PASM cells produced IL-8. A time-course for PASM cells cultured overnight in M199 showed a progressive increase in IL-8 production (Figure 4). Stimulation with TNF-α or IL-1β significantly increased IL-8 production by PASM cells and HASM cells as compared with media control (Figure 5a and 5b, respectively).

**Discussion**

In this study, we describe a simple and economical method to obtain human arterial smooth muscle cells for in vitro studies using chorionic plate arterial explants. PASM cells started growing from explants within 1 week and cultures reached confluence in approximately 4 weeks. At confluence, primary cultures of PASM cells had uniform cell morphology that was characterized by elongated cells in parallel rows.

Immunoblotting and immunostaining indicated that the established primary PASM cell cultures were vascular.
smooth muscle cells and were not contaminated with endothelial cells or fibroblasts. All PASM cell cultures expressed smooth muscle contractile proteins: α-smooth muscle actin, β-tropomyosin, and h-caldesmon. HASM cells showed similar expression to PASM cells, while HUVECs and fibroblasts did not express α-smooth muscle actin or h-caldesmon. PASM cells showed uniform immunostaining of α-smooth muscle actin and lacked immunostaining of a fibroblast-specific antigen.

PASM cells were responsive to inflammatory mediators as evidenced by production of IL-8. A time-course for PASM cells cultured overnight showed a progressive increase in IL-8 production. Stimulation with TNF-α or IL-1β increased IL-8 production by PASM cells and HASM cells, demonstrating that PASM cells respond similar to HASM cells and that they were functionally active and responsive to inflammatory mediators.

Perspectives
These data suggest that PASM cells are an alternative to donor or commercially available arterial smooth muscle cells. Placentas are readily available and an inexpensive source of arterial smooth muscle. Donor age and questionable health problems are not a concern because medical information regarding the health of the mother and sex of the baby are easily obtained. In conclusion, we have established a PASM cell isolation technique, which is a simple and economical method to obtain normal human arterial smooth muscle cells for in vitro studies. PASM cells, like HUVECs, may prove useful for the study of cardiovascular disease.

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References
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