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Isolation of vascular smooth muscle cells from a single murine aorta

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Abstract. The vascular smooth muscle cell plays a significant role in many important cardiovascular disorders, and smooth muscle biology is therefore important to cardiovascular research. The mouse is critical to basic cardiovascular research, largely because techniques for genetic manipulation are more fully developed in the mouse than in any other mammalian species. We describe here a technique for isolating smooth muscle cells from a single mouse aorta. This technique is particularly useful when material is limiting, as is frequently the case when genetically modified animals are being characterized.

Key words: Smooth muscle, Tissue culture, Transgenic, Vascular biology

1. Introduction

There are two common approaches to isolating vascular smooth muscle cells. The first involves explanting vascular tissue and then allowing time for smooth muscle cells to migrate out from the explanted tissue fragments [4]. Using the explant technique, we were able to obtain only 750–1500 cells per murine aorta after two weeks in culture. The explant protocols are therefore generally unsuitable for isolating and characterizing smooth muscle cells from limiting amounts of tissue. The second common approach to isolating vascular smooth cells involves limited enzymatic digestion of vascular tissue followed by plating of the dispersed smooth muscle cells [1, 3]. Using two published versions of protocols that utilize enzymatic digestion, we were unable to isolate any smooth muscle cells at all, despite multiple attempts. We present a protocol utilizing enzymatic digestion that is more detailed than any protocol we were able to find in the literature, and is different in critical respects from those protocols. For example, we found that the specific enzyme preparation used is critically important, and we specify the optimal preparation, whereas other published protocols do not. In addition, we recommend digestion times that in our hands are far superior to the digestion times used in previously published protocols. Our technique allows the isolation of relatively large numbers of cells (at least 6000 cells per murine aorta after 7–10 days in culture) in a predictable manner. We have optimized this technique for use with a single murine aorta, thus allowing the relatively rapid characterization of vascular smooth muscle cells from a particular mouse line when the number of available animals is limited. We therefore expect this technique to be particularly useful in the initial evaluation of genetically modified animals.

2. Materials

I. *Solutions*

- Sodium pentobarbital: 1 gm/ml (catalog #WAB10500, The Butler Company).[1]
- Phosphate buffered saline: (catalog #10010-023, Life Technologies).[2]
- Fungizone solution: 10 µl of 0.25 mg/ml Fungizone (catalog #15290018, Life Technologies)[2] in 10 ml Dulbecco's Modified Eagle Medium (DMEM) (catalog #12430-054, Life Technologies).[2] Filter sterilize. Store at 4 °C, for no more than three weeks.
- Culture medium: DMEM with 10% fetal bovine serum (FBS) (catalog #16000036, Life Technologies),[2] 1% penicillin/Streptomycin (catalog #15140-122, Life Technologies),[2] 1% glutamine (catalog #17-605E, BioWhittaker).[3] Filter sterilize. Store at 4 °C, for no more than four weeks.
- Enzyme solution (for several aortas): 7.5 mg collagenase (type II, product code LS004174, Worthington Biochemical Corporation)[4] in 5.5 ml culture medium. Filter sterilize using 0.22 micron low protein binding filter. Prepare fresh for each isolation procedure.
- 70% Ethanol: 70% (v/v) 200 proof ethanol (catalog #111000200DC05, Pharmco),[8] 30% (v/v) deionized water.

II. *Instruments*

1. The following instruments were obtained from Biomedical Research Instruments (BRI) Inc.[5]
   - Small sharp scissors: BRI #28-1000.
   - Toothed forceps: BRI #10-2440.
   - Straight fine forceps: BRI #10-3150.
   - Angled fine forceps: BRI #10-3110.
   - Microdissecting scissors: BRI #11-1390.
2. A dissecting microscope is also necessary. We used a TMS microscope from Nikon.[9]

III. *General supplies*

- Small tissue culture tubes: Falcon 35-2054, Becton Dickinson.[6]
- 15 ml conical polypropylene tubes: Falcon 352097, Becton Dickinson.[6]
- 48-well plates: Corning Costar 3548, VWR Scientific Products.[7]
- 100 mm Petri dishes: Falcon 353003, Becton Dickinson.[6]
- 3 ml syringes; Falcon 309585, Becton Dickinson.[6]
- 1 ml syringes: BD 309602, Becton Dickinson.[6]
- $26\frac{1}{2}$ gauge needles: BD 305111, Becton Dickinson.[6]

3. Procedures

1. Euthanize mouse by using a 1 ml syringe fitted with a $26\frac{1}{2}$ gauge needle to administer an intraperitoneal injection of 0.25 ml of sodium pentobarbital.
2. Place mouse in supine position on a small styrofoam board. Immobilize by pinning each paw to the styrofoam. Place under a dissecting microscope.
3. Rinse off thorax and abdomen with a 70% ethanol/30% water solution.
4. Incise and remove skin from thorax and abdomen with small sharp sterile scissors and toothed forceps. After skin is removed, dip instruments in 70% ethanol/30% water, then in DMEM.
5. Open thorax to expose heart and lungs. Set scissors and toothed forceps aside and use sterile straight fine forceps and sterile angled fine forceps for the remainder of the dissection.
6. Dissect out the aorta from its origin at the left ventricle to the iliac bifurcation. Leave the aorta attached to the left ventricle.
7. Use a 3 ml syringe fitted with a $26\,{}^{1}/_{2}$ gauge to puncture the left ventricle. Perfuse with 3 ml sterile phosphate buffered saline, so that the aorta is flushed.
8. Remove the aorta, using sterile microdissecting scissors, and place in a 100 mm Petri dish, in a drop or two of Fungizone solution.
9. Dispose of the mouse carcass.
10. Using the straight and angled forceps (the angled forceps to hold down the aorta and the straight forceps to pull off bits of tissue), and working under dissecting microscope, remove adventitia from aorta. The adventitia will appear as ragged bits of tissue coming off the aorta. When it is removed, the aorta will be a smooth tube.
11. Remove aorta from Fungizone and place in a new 100 mm Petri dish, in one or two drops of culture medium.

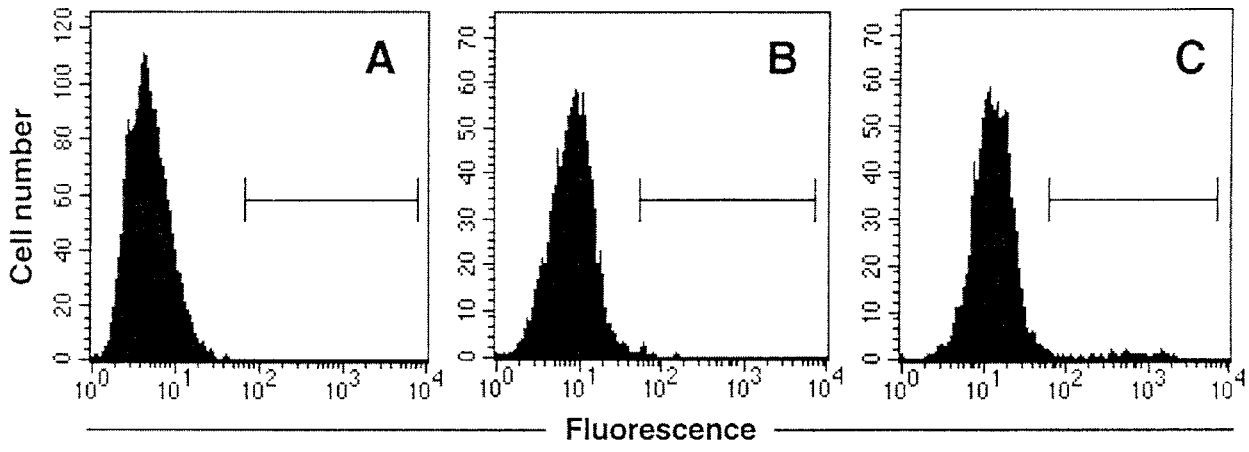

Figure 1. *Less than 1% of isolated cells are recognized by an antibody directed against the endothelial cell-specific CD31 antigen.* Cultured smooth muscle cells were incubated with an anti-CD31 antibody and then with a fluorescein-conjugated secondary antibody. In negative controls using NIH 3T3 fibroblasts (A), or smooth muscle cell preparations in which primary antibody is omitted (B), there is no significant fluorescence (0.06% of cell stain in B). When smooth muscle cell preparations were stained with primary and secondary antibodies, 0.73% of cells stain (C). In each graph, the abscissa is a log scale of fluorescence and the ordinate is cell number. 10,000 live cells were counted to produce each graph.

12. Cut the aorta into pieces that are approximately square and 1–2 mm on a side, using microdissecting scissors or a scalpel. Cover Petri dish and move to a tissue culture hood.
13. Working in hood, place pieces of aorta into a small tissue culture tube containing 100 µl of enzyme solution.
14. Tape tube cap awry so that it still covers tube but allows free exchange of incubator air.
15. Place in standard tissue culture incubator at 37 °C, 5% CO2, for four to six hours.
16. Remove tube from incubator and flick gently to resuspend cells.
17. Add 3 ml culture medium.
18. Transfer to 15 ml conical polypropylene tube and centrifuge for 5 minutes, 300 ×*g* at room temperature in tabletop centrifuge.
19. Aspirate off medium and resuspend cells in 5 ml fresh medium.
20. Centrifuge as before.
21. Resuspend cells in 700 µl medium and transfer to a single well of a 48-well plate.
22. Place in incubator as before and leave undisturbed for 5 days.

General Notes:

Fully grown mice (e.g. at least eight weeks old) provide significantly more smooth muscle cells than young mice (e.g. 3 weeks old).

In our hands, substitution with other types of collagenase yielded inferior results.

There is a risk of contamination, particularly fungal contamination, if steps are not taken to prevent it. Instruments should be sterilized in an autoclave in preparation for the procedure, and the scissors and forceps used to remove skin should be set aside and not used again, as noted in the Basic Protocol section above. It is also advisable to occasionally dip the instruments into 70% ethanol/30% water, then into sterile DMEM, as one performs the dissection. If these steps are incorporated into the protocol contamination should not occur.

Digestion time courses were performed as part of developing this protocol. Every two hours following the initiation of digestion, the sample was agitated, a 2 µl aliquot was taken, and the number of free cells in the aliquot was counted on a hemacytometer. Enzymatic digestion times of four to six hours are optimal. Shorter and longer times result in the isolation of smaller numbers of cells. Cells isolated after four to six hours of digestion are essentially all smooth muscle cells (see Results and Discussion). Cells isolated after shorter and longer periods of digestion were less rigorously analyzed, but based on morphological criteria these preparations seem to also consist exclusively, or nearly exclusively, of smooth muscle cells.

4. Results and discussion

A number of lines of evidence lead to the conclusion that the cell populations isolated by this procedure are almost pure smooth muscle cells. The most likely non-smooth muscle cell type to be isolated using this protocol is the endothelial cell [2]. Flow cytometry studies using antibodies directed against CD31, an endothelial-specific surface antigen, showed that less than 1% of cells reacted to that antibody, indicating that no more than 1% of cells in these cultures were endothelial cells (Figure 1). In addition, when these cell cultures were stained with antibodies directed against smooth muscle-specific proteins (smooth muscle alpha-actin and

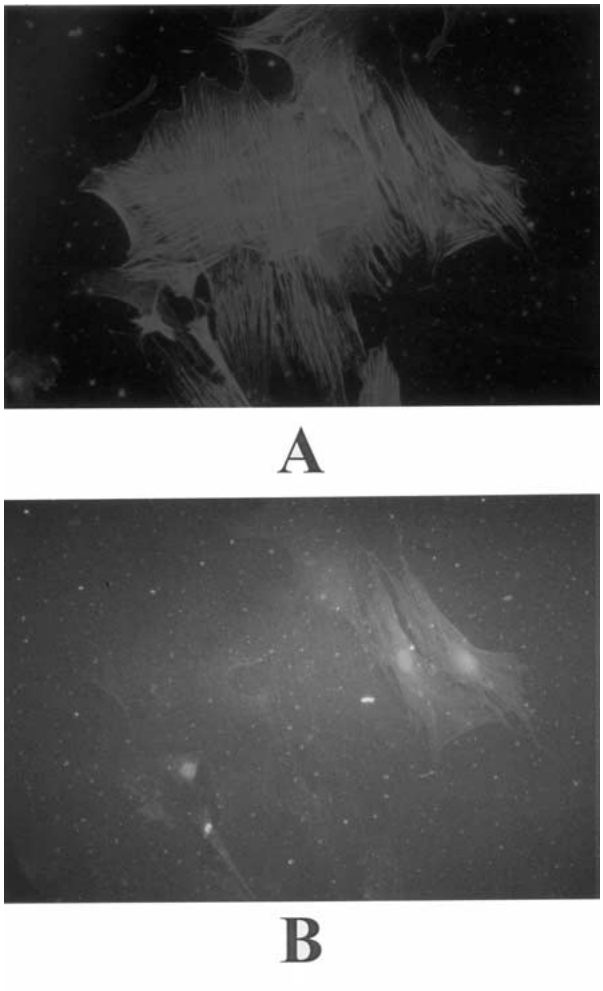

Figure 2. *Fluorescent smooth muscle cells from a transgenic mouse*. Aortic smooth muscle cells were cultured as described, then fixed and stained with primary antibody directed against smooth muscle alpha-actin and secondary antibody conjugated to Texas red. (A) Visualization under appropriate filters on a fluorescence microscope shows the fibrillar pattern typical of smooth muscle alpha-actin. (B) When the filters are changed to allow visualization of yellow fluorescent protein, expression is observed in a subset of smooth muscle cells. (See Results and Discussion for fuller description of the background of this experiment.)

calponin), no nonstaining cells were observed (Figure 2 and data not shown). We therefore regard 99% as a conservative estimate of the purity of the smooth muscle cell population.

The cells from a single aorta were near-confluent (at least 6000 cells/well of a 48-well plate) after 7–10 days. This result was entirely reproducible in many independent cell isolation procedures. At this point certain types of characterization of the cell population (immunological, morphological, growth rate, migration) may be performed. Studies that require larger numbers of cells require longer periods of culture. For example, for flow cytometry, in which 10,000 cells were used in an experiment, culture periods of 12–14 days were typically necessary to analyze cells from a single aorta.

Figure 2 shows an example of this technique's utility. Transgenic mice were made with the yellow fluorescent protein under the control of a smooth muscle-specific promoter, that of the SM22-alpha gene. PCR data showed that the transgene had integrated in seven founder mice. Examination of the founder mice (by examining clipped ears and tails under a fluorescence microscope) provided no evidence of fluorescence. Examination of aortic and femoral artery sections from transgenic F1 mice also showed no fluorescence above background when compared to controls. Aortic smooth muscle cells were then prepared by the technique describe here, and expression of the transgene was easily detected in a subset of smooth muscle cells from one of the seven founder lines, as shown in Figure 2. The ability to use a single mouse in this assay allowed rapid detection of the most useful line, and limited the time during which it was necessary to maintain and propagate less useful lines.

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Notes on suppliers

1. Life Technologies , 9800 Medical Center Drive, P.O. Box 6482, Rockville, MD 20850, USA
2. The Butler Company, 2201 Dividend Drive, Columbus, OH 43228, USA
3. BioWhittaker, 8830 Biggs Ford Road, P.O. Box 127, Walkersville, MD 21793-0127, USA
4. Worthington Biochemical Corporation, 730 Vassar Ave, Lakewood, NJ 08701, USA
5. Biomedical Research Instruments, Inc., 12264 Wilkins Av., Rockville, MD 20852, USA
6. Becton Dickinson, 1 Becton Drive, Franklin Lakes, NJ 07417, USA
7. VWR Scientific Products, 200 Center Square Rd., Bridgeport, NJ 08014, USA
8. Pharmco, 58 Vale Rd., Brookfield, CT 06804, USA
9. Nikon USA, 1300 Walt Whitman Rd., Melville, NY 11747, USA

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