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# CULTURE OF SMOOTH MUSCLE CELLS FROM GUINEA PIG MESENTERIC LYMPHATIC VESSELS

D.G. Wang, Z.Y. Liu, Y.S. Bi, S.Z. Sun, L. Shi, X. Wang, X.H. Zhang

Department of Pathology (DGW,SZS,LS,XW), Shandong University School of Stomatology; Department of Anatomy (ZYL,YSB), Department of Histology and Embryology (XHZ), Shandong University School of Medicine, Shandong Province, Jinan, Peoples Republic of China

*ABSTRACT*

*The in vitro culture of lymphatic smooth muscle cells (SMCs) is a crucial step in studying their function and involvement in disease. Yet there is no efficient approach available so far because of the difficulties posed by the small size of most lymphatic vessels. We present a simple yet efficient method for isolating and culturing SMCs of collecting lymphatic vessels from guinea pig mesenteric tissue. In our approach, thin lymphatic vessels were digested twice from adventitia to media to release SMCs, which were then cultured by traditional methods. The lymphatic SMCs we cultured did not exhibit contact inhibition and demonstrated typical SMCs characteristics under light microscope, electron microscope and by immunohistochemical studies. This method is applicable to the culturing of lymphatic SMCs from other organs and provides useful materials for physiological and pathological lymphatic studies.*

SMCs on lymphatic vessels can propel lymph when they contract. Dysfunction or aging of lymphatic vessels may result in chronic lymphedema, which in turn may stimulate the proliferation of blood and lymphatic vessels (1). SMCs in lymphatic vessels of lymphedematous tissues have also been found to propagate and migrate in the subendothelial layer (2) causing further lymphatic dysfunction that can manifest clinically. Research on lymphatic SMCs has been conducted both *in vivo* (3) and *in vitro* (4). Yet, the progress regarding *in vitro* study of lymphatic SMCs, such as the factors involved in their proliferation and their alterations under physiological and pathological circumstances, has been slow due in part to difficulties in culturing these cells. Although endothelial (5,6) and smooth muscle (7,8) cells of human and animal blood vessels have been cultured *in vitro* successfully, these methods do not apply to the culturing of lymphatic vascular SMCs mainly because the lymphatics are smaller, and their walls are very thin. Here we describe a simple yet efficient approach for isolating and culturing SMCs of collecting lymphatic vessels from guinea pig mesenteric tissue.

## *MATERIALS AND METHODS*

### *Reagents*

Medium consisted of DMEM (Gibco) supplemented with 100 u/ml penicillin, 100 g/ml streptomycin, and newborn cattle serum (NCS) (Dalian) at 20% NCS for primary cells and 15% NCS for subculture were obtained from Sigma with trypsin(1:250) (Sigma). Mouse monoclonal anti-α-smooth muscle actin and sheep anti-mouse IgG-FITC were

obtained from Sigma. Guinea pigs (Hartley) were housed and maintained at the Laboratory Animal Center of Shandong University under an approved protocol.

*METHODS*

*Primary Cells*

Guinea pigs weighing approximately 400g (not obese), female or male, were anesthetized with an intraperitoneal injection of 2% pentobarbital sodium (2 ml) and then the abdomin was immersed in 70% ethanol for five minutes to sterilize the skin and reduce fur interference. Skin and peritoneum were cut to expose the peritoneal cavity. Mesenteric collecting lymphatic vessels were easily distinguished from accompanying blood vessels by semitransparent or milky white color and isolated with sharp sterile scalpel and scissors. Efforts were made to reduce additional tissues around the lymphatic vessels. Approximately 7-8 segments of lymphatic vessels of 2.5-3.5 cm in length were obtained from one guinea pig. The segments were washed 3 times with PBS to remove fat and blood, digested with trypsin (0.25%) at 37 for 15 minutes with shaking, and the trypsin solution containing mesentery and adventitia of lymphatic vessels was discarded. The SMCs in media were then digested with a new trypsin solution for 30-45 min, and undigested vessels were discarded. Trypsin was inactivated by the addition of NCS, and the cell suspension was centrifuged at 1000 rpm for 7 min. The pellet was re-suspended in DMEM to a concentration of $5x10^5$-$7.5x10^5$ cells/ml and seeded into one tissue culture flasks ($25cm^2$/50 ml) with 5-7 ml medium. The cells were maintained at 37 in a humidified incubator with 95% air and 5% $CO_2$.

After a minimum of 2 days in culture, cell divisions could be observed under inverted phase contrast microscope. Medium was changed every 2 to 3 days based on pH change, and the cells reached confluence in 6-7 days and could be subcultured before they became completely confluent.

*Electron Microscopy*

When near 100% confluence, cells were trypsin digested, washed in PBS (0.1M) three times, fixed in 2.5% (w/v) glutaraldehyde for 3h followed by 3 PBS washes, fixed overnight in 2% (w/v) osmium tetroxide, and washed 3 times in PBS. The specimen was then dehydrated in a gradient of ethanol, immersed in epoxypropane for 30 min, then immersed in a series of epoxypropane/Epon-812 solutions: 3:1 for 1 h, 1:1 for 1h, 1:3 for 2h, and pure Epon-812 for 5h or overnight. The resin embedded specimen was heat polymerized at 37 for 12h, 45 for 12h, and 60 for 24h. Ultrathin sections were cut using an ULTRACUT E and stained with uranyl acetate and lead acetate. Sections were viewed with a JEM 1200 EX transmission electron microscope at 80 KV.

*Immunohistochemistry*

The cells were stained with -smooth muscle actin to confirm their smooth muscle origin. Briefly, cells were grown on glass cover slips for 1 week, washed in PBS three times, fixed for 5-10 min in acetone at 4°C, and rinsed twice for 10 minutes in PBS. Then the cells were incubated with mouse monoclonal anti-human and animal -SM actin (1:400) at 4°C overnight followed by rinsing twice over a 5 minute period in PBS, and sheep anti-mouse IgG-FITC was applied at 37°C for 1h followed by washing twice in PBS. Slides were coversliped with 50% (v/v) glycerine in PBS and viewed with a fluorescence microscope.

*RESULTS*

Primary cell cultures consisted of SMCs and non-SMCs such as fibroblasts, fat cells, blood cells, and endothelial cells. After five subcultures, fat, blood, and endothelial cells were dead and discarded with medium change or outgrown by SMCs. However, it was difficult to get rid of fibroblasts in the

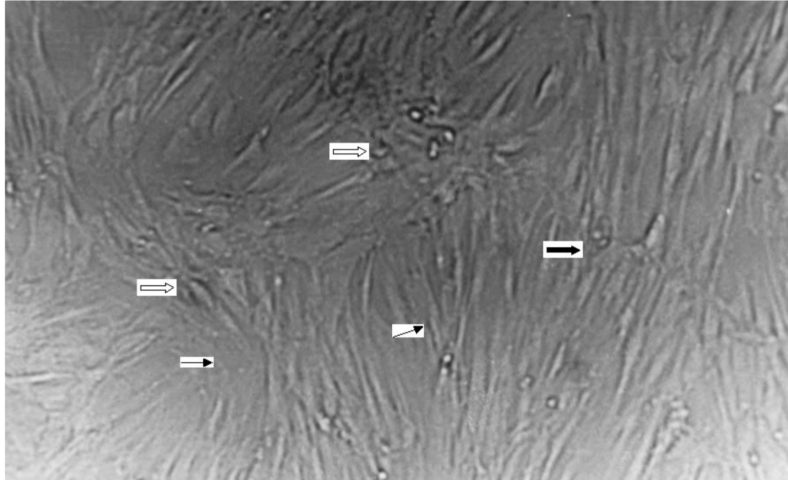

*Fig 1. Mesenteric lymphatic smooth-muscle cells, 5th Passage, display loss of contact inhibition giving an appearance of mountain peaks (⇨), ridges (➡) and valleys (⟶) under low magnification (40x).*

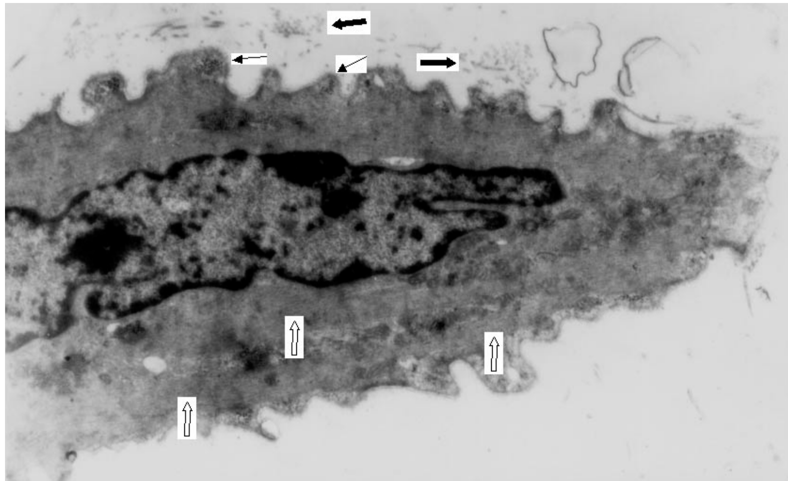

*Fig 2. Transmission electron micrograph of SMCs displaying membrane projections, myofilament bundles (⇨), secretory granules (⟶), and cohesion (➡) around the cells (6000x).*

culture system. Fibroblasts could be reduced by prolonging the first digestion because they mainly lie in adventitia. The purity of SMC was up to 95% (cells with myofilament as a % of total cells) by electron microscopy counting, and 99.5% were viable based on trypan blue exclusion at 5th passage. The cells showed characteristics of SMCs when examined with inverted phase contrast microscopy exhibiting the shape of long spindles. The nucleus lay in the center of the cell and had a shape of ellipse or rectangle with several nucleoli. At confluence, cells did not exhibit contact inhibition *(Fig. 1)* giving an appearance of mountain peaks, ridges, and valleys under low magnification.

Under transmission electron microscopy, cells took the shape of short spindles with an oval nucleus. They exhibited many projections on the membrane, and the cytoplasm was largely filled with myofilament bundles in parallel with the long axis of the cell.

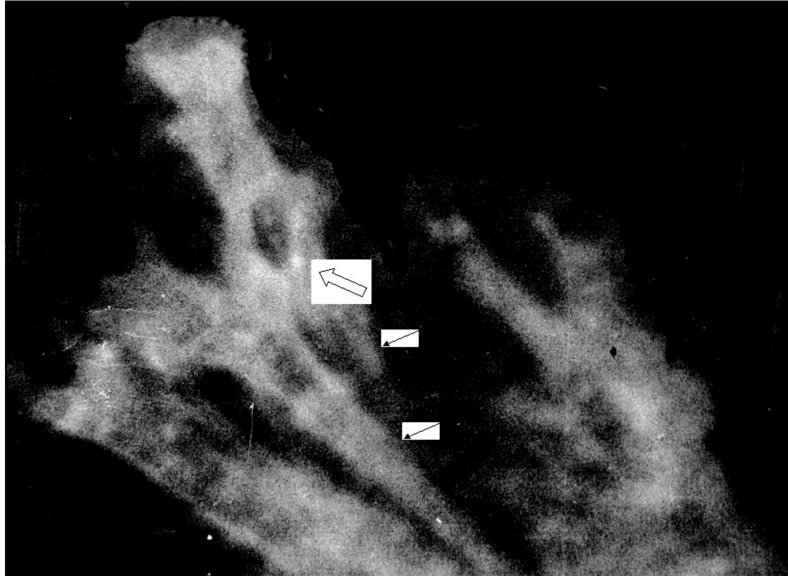

*Fig 3. Anti α-smooth muscle actin antibody immmunohistochemical stain displaying deep green rays (⇨) circling the nucleus, and fluorescent myofilaments (→) parallel with the long axis of cells (x400).*

Dense spots could be seen on the inner membrane of the cell. There were secretory granules in the cytoplasmic projections and collagen scattered around the cell *(Fig 2)*.

The immunofluorescence assessment of α-SM actin was carried out with a 2-step antibody staining technique *(Fig. 3)*. The purity of SMC was up to 92% (cells with fluorescence as % of total cells).

## *DISCUSSION*

We have presented a simple but efficient technique for culturing SMCs derived from colleting lymphatic vessels. The rapidity, reproducibility, and ease of this approach compared to standard/traditional methods is particularly remarkable.

There have been several methods used to culture SMCs from blood vessels with the main one being blood vessel enzyme-dispersal explants method (9). Applying these methods to lymphatic vessels, Johnston (10) cultured SMCs from collecting lymphatic vessels (diameter 0.5-3 mm) in cattle mesenteric tissue. He infused enzymes into the vessels to digest the endothelial cells, then the SMCs, and cultured them separately. His method is only suitable for lymphatic vessels thick enough to infuse enzymes and cannot be used with thin vessels such as the collecting lymphatic vessels in guinea pig mesenteric tissue. Most small animal collecting lymphatic vessels are too thin to be microdissected directly or to infuse enzymes.

It is well known that SMCs of lymphatic vessels in different organs and tissues have different physiological properties. Thus, it may not be appropriate to use the SMCs of thoracic duct or right lymphatic trunk to study the SMCs of peripheral lymphatic vessels. This makes culturing techniques for SMCs from very thin lymphatic vessels a critical issue. The method reported in this study was successful in culturing the SMCs derived from guinea pig mesenteric lymphatic vessels (diameter 100-250 μm with wall thickness 4-20 μm). However, the data obtained from these maintenance guinea pig vessels may not apply to larger vessels or to those from other species (including humans).

Cultured SMCs from veins (11) and arteries (12) can be classified into two categories based on their distinct morphology: (1) spindle-shaped or fusiform; (2) epithelioid-shaped or stellate. The spindle-shaped is the contractile phenotype, while the epithelioid-shaped is the synthetic phenotype. The contractile phenotype has more myofilaments and fewer organelles than the epithelioid-shaped phenotype. Potentially, the two phenotypes contribute differently to vascular physiological functions and are involved in different pathological processes such as, for example, arteriosclerosis. Differences have been reported in characteristics such as contractility, chemotaxis, and mitogenic response to various stimuli (13-15). The lymphatic SMCs that we cultured contain both myofilaments and secretory granules. Is this an integration of both synthetic and contractile phenotypes in an immature intermediate phase of SMC, or do these lymphatic SMCs exercise both of these two functions? The availability of these isolates in culture will allow future studies to elucidate these issues.

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*REFERENCES*

1. Casley-Smith, JR, L Clodius, NB Piller: Tissue changes in chronic experimental lymphedema in dogs. Lymphology 13 (1980), 130-141.
2. Boggon, RP: The Ultrastructure of Normal and Abnormal Lymph Vessels. M. Chir. Thesis. Cambridge University, 1971, 214-218
3. Hollywood, MA, KD Cotton, KD Thornbury, et al: Tetrodotoxin-sensitive sodium current in sheep lymphatic smooth muscle. J. Physiol. 503 (1997), 13-20.
4. Ohhashi, T, S Yokoyama: Nitric oxide and the lymphatic system. Jpn. J. Physiol. 44 (1994), 327-342.
5. Gimbrone, MA, RS Cotran, J Folkman: Human vascular endothelial cells in culture. Growth and DNA synthesis. J. Cell Biol. 60 (1974), 673-684.
6. Slater, DN, JM Sloan: The porcine endothelial cell in tissue culture. Atherosclerosis 21 (1975), 259-272.
7. Ives, HE, GS Schultz, RE Galardy, et al: Preparation of functional smooth muscle cells from the rabbit aorta. J. Exp. Med. 148 (1978), 1400-1413.
8. Chamley-Campbell, J, GR Campbell, R Ross: The smooth muscle cell in culture. Physiol. Rev. 59 (1979), 1-61.
9. Hu, D, H Liu, C Ma: Cultivation of intrapulmonary arteriolar smooth muscle cell from rabbit. Chin. J. Appl. Physiol. 13 (1997), 21 24.
10. Johnston, MG, MA Walker: Lymphatic endothelial and smooth muscle cells in tissue culture. In Vitro 7 (1984), 497-509.
11. Wang, Z, PJ Rao, MR Castresana, et al: A method to isolate morphologically distinct clones of smooth muscle cells from human saphenous vein. Meth. Cell Sci. 24 (2002), 131-137.
12. Wang, Z, PJ Rao, SD Shillcutt, et al: Phenotypic diversity of smooth muscle cells isolated from human intracranial basilar artery. Neuroscience Letters 351 (2003), 1-4.
13. Bochaton-Piallat, ML, P Ropraz, F Gabbiani, et al: Phenotypic heterogeneity of rat arterial smooth muscle cell clones: Implications for the development of experimental intimal thickening. Arterioscler. Thromb. Vasc. Biol. 16 (1996), 815-820.
14. Li, S, YS Fan, LH Chow, et al: Innate diversity of adult human arterial smooth muscle cells: cloning of distinct subtypes from the internal thoracic artery. Circ. Res. 89 (2001), 517-525.
15. Villaschi, S, RF Nicosia, MR Smith, et al: Isolation of a morphologically and functionally distinct smooth muscle cell type from the intimal aspect of the normal rat aorta. Evidence for smooth muscle cell heterogeneity. In Vitro Cell. Dev. Biol. Anim. 30A (1994), 589-595.

**Professor Zhiyu Liu, MD**
**Department of Anatomy**
**Shandong University School of Medicine.**
**Shandong, Jinan, 250012**
**P. R. CHINA**
**Phone: 0531-8382573**
**Fax: 0531-8382721**
**Email: lymph@sdu.edu.cn**