Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Cultivation and Characterization of Pulmonary Microvascular Endothelial Cells from Chicken Embryos

Jianfeng Gao, Ding Zhang, Muhammad Shahzad, Kerong Zhang, Liru Zhao and Jiakui Li*

College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, P.R. China
*Corresponding author: lijk210@sina.com

ARTICLE HISTORY

Received: June 12, 2012
Revised: November 17, 2012
Accepted: December 29, 2012

Key words:
Chick embryos
Immunocytochemistry
PMVECs
Primary culture

ABSTRACT

To improve the understanding on the biological properties of endothelial cells (ECs), a method for the isolation and identification *in vitro* culture of avian pulmonary microvascular endothelial cells (PMVECs) is described. The isolated and cultured cells from chick embryos were identified by cellular morphology and immunocytochemistry. The results showed that the cultured cells exhibited typical cobblestone morphology viewed under an inverted microscope; and were bound with *Bandeiraea simplicifolia* lectin and stained positive for CD31 and factor VIII-related antigen. In conclusion, the findings of present study for the isolation and cultivation of PMVECs may allow more detailed analysis of their biological properties, and provide a valuable model for studying pathological processes including pulmonary hypertension, ascites and pulmonary vascular remodeling in broiler chickens.

To Cite This Article: Gao J, D Zhang, M Shahzad, K Zhang, L Zhao and J Li, 2013. Cultivation and characterization of pulmonary microvascular endothelial cells from chicken embryos. Pak Vet J, 33(3): 300-303.

INTRODUCTION

Endothelial cells (ECs) form two extensive tubular networks, the blood and lymphatic vasculatures that transport a large variety of molecular and cellular cargo. The formation and function of these vessels support the growth of organs during development and early postnatal life, but also help to defend diseases, maintain the body temperature and stabilize homeostatic balance in the adult (Eilken and Adams, 2010).

During embryogenesis, the earliest stages of vascular development occur when hematopoietic cell and ECs precursors, hemangioblasts, migrate into blood islands differentiating into ECs. Thus, newly formed plexuses grow as a result of angiogenesis, that is, vascular sprouting by single ECs within a preexisting capillary plexus (Caolo *et al.*, 2012). ECs are highly heterogeneous in terms of differentiation and proliferation status. They can serve as an identity in blood or lymphatic vessels, location within a specific part of the vasculature, and organ-specific specialization (Adams and Alitalo, 2007).

ECs and its roles in the disease process have been extensively studied in mammals (Ge *et al.*, 2006; Banumathi *et al.*, 2009). Stimulation of ECs or an uncontrolled ECs response are common events in many pathologic processes including atherosclerosis, vasculopathy, hypertension, congestive heart failure, inflammatory processes, and pulmonary arterial hypertension (Sakao *et al.*, 2009). However, to the best of our knowledge, few studies regarding cultivation and identification of avian PMVECs have been reported (Han *et al.*, 2009). In this study, we described a simple and reliable method for cultivation of chick embryos PMVECs based on tissue culture method modified from the techniques previously applied to mammalian cells, and characterized their biological properties. This technique will enable the future researchers to culture avian PMVECs, providing a valuable in vitro model for studying pathophysiological processes, including pulmonary hypertension, ascites and pulmonary vascular remodeling in broiler chickens.

MATERIALS AND METHODS

PMVECs isolation and culture: Isolation and culture of avian PMVECs were done as described by Gao *et al.*, (2012). Ten 17-day-old embryonated broiler eggs were procured and perfused with PBS. After stripping off the pleura, the peripheral lung tissues were sharply dissected, minced finely and placed in a CO_2 incubator for 3h. M199 complete culture medium was added to infiltrate the pulmonary tissue and transferred into a 5% CO_2 incubator for further expansion. On adding M199 complete culture medium after 24h, the tissue pellets were subsequently removed at 60h.

Hematoxylin-Eosin (H&E) staining: The cultured cells were rinsed 3 times at 4°C PBS, and then fixed with 3.7% paraformaldehyde for 20min. After staining with H&E, the samples were observed under an inverted fluorescence microscope.

Staining with *Bandeiraea simplicifolia* lectin-fluorescein isothiocyanate conjugate (FITC-BS-I): The FITC-BS-I reaction was performed as described by King *et al.* (2004). The cells were washed 3 times with PBS at 4°C and fixed with 3.7% paraformaldehyde for 20min. Samples were permeabilized with 0.25% Triton X-100 and 0.25% Tween-20 in PBS and then blocked with 10% goat serum to prevent non-specific binding. The cultured cells were added with 20 μg/ml of FITC-BS-I and incubated at 4°C overnight. ProLong Gold Anti-fade reagent with 4'-6'-diamidino-2-phenylindole dihydro-chloride was used for nuclear counterstaining and were observed under inverted fluorescence microscope.

Immunofluorescence staining with anti-CD31: Immuno fluorescence staining was carried out with anti-CD31 according to the method as described by Hermann *et al.,* (2000). The cells were fixed in fresh methanol at -20°C for 20min. After rinsing 3 times with PBS, they were permeabilized for 20min with 0.25% Triton X-100 and 0.25% Tween-20. By blocking with PBS containing 10% goat serum to prevent non-specific binding, the cells were exposed to CD31 monoclonal antibody overnight. FITC-conjugated goat anti-mouse IgG diluted at 1:100 and DAPI were used as the secondary antibody and nuclear counter stain respectively. Images were then analyzed under an inverted fluorescence microscope.

Immunocytochemical staining for factor VIII-related antigen: The immunocytochemical staining of factor VIII-related antigen was employed from the protocol of Yablonka-Reuveni (1989). The cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.25% Triton X-100. After rehydrating with 5% bovine serum albumin, the cells were exposed to rabbit anti-factor VIII-related antigen. On washing 3 times with PBS, they were exposed to goat anti-rabbit IgG. The cells were then stained with 3, 3'-diaminobenzidine and the negative control group received only the secondary antibody. The cells were analyzed under inverted fluorescence microscope.

RESULTS

Cell growth and morphological characterization: Our findings showed that after 24 h of incubation, a few cultivated cells had migrated out of the tissue mass (Fig. 1A, arrow), and extensive proliferation was evident after 48 h. The purified cells formed confluent monolayers within 2-3 days and showed typical cobblestone morphology under an inverted microscope (Fig. 1B). These cells resembled ECs, forming a monolayer of uniform, flat, closely-packed, polygonal cells observed by H&E staining (Fig. 1C).

Immunofluorescence staining: The cultured cells combined with FITC-BS-I and emitted bright green fluorescence (Fig. 2A). Moreover, these cells displayed anti-CD31 immunoreactivity with green fluorescent stain in the cytoplasm (Fig. 2B).

Immunocytochemical staining for factor VIII-related antigen: The immunolocalization of factor VIII-related antigen showed a typical granular cytoplasmic pattern (Fig. 3B) and no staining was observed in the control group (Fig. 3A).

DISCUSSION

Pulmonary hypertension accompanied by ascites is a prominent cause of morbidity and mortality in chickens produced for meat (Julian, 1998). Previous studies have shown that ECs and endothelin-1 secretion play dramatic roles in the pathogenesis of PH, ascites and vascular remodeling (Hamal *et al*., 2010; Alvarez-Medina *et al*., 2012). A suitable and reliable *in vitro* model is needed for studies aiming at improving the understanding of the molecular pathophysiology of these processes. The isolation of cells from the capillary endothelium posed challenges not encountered in the isolation of human umbilical vein ECs. Despite considerable researches, the isolation and cultivation of microvascular ECs from solid tissues remain difficult (Hewett *et al*., 1993). Numerous studies have reported the production of EC cultures by enzymatic digestion (Banumathi *et al.,* 2009; Haribalaganesh *et al.,* 2010). However, the purification of ECs with trypsin can result in cell deformation, as well as affecting cell proliferation and receptor expression, and shortening of the cell lifespan (Bull *et al.,* 1990). Moreover, Li *et al.* (2008) reported that the vitality and growth rate of PMVECs cultured with tissue culture method were higher than those cultured with the enzyme digestion culture method. We therefore aimed to establish a simple and reliable method for isolating and culturing ECs from lung tissue.

Tissue culture represents a practical method for growing tissues and/or the separating of cells from the organs (Singh *et al.,* 2011). In previous studies use of tissue culture method to isolate PMVECs from the rat lungs, were made without purification (Li *et al.,* 2002). In present study, we described a technique for making highly purified PMVECs from avian pulmonary tissue. The endothelial nature of the isolated cells was confirmed by morphological examination and by staining for the characteristic EC markers, *Bandeiraea simplicifolia* lectin, CD31, and factor VIII-related antigen. Our findings showed that the cultured cells formed cobblestone morphology, stained positively for factor VIII-related antigen, displayed immunoreactivity of anti-CD31, and bound the fluorescent endothelial cell-specific lectins, FITC-BS-I which were consistent with the previous studies. Staining of ECs in avian embryos with antisera to factor VIII-related antigen has been observed (Yablonka-Reuveni, 1989). Also, King *et al.* (2004) suggested that *G. simplicifolia* in particular interacted with PMVECs *in vivo* and *in vitro* selectively. Furthermore, another study displayed that over 95% of the cultured yolk sac endodermal endothelial cells were PECAM-1-positive (CD31) in their experiments (Hermann *et al.,* 2000). These observations suggested that the isolated cells manifested typical ECs characteristics and the cultured

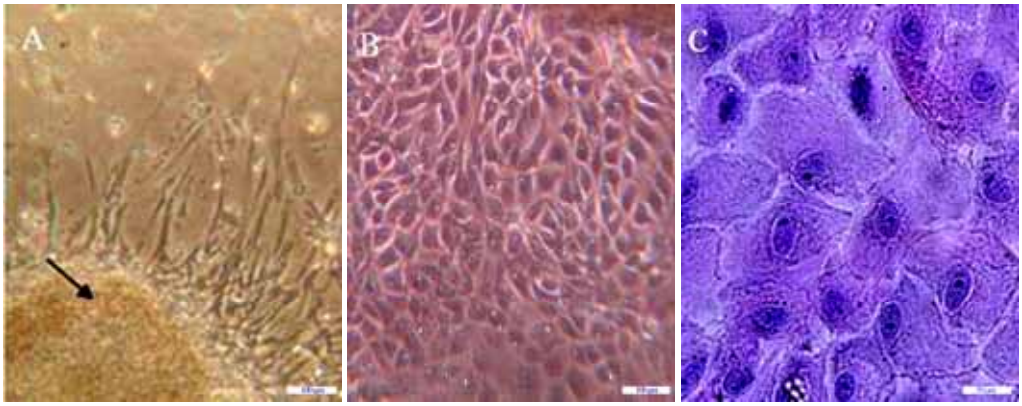

Fig. 1: Tissue pellet and its confluent PMVEC monolayer under Inverted fluorescence microscope. (A) A few cells were visible around the tissue pellet (arrow) after 24 h of culture. Scale bar = 100 μm. (B) Confluent PMVEC monolayer exhibited typical "cobblestone" morphology. Scale bar = 100 μm. (C) PMVEC visualized by H&E staining exhibited a distinct morphology with obvious nucleus. Scale bar = 50 μm.

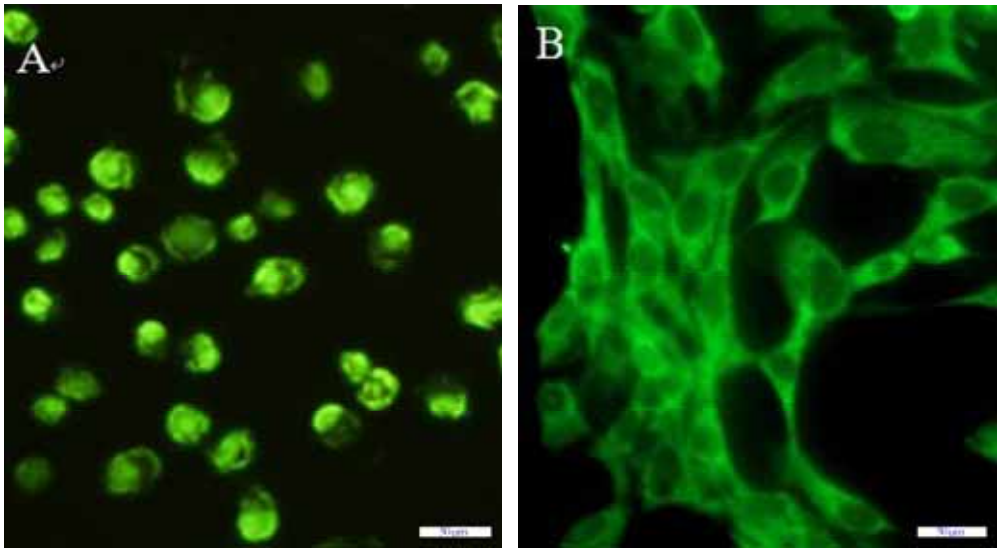

Fig. 2: Immunostaining of PMVECs by FITC-BS-I and anti-CD31. (A) Cultured cells bound with FITC-BS-I and emitted bright green fluorescence. (B) Anti-CD31 immunoreactivity was detected in the cytoplasm in the treated group. Scale bar = 50 μm.

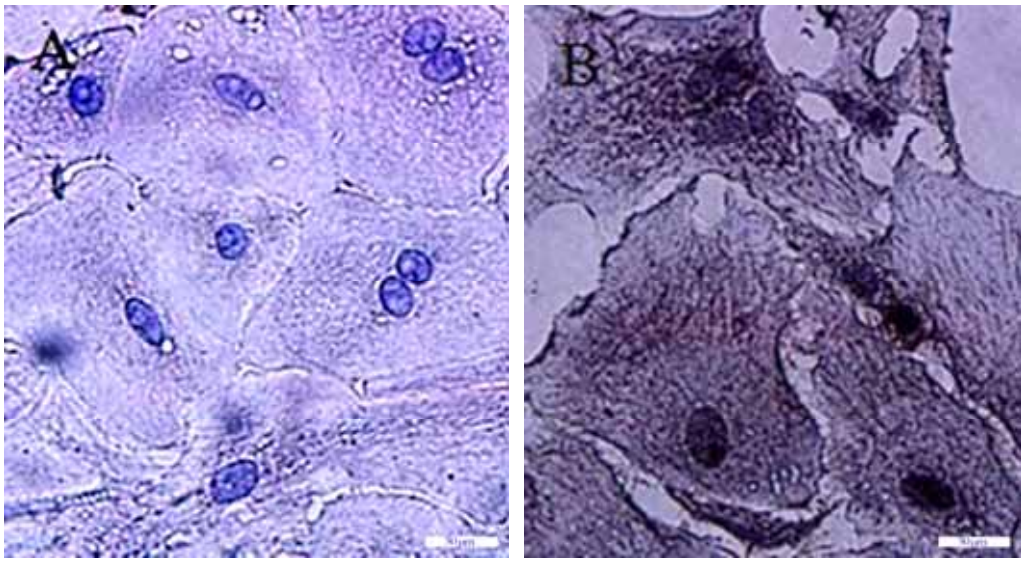

Fig. 3: Immunocytochemical staining for factor VIII-related antigen. (A) Staining was negative in the control group. (B) Cultured cells demonstrated strong immunostaining for factor VIII-related antigen, which showed a typical granular cytoplasmic pattern. Scale bar = 50 μm.

cells were PMVECs. Compared with other methods using enzyme digestion (Ge *et al*., 2006), the protocol proposed by the present study requires less labor and time. Additionally, we found that 16-18-day-old chick embryos were better suitable for isolation of PMVECs which might be due to the full development of the lungs and less blood supply at this stage, the latter being affected the cell migration out of the tissue explants.

In conclusion, we have successfully established for the first time a relatively simple, effective and less time-consuming method for the isolation and cultivation of PMVECs from chick embryos using tissue culture method. This method would provide a valuable *in vitro* technique for studying pathophysiological processes, including pulmonary hypertension, ascites and pulmonary vascular remodeling in broiler chickens.

Acknowledgment: The authors would like to thank the PhD candidates Zhaofang Xi, Dongyang Liu, Liming Wu and Songrui Liu as well as the MSc candidate Rong Deng for their helpful suggestions. This study was supported by the National Natural Science Foundation of China (No. 30972222) and the Tibet Autonomous Region Science Fund.

REFERENCES

Adams RH and K Alitalo, 2007. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol, 8: 464-478.

Alvarez-Medina DI, A Hernandez and C Orozco, 2012. Endothelial hyperpolarizing factor increases acetylcholine-induced vasodilatation in pulmonary hypertensive broilers arterial rings. Res Vet Sci, 92: 1-6.

Banumathi E, R Haribalaganesh, SS Babu, NS Kumar and G Sangiliyandi, 2009. High yielding enzymatic method for isolation and culture of microvascular endothelial cells from bovine retinal endothelial cells. Microvasc Res, 77: 377-381.

Bull HA, MA Rustin, J Spaull, J Cohen, E Wilson-Jones and PM Dowd, 1990. Pro-inflammatory mediators induce sustained release of prostaglandin E2 from human dermal microvascular endothelial cells. Brit J Dermatol, 122: 153-164.

Caolo V, DGM Molin and MJ Post, 2012. Notch regulation of hematopoiesis, endothelial precursor cells, and blood vessel formation: orchestrating the vasculature. Stem Cells Int, doi:10.1155/2012/805602

Eilken HM and RH Adams, 2010. Dynamics of endothelial cell behavior in sprouting angiogenesis. Curr Opin Cell Biol, 22: 617-625.

Gao JF, D Zhang, KR Zhang, MY Liu, ZQ Han and JK Li, 2012. Effects of selenium supplementation on expression of endothelin-1 and its receptors in pulmonary microvascular endothelial cells from chick embryos. Biol Trace Elem Res, 150: 173-177.

Ge SJ and SP Joel, 2006. Isolation and culture of microvascular endothelial cells from murine spinal cord. J Neuroimmunol, 177: 209-214.

Hamal KR, RF Wideman, NB Anthony and GF Erf, 2010. Differential expression of vasoactive mediators in microparticle-challenged lungs of chickens that differ in susceptibility to pulmonary arterial hypertension. Am J Physiol Regul Integr Comp Physiol, 298: 235-242.

Han YY, SD Liu and Y Yang, 2009. Cultivation and identification of the chick embryo pulmonary artery endothelial cells and smooth muscle cells. J China Agr Univ 14: 17-21.

Haribalaganesh R, E Banumathi, S Sheikpranbabu, V Deepak, N Sirishkumar and S Gurunathan, 2010. Isolation and characterization of goat retinal microvascular endothelial cells. In Vitro Cell Dev Biol-Animal, 46: 529-537.

Hermann M, MG Mahon, KA Lindstedt, J Nimpf and WJ Schneider, 2000. Lipoprotein receptors in extraembryonic tissues of the chicken. J Biol Chem, 275: 16837-16844.

Hewett PW and JC Murray, 1993. Human microvessel endothelial cells: isolation, culture and characterization. In Vitro Cell Dev Biol-Animal 29A: 823-830.

Julian RJ, 1998. Rapid growth problems: ascites and skeletal deformities in broilers. Poult Sci, 77: 1773-1780.

King J, T Hamil, J Creighton, B Wu, P Bhat, F McDonald and T Stevens, 2004. Structural and functional characteristics of lung macro and microvascular endothelial cell phenotypes. Microvasc Res, 67: 139-151.

Li F, R Yang, Q Huang, J Qin, S Cai and Y Wu, 2002. Investigation on culture of rat pulmonary microvascular endothelial cells and their viscoelasticity. J Biomed Eng, 19: 36-39.

Li M, MH Yang and Y Liu, 2008. Comparison and improvement of rat pulmonary microvascular endothelial cell culture methods. J Clin Rehab Tissue Eng Res, 31: 6141-6144.

Sakao S, K Tatsumi and NF Voelkel, 2009. Endothelial cells and pulmonary arterial hypertension: apoptosis, proliferation, interaction and transdifferentiation. Resp Res, 10: 95.

Singh M, X Ma, E Amoah and G Kannan, 2011. In vitro culture of fibroblast-like cells from postmortem skin of Katahdin sheep stored at 4°C for different time intervals. In Vitro Cell Dev Biol Anim, 47: 290-293.

Yablonka-Reuveni Z, 1989. The emergence of the endothelial cell lineage in the chick embryo can be detected by uptake of acetylated low density lipoprotein and the presence of a yon Willebrand-like factor. Dev Biol, 132: 230-240.