Isolation and culture of endothelial cells from embryonic rat yolk sac

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Abstract

Yolk sac blood islands are the first morphologic evidence of hematopoietic development during mammalian embryogenesis, and visseral yolk sac mesoderm gives rise to the first embryonic blood cells within a rich endothelial network. Present study reports the isolation and culture of endothelial cells from 11.5 days old embryonic rat yolk sac. The embryos were dissected from 11.5 days pregnant Wistar rat (*Rattus norvegicus*) and the external yolk sac membrane and embryos were removed under aseptic condition. After washing three times with Calcium-Magnesium free Hank's balanced salt solution (CMF-HBSS), the tissue was minced, and fragments were incubated in CMF-HBSS containing 2mg/ml Trypsin, 100mg/ml collagenase I and 40mg/ml DNAse at 37°C until the tissue was completely dispersed. The digestion effect was then neutralized by fetal bovine serum at 1:3 (v/v). The cell suspension was centrifuged at 1000 rpm for 10 min., the supernatants were discarded and the cell pellets resuspended in Dulbecco modified Eagle medium containing 15% fetal bovine serum, 1.25mg/ml amphotericin B, 25mg/ml gentamycin sulphate and 100mg/ml endothelial cell growth supplement. The resuspended cells were plated in two diverse 25cm² culture flasks for overnight differential adherence at 37°C. The non-adherent cells were removed by gentle aspiration and adherent cells refed with fresh medium. The cells were transferred using 1ml of 0.2% Trypsin when cultures reached near-confluence. The cultured yolk sac endothelial cells had characteristic cobblestone appearence and positive immunofluorescent staining for von Willebrand Factor (vWF). Weibel–Palade bodies, the major ultrastructural marker for endothelium, were also detected in cultured cells by electron microscopy.

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Introduction

Embryogenesis is an important process in biology. During this process, especially in the organogenesis period, there is extensive proliferation and differentiation. The organogenesis is also a period of the establishment of the vascular system. The formation of new blood vessels is a complex and tightly controlled process in normal embryonic development, the female reproductive cycle and wound healing. However, uncontrolled angiogenesis plays a critical role in pathologic disorders. Vessel formation in the mammalian embryo is accomplished via two different processes: vasculogenesis, the formation of blood vessels *in situ* from angioblast, and angiogenesis, the formation of blood vessels by sprouting from pre-existing vessels (1). Vasculogenesis seems to be restricted to early developmental periods, whereas angiogenesis can occur during whole life, but also occurs in the early embryo (2, 3).

Early morphological studies showed that the vascular system is the earliest system to begin development in order to take a functional role in providing metabolic requirements for a rapidly growing embryo (4). During the early stage of embryonic development, many organ systems begin their development in the absence of vascular tissue. Both the vascular endothelium and blood cells ultimately stem from groups of mesenchymal cells located in diverse embryonic regions. In the rat, the primordium of blood vessel appears around the 9th day of gestation, when the mesodermal germ layer is formed. The circulatory system in

the visceral yolk sac establishes by the 12th day of gestation when nutritional and informational molecules are transported via the vessels from the yolk sac to the embryo (5).

The vascular endothelium is a continuous single-cell-thick lining of the cardiovascular system. Although, the basic morphology of endothelial cells is similar in blood vessels of all sizes, more detailed comparative studies have shown that there are some physiological, metabolical and immunological differences between the endothelial cells from various tissues. The heterogeneity of endothelial cell populations is related to the organ of origin, vessel origin, and whether embryonic or postembryonic tissue is used (6, 7). It would clearly be useful to be able to culture the endothelial cells from all the various sizes and types of blood vessels. This would allow us to examine their biochemical and physiological properties and to study the diseases. Although, rapid progress has been made in developing culture techniques for cells from adult large vessels (8-10) and capillaries (11-13) from different species and different organs, there has been relatively little progress in fetal and embryonic endothelial cell culture.

The aim of this study is to isolate and culture the endothelial cells from embryonic rat yolk sacs as early as possible by modifications of present culture techniques.

Materials and Methods

Isolation and culture of embryonic yolk sac endothelial cells

Female Wistar rats (*Rattus norvegicus*) each weighing about 200 g (from the breeding unit of the animal house at Queen's Medical Centre, Nottingham University) were terminally anaesthetised with ether on day 11.5 of gestation. Under aseptic conditions the entire uterus was removed, placed in Calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) (from Gibco, BRL) and cut into individual conceptuses. After dissection of uterus and decidual tissues, the visceral yolk sacs were torn and embryos were removed. Yolk sacs were cleaned from ectoplacental and amniotic tissue. 40 visceral yolk sacs were excised and divided in two groups, 20 yolk sacs per group.

While the first group was homogenised in 10ml CMF-HBSS using a loose fitting homogeniser (Potter-Emelyerm) to give 1 or 2mm² fragments, the second group was placed in a sterile Petri dish, and using a sterile scalpel, the yolk sacs were minced to 1-2mm² fragments. The homogenate was centrifuged at 1000 rpm for 10 minutes. The fragments obtained from the second group were washed three times with CMF-HBSS and stirred at 37°C in CMF-HBSS containing 2mg/ml Trypsin, 100mg/ml Collagen I and 40mg/ml DNAse (all from Sigma) until the tissue was completely dispersed. The digestion effect was then neutralized by the addition of Fetal bovine serum (FBS) (from TCS) at 1:3 (v/v), and the cell suspension passed through 100mm mesh nylon bolting cloth. The filtered mixed cell suspension was centrifuged at 1000 rpm for 10 min. The supernatants were discarded and the cell pellets from both groups were resuspended in Dulbecco's modified Eagle's

minimal essential medium (DMEM) (Gibco, BRL) with 1.25mg/ml amphotericin B, 25mg/ml gentamycin sulphate, 100mg/ml ECGS, 100mg/ml heparin sulphate (all from Sigma) containing 15% FBS. The resuspended cells were plated in two diverse 25cm² culture flasks (from Nunc), which were also coated with 5mg/cm² of fibronectin (From Sigma) according to the manufacturer's directions, for overnight differential adherence at 37°C. The non-adherent cells were removed by gentle aspiration and adherent cells refed with fresh medium. Culture flasks were incubated with 5% CO_2 and 95% air at 37°C, and fed every two days with fresh medium until the endothelial cells became confluent.

When the cells became confluent, the medium was removed and the cells were washed twice with CMF-HBSS. The endothelial cells were dissociated using 1ml of 0.2% Trypsin in CMF-HBSS for 5 minutes at 37°C. The enzymatic effect of the trypsin was stopped by the addition of 9ml of fresh culture medium. After the cell suspension was pelleted at 1000 rpm for 10 minutes, the pellet was resuspended in 10ml of fresh culture medium and each 5 ml of the cell suspension was plated in 25 cm² culture flasks for the second passage.

Cell proliferation and migration were checked every day and photographs were taken using a phase-contrast microscope (Nikon, Diaphot).

Immunofluorescence microscopy

After obtaining the cell suspension from embryonic yolk sacs for plating in culture flasks, some cells were separated and 50ml cell suspension per well was plated onto fibronectin subbed multiwell glass slides (Flowlabs) and incubated, until confluence, at 37°C. When the cells reached to confluence, the slides were washed in PBS (containing 0.1% BSA) twice, 2 minutes for each wash, and fixed in 4% formaldehyde in PBS for 10 minutes at room temperature. After washing in PBS twice, the tissues were permeabilised in 0.05% Triton-X-100 (from BDH) in PBS for 10 minutes at room temperature. The slides were washed by three changes of PBS and excess PBS was removed from the slides using a piece of filter paper. 1:200 diluted 30ml anti vWF (rabbit anti human vWF antibody) (from Sigma), as a primary antibody, was added to each experimental well. 30ml of PBS was also added to each control well, and the slide incubated overnight at 4°C. After removal of excess antibody from the wells and washing in PBS three times, 30ml of secondary antibody which diluted 1:500 (FITC-conjugated anti rabbit IgG from Sigma) was added to all wells and the tissue incubated for 1 hour at 37°C.

Following the removal of excess antibody and washing three times with PBS, 10 minutes each, the slides were counterstained with propidium iodide, 1:3 dilution, for 5 minutes at room temperature and washed three times, two minutes each, with PBS. After drying, the slides were mounted using a mounting medium for fluorescence (Vectashild, VECTOR) and glass cover-slips. The slides were viewed using immunofluorescence microscope (Nicon, AFX) and the photographs were taken as quickly as possible.

Electron microscopy

11.5 days old embryonic yolk sac and confluent endothelial cells from the culture flask were fixed in 2% glutaraldehyde and 1% paraformaldehyde for two hours. After washing in PBS overnight, the cells were post-fixed in 1% osmium tetroxide in Millonig's buffer for one hour. The cells were then dehydrated in a 50%, 70%, 90% and 100% ethanol consecutively for 20 minutes each. This was followed by a change in 1,2-epoxypropane for 30 seconds. 1,2-epoxypropane dissolves tissue culture plastic allowing the cell layer to be pulled clear with forceps. The cells were removed and centrifuged at 13 000 rpm for 5 minutes in an Eppendorf tube containing 1,2-epoxypropane. The pellet was left for 15 minutes, and then it was transferred into a 1:1, epoxypropane-araldite mixture and left overnight in a fume cupboard to ensure adequate infiltration. After two changes in 100% araldite, the specimen was embedded with 100% araldite and left in an oven at 60°C for a period of 48 hours for polymerisation to occur.

The Araldite-embedded specimens were trimmed in a chuck on a Ultracut E ultramicrotome (Reichert). 1mm sections were cut for light microscopy and they were mounted on a slide and stained with toluidine blue for 2 minutes on a hotplate and viewed using a light microscope to determine the precise region being sectioned. The blocks were further trimmed to produce a trapezoid cutting face. Ribbons of sections of gold (90-150nm) or silver (60-90nm) interference colours were cut using a glass knife (made with an LKB 7800 knife maker). Sections were collected in troughs filled with distilled water, and after flattening with 100% chloroform vapour, were picked up on 3.05mm 200 mesh hexagonal grids (Taab Ltd.). Sections were stained using uranyl acetate and lead citrate, and examined using a Philips EM 410 Transmission Electron Microscope.

The cultured cells were confirmed structurally as endothelial cells by the presence of Weibel-Palade bodies (WPB). Human umbilical cord (generous gift from Dr L. Leach from Nottingham University) and 11.5 days old embryonic visceral yolk sac vessels were used as controls.

Results

Embryonic yolk sac endothelial cells in primary culture

It was found that enzymatic dissociation of embryonic yolk sac endothelial cells was more reliable method than homogenisation. After overnight incubation of the homogenised cells, some adhered as individual cells whilst a few small groups of cells (which were from vessel pieces) were seen in the culture dish. The small groups of cells became a colony on the second day and the cells reached confluence between 7-10 days. Using equal amounts of yolk sacs for enzymatic dissociation and after overnight incubation, it was observed that there was a number of adherent endothelial cells in culture flask and they reached confluence earlier, in 3-6 days, than homogenised cells. It was also observed that the enzyme cocktail was more efficient than Trypsin-EDTA solution for obtaining embryonic yolk sac endothelial cells. Whilst an effect with the enzyme cocktail
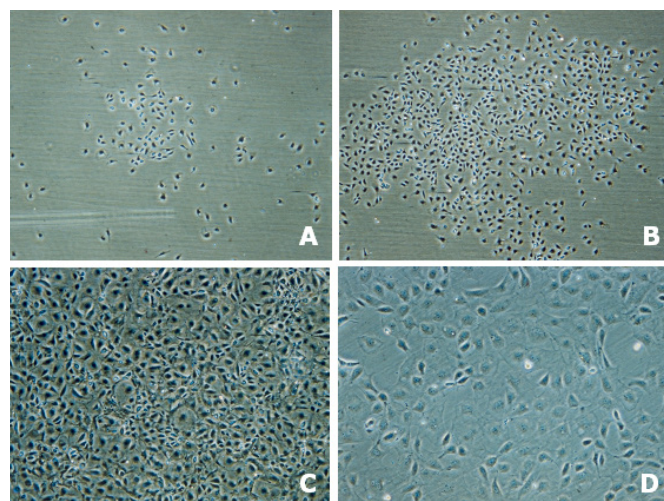

Figure 1. Phase-contrast micrograph of yolk sac endothelial cells in culture prepared by enzyme cocktail treatment. (A) *After overnight incubation (X120)*, (B) *36 hour after plating (X120)*, (C) *72 hour after plating (X120)*, (D) *72 hour after plating (X160)*.

(1ml per yolk sac) was seen within 15 minutes, and these cells became confluent within 3 to 4 days after plating, the reaction using the same amount Trypsin-EDTA solution was slower (30-45 minutes), and the resultant cells grew slowly and did not become confluent before day 6 after plating.

The enzyme cocktail dissociated yolk sac endothelial cells were grown to confluence in between three and seven days after plating on fibronectin coated 25cm² culture flasks, and incubating in DMEM with 15% FBS including 100mg/ml ECGS, 100mg/ml heparin sulphate and antibiotics at 37°C. The confluence time was dependent upon the number of cells seeded in the 25cm² flask. The cells from 10-15 yolk sacs reached confluence between days 5 and 7, whereas the cells from 20 yolk sacs reached confluence 3 or 4 days after plating. When 20 embryonic yolk sacs were used per 25cm² culture flask, some cell colonies were seen within 24 hours after plating (**Fig. 1A**). The cells appeared flat, had polygonal shapes, and were closely apposed to one another. The second 24 hours showed a rapid migration and proliferation period. Large cell colonies were seen during this period, and the cells exhibited the "cobblestone" appearance characteristic of endothelial cells (**Fig. 1B**). Migration and proliferation continued as the cells became confluent, after a 72 hours culture period (**Fig. 1C and D**).

Fate of cultured embryonic yolk sac endothelial cells

The proliferation and migration of the embryonic yolk sac endothelial cells was rapid when the cells were plated from 20 embryonic yolk sacs into 25cm² fibronectin coated culture flasks in the DMEM with 15% FBS including 100mg/ml ECGS, 100mg/ml heparin sulphate and antibiotics at 37°C. The cells became confluent after 72 hours. The confluent cells were disaggregated, using 1ml of 0.2% Trypsin per flask for 5 minutes at 37°C, and plated in two 25cm² culture flasks, when a similar growth pattern occurred to that seen during the first passage, and cells became confluent three or four days after plating.
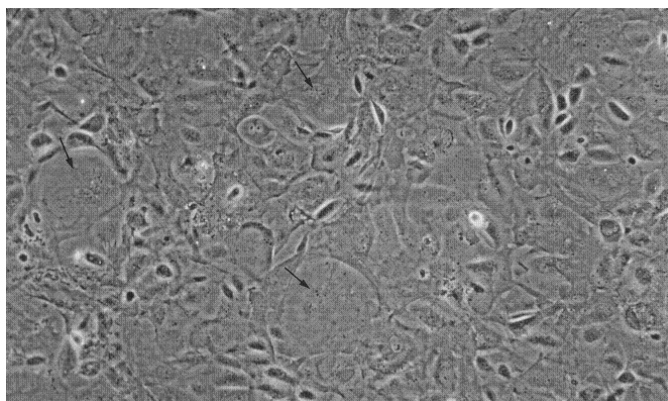
Figure 2. Phase-contrast micrograph of third passage yolk sac endothelial cells in culture. *Arrows indicate polynuclear endothelial cells (X320).*

However, when the cells were plated for a third passage, a considerable number of the cells did not adhere to the culture flask, and the adhered cell proliferation rate was quite slow. Although, there was nuclear division, cytoplasmic division did not always occur. Two or three nuclei were seen in a cell and cell volume was quite large, and some lipid droplets were also seen in these large cells (**Fig. 2**). These cells showed stem cell like growth potential and a stable phenotype in culture. There were always floating cells in the culture medium and the cells never became confluent.

Immunofluorescence microscopy

When primary cultures of 11.5 days old yolk sac endothelial cells were treated with anti vWF, a granular pattern of staining was observed in the perinuclear region (**Fig. 3A, B-control**) typical of that seen in endothelial cells from different species and organs (9, 11). A variability of intensity of the reaction from cell to cell was noted in the primary culture. All cells exhibited some degree of specific staining when compared to controls. It was also noted that a single cell which has different shape showed a greater staining density than did groups of cells (**Fig. 3C, D, E**).

Electron microscopy

Under the electron microscope, the cultured embryonic yolk sac endothelial cells had the general characteristics of vascular endothelium, when they were compared to the vessels within the 11.5 days old embryonic yolk sacs. The specific endothelial organelles (WPB) were evident in both *in vivo* 11.5 days old yolk sac vessels and *in vitro* endothelial cell culture, using human umbilical cord vessel as a control (**Fig. 4**).

Discussion

The formation of new blood vessels is a complicated process which involves the migration, proliferation, and organisation of endothelial cells. This complicated process of endothelial cells involves in a wide range of biological processes which include reproduction, development and wound healing (14), as
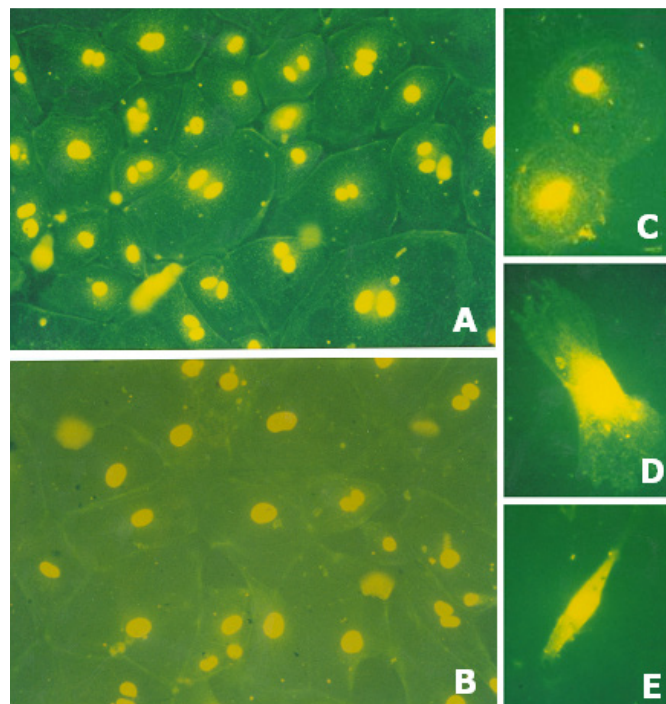

Figure 3. Immunofluorescence micrograph of isolated 11.5 days embryonic yolk sac endothelial cells (3 days after passage) stained with vWF. (A *and* B: *control),* (C, D, E) *different shape of endothelial cells (X400).*
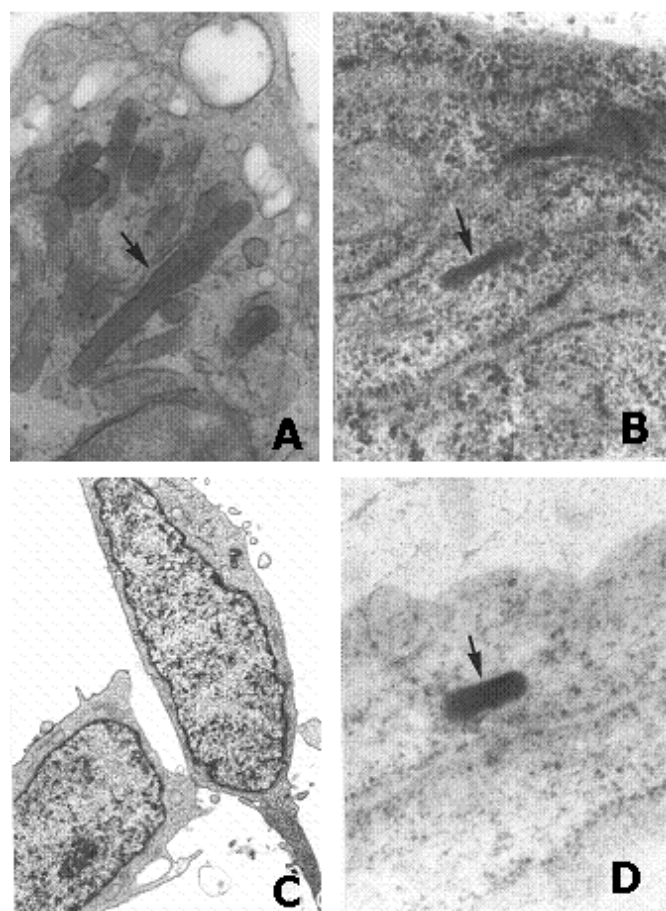

Figure 4. Electron micrograph of in vivo and in vitro endothelial cells from embryonic rat yolk sac. (A) *Human umbilical cord vein (X29000),* (B) *11.5 days in vivo yolk sac vessel (X29000),* (C) *two cultured endothelial cells (X3000),* (D) *higher magnification of cultured endothelial cell* (X29000). *Arrows show Weibel-Palade bodies.*

well as pathological processes such as inflammatory disorders of skin and joints (15), diabetic retinopathy and tumour invasion (16). This has led to major efforts over the past twenty years to isolate and culture endothelial cells from both animal and human tissues, in order to further investigate their role. It is becoming apparent that there is marked endothelial heterogeneity, related in some way to the function of the organ in which the cells are found. The heterogeneity could be in cell morphology, surface antigen expression or growth factor expression (16). Yolk sac blood islands are the first morphologic evidence of hematopoietic development during mammalian embryogenesis and visceral yolk sac mesoderm gives rise to the first embryonic blood cells within a rich endothelial network (17). Examination of embryonic visceral yolk sac endothelial cells may be important for the understanding of both the heterogeneity of endothelial cells and pathologic angiogenesis.

Previous literatures reported some vertebrates' embryonic yolk sac endothelial cell cultures, such as quail (18), transgenic mouse (19), murine (20), porcine (21) and human (22). This work represents probably the first description of primary culture of embryonic rat yolk sac endothelial cells. The cells derived from 11.5 days old embryonic rat yolk sac vessels were maintained until the third passage. Confluent monolayer cultures were identified as endothelial in origin by their *in vitro* growth behaviour, ultrastructural, and immunolabelling characteristics.

One of the major problems in establishing microvascular endothelial cell cultures has been obtaining and maintaining the endothelium. Many isolation techniques have been applied to obtain a pure microvessel endothelial cell culture. Most methods depend upon physical dissociation of the endothelial cells by either homogenising or mincing the tissue before incubating it with proteolytic enzymes. The incubation time is depending on the tissue in question and the time to release the endothelial cells (16). Another isolation technique uses immuno-magnetic beads (23) which requires cell specific antibodies or markers. The cellular structure of the yolk sac, which has only a few cell types, makes it easier to obtain yolk sac endothelial cells. As it was seen for cells from other sources, only yolk sac endothelial cells adhere to plastic culture flasks. Present work indicated that the yolk sac endothelial cells dissociate more easily using an enzyme cocktail, containing Trypsin, Collagenase I and DNAse, than the homogenising technique, and the cocktail treated cells become confluent earlier than homogenised cells.

The cells and tissues of the embryo are different in many respects from the cells and tissues of the adult organism. It was reported that embryonic cells undergo rapid rates of growth and differentiation and consequently may have unique energy requirements, metabolism, synthesis of macromolecules, and enzymatic pathways (24). Earlier studies attempting to culture embryonic endothelial cells showed that rat, mouse, and chick embryo cells were very sensitive to manipulation and failed to survive one passage (11). It has also been reported that there was a difference in the viability of capillary endothelial

cells from several different species. For example, while bovine endothelial cells grow rapidly and survive repeated passage, human capillary endothelial cells grow slowly and required additional growth factors (fibroblast growth factor, endothelial cell growth supplement) (11). In the present study, the morphology of *in vitro* embryonic yolk sac endothelium was normal, and rapid cell proliferation and migration were seen until the third passage. In the third passage, the endothelial cells changed their morphology and started to lose viability. The embryonic endothelial cells probably have different requirements at this stage than those which were used in first and second passages.

It has been reported that endothelial cells have marked variability from tissue to tissue and from one species to another, and this heterogeneity is evident morphologically and functionally (25). There is also heterogeneity in the response of endothelial cells to antigens. Therefore, a general endothelial cell marker has not been found yet. Immunochemical staining for vWF, which was found only in endothelial cells, platelets, and megakaryocytes (26), is considered the most reliable marker for endothelial cells in culture (27). vWF reacts specifically with the cytoplasm of human endothelial cells of normal, reactive and neoplastic blood and lymphatic vessels. It also reacts with endothelial cells from several mammalian species. Although rat capillary endothelial cells from different organs were shown to be positive for vWF (9, 11,12, 28, 29), it was also reported that capillary endothelial cells from lung (30), kidney, lymphatic (31) and liver sinusoid were either very weakly positive or negative. Comparative study of some endothelial cell marker on the embryonic and extra-embryonic rat tissues showed that vWF expression was positive on macro and microvessels from both tissues (32). In the present study, it was also found that cultured rat yolk sacs endothelial cells were positive and expressed vWF in perinuclear granules. Besides the biochemical identification of yolk sac endothelial cells by vWF, they were distinguished *in vivo* and *in vitro* at the ultrastructural level by the presence of a unique organelle, the WPB.

Taking the above information into account, it could be said that it is possible to isolate and culture of embryonic yolk sac endothelial cells. The cell growth may be increased by using endothelial cell mitogens in the culture medium. Finally, these subject needs more experimental work to understand how the embryonic endothelial cells are grown in optimum conditions, how and which angiogenic factors affect embryonic endothelial cell growth?

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