Journal of Biochemistry and Molecular Biology, Vol. 31, No. 2, March 1998, pp. 156–160

JOURNAL OF
Biochemistry
and
Molecular Biology

Primary Culture of Bovine Capillary Endothelial Cells for *In Vitro* Angiogenesis Assay

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Received 6 November 1997

In this study, we cultured bovine capillary endothelial cells from adrenal cortex and compared these cells with capillary endothelial cells obtained from bovine adrenal medulla on morphological and cytokinetic properties. We demonstrated that bFGF and gelatin matrix were required for the growth of adrenal cortex-derived capillary endothelial cells over middle passage, but not for the growth of adrenal medulla-derived capillary endothelial cells. Also, we showed that the growth of adrenal cortex-derived capillary endothelial cells must be stimulated by bFGF and the gelatin matrix for the measurement of *in vitro* angiostatin activity. These data indicate that adrenal cortex-derived capillary endothelial cells over middle passage are more suitable than adrenal medulla-derived capillary endothelial cells for *in vitro* angiogenesis assay.

Keywords: Angiogenesis, Angiostatin, Bovine capillary endothelial cells, bFGF.

Introduction

Angiogenesis is the process of blood vessel formation that sprouts from existing blood vessels (Folkman *et al.*, 1996). Blood capillaries are primarily composed of endothelial cells, which are usually quiescent in the adult mammal under physiological conditions (Hanahan *et al.*, 1996). Angiogenesis is required for a variety of physiological processes such as embryonic development, wound healing, and tissue as well as organ regeneration. Outgrowth of new blood vessels under pathological conditions can lead to the development and progression of diseases such as tumor growth, diabetic retinopathy, tissue and organ malformation, and cardiovascular disorders (Folkman *et al.*, 1992). The switch of angiogenesis phenotype depends upon the net balance between up-regulation of angiogenic stimulators and down-regulation of angiogenic suppressors (Hanahan *et al.*, 1996).

A variety of growth factors can stimulate angiogenesis *in vitro* and *in vivo* (Folkman *et al.*, 1995). Of the known angiogenic factors, fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) are most commonly expressed (Ferrara *et al.*, 1989; Gospodarowicz *et al.*, 1989; Senger *et al.*, 1990; Nguyen *et al.*, 1994; Cao *et al.*, 1996). Tumor cells overexpress one or more of these the angiogenic factors that may function synergistically in promoting tumor growth. Also, expression of the angiogenic inhibitor must be simultaneously down-regulated (Good *et al.*, 1990; Van Meir *et al.*, 1994). In the case of wound healing, the multiple factors including the angiogenic stimulator (FGF) and inhibitor (TGF) are called into play to bring about regrowth of damaged tissue and a functional vascular bed (Lynch *et al.*, 1989).

The strategy to discover a new angiogenic factor depends upon generation of neovascularization in the chick chorioallantoic membrane assay and in the corneal assay *in vivo*, and endothelial cell tube formation and migration assay *in vitro*. However, these methods are not suitable for the assay system to purify angiogenic factor from crude materials. Recently, O'Reilly *et al.* developed a simple *in vitro* angiogenesis assay system using bovine capillary endothelial cell proliferation and purified potent angiogenic inhibitors, including angiostatin and endostatin (O'Reilly *et al.*, 1994; O'Reilly *et al.*, 1997). These proteins suppressed neovascularization in the mouse corneal assay and growth of tumor metastases.

In the present report, we describe the primary culture of capillary endothelial cells from bovine adrenal cortex. We also showed that adrenal cortex-derived capillary endothelial cells differ from adrenal medulla in respect of several biological properties. Also, we report that adrenal cortex-derived BCE cells are more suitable than adrenal medulla-derived capillary endothelial cells for the *in vitro* angiogenesis assay system.

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Materials and Methods

Preparation of gelatin-coated dishes Nunc (no. 150350) tissue culture dishes were flooded with 1.5% (w/v) gelatin (Difco Laboratories) made up in magnesium- and calcium-free phosphate-buffered saline (PBS). The dishes were allowed to stand at 4°C overnight. Just before use, the gelatin was aspirated, and the dishes were washed once with serum-free medium.

Cell culture Capillary endothelial cells of adrenal cortex were cultured from bovine adrenal grands according to the modified method of Folkman *et al.* (1979) and Gospodarowicz *et al.* (1986). The five adrenal glands were obtained as cleanly as possible from slaughtered calves. The adrenal cortex was extricated from the gland and cut into 1 mm pieces. The sliced tissues were then incubated in 0.5% collagenase (at room temperature for 1 h. The detached capillary segment and endothelial cell aggregates were suspended in culture medium (DMEM containing 10% bovine calf serum, 2 mM L-glutamine, 10 units/ml penicillin G, and streptomycin sulfate) and plated into gelatinized dishes. After 2 to 4 days, the isolated colony of endothelial cells were mechanically removed using a pipette equipped with a microtip. Endothelial cell aggregates were seeded onto gelatinized dish and grown in culture medium containing 3 ng/ml recombinant human bFGF (R&D Inc.).

Bovine adrenal medulla-derived capillary endothelial (EJG) cells (Banrejee *et al.*, 1985) were purchased from American Type Culture Collection (Rockvill, USA) and were maintained in EMEM. The media contained 10% fetal bovine serum, 2 mM L-glutamine, 10 units/ml penicillin G, and streptomycin sulfate. All media components were from Gibco/BRL, Inc. (Grand Island, NY).

Endothelial cell proliferation assay The endothelial cell proliferation assay was performed according to the method of O'Reilly *et al.* (1994). BCE cells were maintained in DMEM containing 10% heat-inactivated bovine calf serum, 3 ng/ml recombinant human bFGF under 10% CO_2. Cells growing in gelatin-coated 6-well plates were dispersed in 0.05% trypsin solution and resuspended with DMEM containing 10% bovine calf serum. Approximately 12,500 cells in 0.5 ml were added to each well of gelatinized 24-well plates and incubated at 37°C for 24 h. The medium was replaced with 0.25 ml of fresh DMEM containing 5% bovine calf serum, and samples were added to each well. After 30 min of incubation, media were added to obtain a final volume of 0.5 ml DMEM containing 5% bovine calf serum, and bFGF was added to a concentration of 1 ng/ml. After 72 h of incubation, cells were trypsinized and the cell number was determined using a hemocytometer. To ensure that any inhibition observed was not due to detachment of the cells from the plate, all wells of the assay were examined under an inverted microscope for evidence of cell detachment prior to cell counting.

Purification of angiostatin Plasminogen (Pg) was purified according to the modified method of Deutsch and Mertz (1970). The blood (2 liter) was treated with 0.85% sodium citrate and centrifuged at 1000 × *g* for 30 min to remove blood cells and insoluble materials. The sample, diluted 1 : 2 with PBS, was applied to a lysine-Sepharose column equilibrated with PBS. The column was washed by 0.3 M NaPi, pH 7.0 containing 3 mM EDTA and then eluted with 0.2 M aminocaproic acid (ACA), pH 7.4. Pg pools were identified with SDS-PAGE. The eluent was diluted with an equal volume of chloroform and the aqueous phase was removed and dialyzed extensively using 20 mM Tris-HCl, pH 7.4.

Angiostatin was produced from the purified Pg by a limited proteolytic digest according to the method of O'Really *et al.* (1996). Briefly, porcine pancreatic elastase (Sigma Inc.) was added (0.8 units/mg) to 100 mg of Pg in 20 mM Tris-HCl, pH 7.4. The solution was incubated at 37°C for 5 h and then loaded onto a lysine-Sepharose column that had been equilibrated with 50 mM NaPi, pH 7.0. The column was washed and subsequently eluted with 0.2 M ACA. The eluent was dialyzed extensively against PBS followed by water. Purified proteolytic angiostatin was analyzed on SDS-PAGE followed by Coomassie staining and N-terminal sequencing.

Results and Discussion

Primary culture of capillary endothelial cells from bovine adrenal cortex Bovine adrenal cortex-derived capillary endothelial (BCE) cells have a characteristic appearance that distinguishes them from other cell types, including adrenal medulla-derived capillary endothelial (EJG) cells.

By 2 to 3 weeks, sheets of BCE cells filled the dish and the cells all had the typical appearance of endothelium in a confluent monolayer, i.e., a hexagonal or cuboidal shape with a large round nucleus and very little overlapping of adjacent cells (Fig. 1A). To demonstrate the spontaneous angiogenesis *in vitro*, we cultured BCE cells on gelatinized dish in medium containing bFGF during 3 weeks after the formation of endothelial monolayer. Capillary endothelial cells reorganized and formed tubular networks. The tube became elevated above the surface of the culture dish as they stretched from one multicellular cell aggregate to another (Fig. 1C).

However, EJG cells became more flattened and elongated and exhibited more tight contact between cells (Fig. 2). Also, when the cells were cultured on gelatinized dish during the 3 weeks after the formation of the endothelial monolayer, these did not form tubular networks even for a long time period (data not shown). These results indicate that BCE cells are distinguished from EJG cells on morphological properties.

Cytokinetics of endothelial cells When BCE cells under passage 5 were grown on a gelatinized dish, the cells grew rapidly with a doubling time of 24 h and the growth was not dependent upon bFGF concentration. However, when the cells over passage 15 were grown on a gelatinized dish, these grew with a doubling time of 70 h and the growth was stimulated to a doubling time of 28 h by 1 ng/ml bFGF (Fig. 3). When the cells over passage 15 were grown on tissue culture plastic without gelatin coating, these grew with a doubling time of 70 h and the growth was stimulated to the doubling time of 44 h by 1 ng/ml bFGF (Fig. 4).

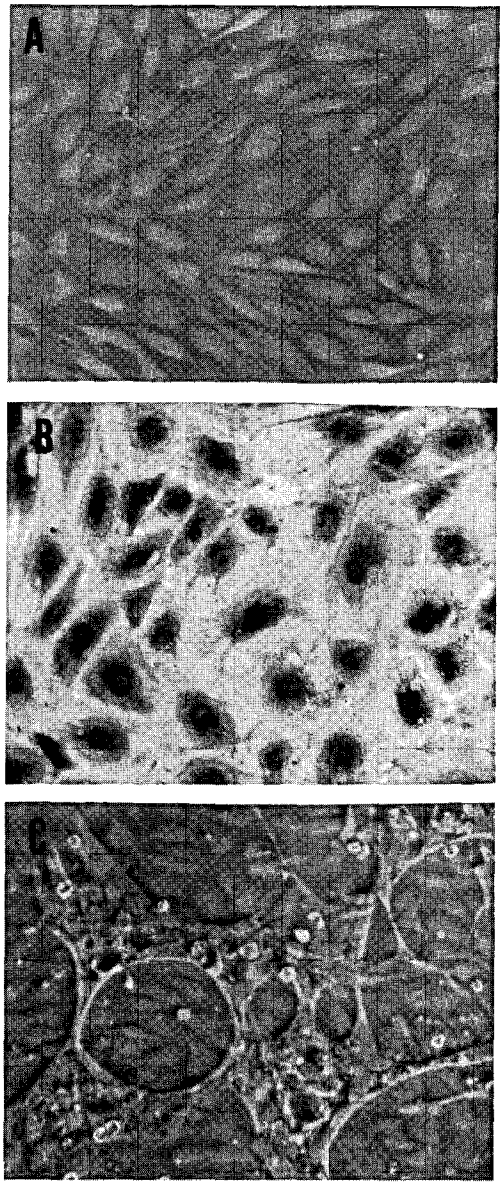

Fig. 1. Adrenal cortex-derived bovine capillary endothelial (BCE) cells. (A) BCE cells growing with 1 ng/ml bFGF. (B) BCE cells were visualized by Giemsa staining. (C) Formation of tubular networks in medium supplemented with 1 ng/ml bFGF. All photographs are at the same magnification of ×400.

On the other hand, when EJG cells were grown either on a gelatinized dish or tissue culture plastic, the cells grew slowly with a doubling time of 7 days and the growth was not dependent upon bFGF or gelatin matrix irrespective of passage number (Fig 3). These results indicate that bFGF and the gelatin matrix were required for the growth of BCE cells over middle passage but not for the growth of EJG cells.

Effect of angiostatin on endothelial cell growth It was reported that angiostatin, an angiogenesis inhibitor,

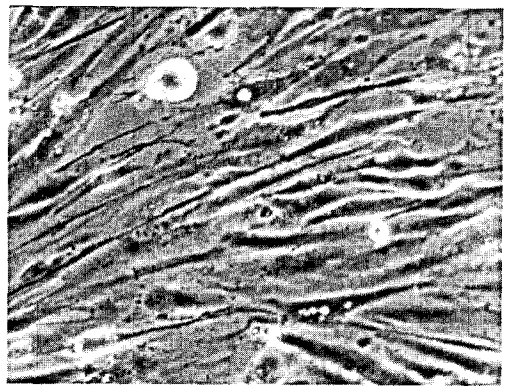

Fig. 2. Adrenal medulla-derived bovine capillary endothelial (EJG) cells (magnification ×400).

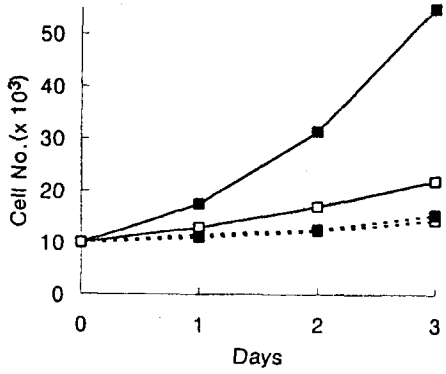

Fig. 3. Effect of bFGF on BCE and EJG cell growth on gelatinized 24-well plate. Ten thousand BCE cells (—) and EJG cells (.....) were seeded on gelatinized 24-well plates in culture medium containing 1 ng/ml bFGF (■—■, ■·····■) or without bFGF (□—□, □·····□). Cell numbers represent the average of three experimental samples.

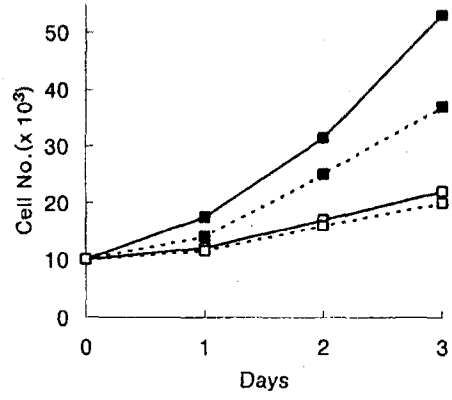

Fig. 4. Effects of the bFGF and gelatin matrix on BCE cell growth. Ten thousand BCE cells were seeded on gelatinized 24-well plate in culture medium containing 1 ng/ml bFGF (■—■) or without bFGF (□—□). Ten thousand BCE cells were seeded on tissue culture plastic in culture medium containing 1 ng/ml bFGF (■·····■) or without bFGF (□·····□). Cell numbers represent the average of three experimental samples.

suppressed capillary endothelial cell growth *in vitro* and growth of tumor metastases *in vivo*. Angiostatin consists of the first four of five kringle domains of plasminogen (1–4 kringle domain) and generates from cleavage of plasminogen by an elastase-like protease (Dong *et al.*, 1997) or the combination of urokinase and a free sulfhydryl donor (Gately *et al.*, 1997) To examine angiostatin effect on the growth of both cell lines, we purified human angiostatin as described under Materials and Methods (Fig. 5A). When BCE cells over passage 15 were stimulated by 1 ng/ml bFGF on gelatinized 24-well plates and then treated with angiostatin, the concentration of half-maximal inhibition for angiostatin was about 2 μg/ml (Fig. 5B). However, when the cells under passage 5 were used, or when the cells over passage 15 were grown on tissue culture plastic, there was very little growth inhibitory effect even in the presence of 40 mg/ml angiostatin (data not shown). Also, in the case of EJG cells, we failed to demonstrate angiostatin activity in 1 ng/ml bFGF and gelatin matrix (Fig. 5B). These results indicate that the growth of BCE cells must be stimulated by bFGF and the gelatin matrix for the measurement of angiostatin activity, and that these cells over middle passage are more suitable than the EJG cells for the *in vitro* angiogenesis assay.

There are several potential applications of this capillary endothelial culture. Tumor angiogenesis takes place at the level of the capillary endothelium, not the aortic endothelium. Capillary endothelial cells may also be valuable for the study of metastatic mechanisms. Endothelial cells present heterogeneity in terms of morphology, marker expression, and function as evident from the analysis of the vascular bed of different organs and endothelial cells obtained from different compartments in the same organ, and macrovascular versus microvascular endothelial cells (Mantovani *et al.*, 1997)

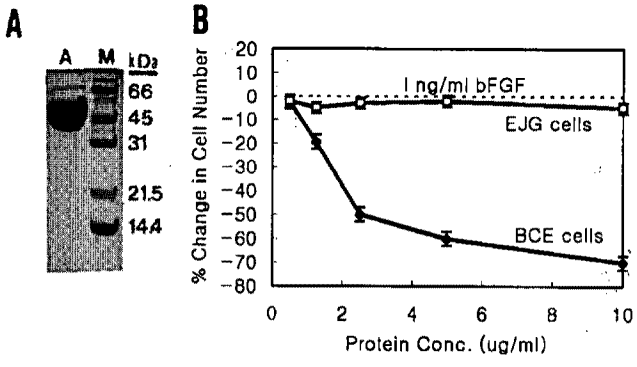

Fig. 5. Comparison of BCE and EJG cell growth inhibition by angiostatin. Panel A; A: Purified angiostatin; M: molecular weight marker (Bio-Rad, Richmond, USA). Panel B; angiostatin were assayed on BCE cells (◆) and EJG cells (□) in the presence of 1 ng/ml bFGF in a 72 h proliferation experiment as described under Materials and Methods. Values represent the means of three determinations (±S.E.) as percent inhibition.

In this study, we compared both capillary endothelial cell lines obtained from different compartments in the bovine adrenal grand on morphological and cytokinetical properties, and demonstrated that adrenal cortex-derived capillary endothelial cells were more suitable for the *in vitro* angiogenesis assay than adrenal medulla-derived capillary endothelial cells for angiostatin activity comparison. Furthermore, to-date, the cells that we cultured have been carried for as long as 5 months without a significant change in morphology or rapid growth by bFGF. These cells have also survived during freezing, storage, and thawing. Thus, the cloned capillary endothelial cells may be useful as an *in vitro* assay system to detect stimulators as well as inhibitors of angiogenesis.

Acknowledgments This study was supported (in part) by the Korea Science and Engineering Foundation (KOSEF) through the Bioproducts Research Center at Yonsei University (project no. 941-0500-023-2).

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