1044-7393/93/2001—0033 $03.20

# ECGF and Heparin Determine Differentiation of Cloned Cerebral Endothelial Cells In Vitro

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Received June 9, 1992; Accepted December 1, 1992

## ABSTRACT

Protein expression patterns of morphologically different cloned capillary endothelial cells from porcine and murine brain cortices were examined. Type I cells, grown in medium containing heparin and endothelial cell growth factor (ECGF), exhibited a polygonal, cobblestone appearance and appeared to replicate the cells of the blood–brain barrier endothelium. Type II cells, grown in medium without heparin and ECGF, were elongated and appeared to replicate capillaries in central nervous system tissue.

Cells of both phenotypes stained positive by the specific endothelial cell marker *Bandeiraea simplicifolia* lectin. The expression of α smooth-muscle actin (mRNA and protein) was taken as a marker for type II cells. By use of 2-D gel images and the GELLAB II system, a data base was created revealing that two proteins (90 kDa, pI 5.1, and 35 kDa, pI 5.7) were exclusively expressed in type I cells. Furthermore, the synergistic action of ECGF and heparin in respect to the phenotypic determination of cerebral endothelial cells was demonstrated.

**Index Entries:** Cerebral endothelial cells; endothelial cell growth factor; heparin; blood–brain barrier; phenotypic switch.

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## INTRODUCTION

The blood–brain barrier (BBB) is created by a continuous layer of endothelial cells that forms the capillary walls in the central nervous system. Specific properties of the cerebral endothelial cells (cEC), such as tight junctions, less transcytosis, and a selective membrane transport system, are the basis for proper BBB function, i.e., the maintenance of a stable chemical environment in the CNS (Bradbury, 1979; Pardridge, 1983). Breakdown or "leakiness" of the BBB has been implicated in several brain diseases, including tumors and strokes as well as degenerative dementia (Müller-Hill and Beyreuther, 1989) and multiple sclerosis (Lossinsky et al., 1989).

A few years ago, Stewart and Wiley (1981) showed that the neural environment of the cEC plays an important role in the induction and maintenance of the BBB. Since then, there have been many findings (Janzer and Raff, 1987; Joó, 1992). To this end, morphological characteristics, as well as biochemical and functional properties, have been used as parameters for BBB function (Rubin et al., 1991).

We have recently shown that the induction of BBB-related γ-glutamyltranspeptidase and $Na^+,K^+$-ATPase activities in cloned cEC depends entirely on whether endothelial cells growth factor (ECGF) and heparin have been added to the culture medium (Bauer et al., 1990; Tontsch and Bauer, 1991). cEC grown in ECGF/heparin-supplemented medium show a cobblestone-like morphology and proliferate at a high rate. On contact with glial and neuronal plasma membranes, cells of this phenotype (type I cEC) respond with an increase in BBB-associated enzyme activities. Removal of growth factor from the culture medium results in a spindle-formed phenotype (type II cEC), a low proliferation rate and a loss of responsiveness to neural stimulation (Bauer et al., 1990).

In this article we demonstrate that cells of the two phenotypes differ also in their protein expression pattern, as revealed by 2-D gel analysis. Moreover, our data indicate that ECGF and heparin act synergistically with respect to the phenotypic determination of cloned cEC.

## MATERIALS AND METHODS

### *Cell Culture*

Microvessels from murine and porcine brain cortices were isolated as has been described earlier (Tontsch and Bauer, 1989). Capillaries were digested with 0.075% collagenase type I (Sigma, Deisenhofen, Germany) at room temperature for 10 min and plated onto culture dishes (Nunc, Roskilde, Denmark) in medium M199 (Seromed, Berlin, Germany) sup-

plemented with 10% FCS, with or without heparin (100 μg/mL, Sigma) and ECGF α (4 ng/mL) (Collaborative Research, Bedford, MA). Medium was changed every 3 d. Capillaries cEC were cloned by limiting dilution and tested for expression of specific endothelial cell markers (Tontsch and Bauer, 1989).

## Immunofluorescence

cEC were plated onto microdishes and grown for 3 d. Cells were then washed with Dulbecco's phosphate buffered saline (DPBS) and fixed with methanol:glacial acetic acid (95:5) at −20°C for 10 min. Fixed cells were washed with DPBS, blocked with 0.5% horse serum in DPBS at 37°C for 10 min, and incubated with an antismooth-muscle α-actin antibody (Biomakor, Rehovot, Israel) (1:300) at 37°C for 30 min. Thereafter, cells were washed three times with DPBS and incubated with FITC-conjugated rabbit antimouse antibody (1:50) (Dakopatts, Glostrup, Denmark) and TRITC-conjugated *Bandeiraea simplicifolia* lectin (Sigma) (100 μg/mL) at 37°C for 30 min, washed with DPBS again, and mounted in Gelvatol 20/30 (Monsanto, Springfield, MA). Immunofluorescence was assessed with a Zeiss Photo III photomicroscope.

## Immunoblot

cEC were grown in different media for 3 d. Then 50 mL of an extraction buffer (50 m*M* Tris-HCl, pH 7.4; 0.154*M* NaCl; 1 m*M* phenylmethanesulfonyl fluoride (PMSF); 1 m*M* EDTA; 0.5% Triton X-100; 0.05% SDS) was added. Cells were scraped off the Petri dishes with a rubber policeman and electrophoresed in a 12% polyacrylamide minigel. Proteins were blotted on polyvinylidene difluoride (PVDF) membranes according to the instructions of the manufacturer (Millipore, Bedford, MA). The antibodies used were: antismooth-muscle α-actin (from Biomakor, diluted 1:500); and alkaline phosphate conjugated rabbit antimouse antibodies (Dakopatts, diluted 1:500).

## Northern Analysis

Total RNA from cEC was isolated according to the method of Chomzynski and Sacchi (1987). Ten micrograms of RNA were denatured with formaldehyde and electrophoresed in a 1% agarose gel. The RNA was blotted on a zetaprobe filter (Biorad, Vienna, Austria) overnight, baked in vacuum at 80°C for 2 h, and prehybridized in Church buffer (Church and Gilbert, 1984) at 65°C for 1 h. The filter was hybridized with a $^{35}$S-labeled probe specific for actin genes (Kocher and Gabbiani, 1987) at 65°C overnight. The hybridized filter was then washed and exposed to X-OMAT (Kodak, Rochester, NY) films at −70°C.

### 2-D Gel Electrophoresis

cEC were cultivated in different media (*see* legend of Fig. 1) for 3 d. The media were then replaced by a methionine-free MEM medium supplemented with 20 μCi $^{35}$S-methionine for 14 h. Thereafter, the media were removed and the cells were washed three times with DPBS and lysed in 50 μL lysis buffer (Bravo, 1984). Aliquots were taken and counted in a Tricarb Scintillation counter (Packard, Zurich, Switzerland), and 100,000 cpm of each was loaded onto the first dimension gels.

2-D gel electrophoresis was carried out according to Bravo (1984). Thereafter, the gels were incubated in acetic acid for 30 min and in 10% 2,5-diphenyloxazole (PPO) in acetic acid for another 30 min. To obtain good resolution, the gels were shrunk in a 30% polyethylene glycol (mol wt 20,000) (Merck, Darmstadt, Germany) solution at 50°C for 30 min and exposed to X-OMAT films at −70°C.

### 2-D Gel Analysis

2-D gels were analyzed using the GELLAB II gel analysis system (Lemkin and Lester, 1989; Stoeckli et al., 1989), which is an integrated collection of computer programs for exploratory data analysis of multiple 2-D gel images. The images were created by a datacopy 612 F CCD scanner. To construct the data base, an R(eference) gel is chosen, and every gel image is segmented into a spot list. All spot lists are compared (paired) with the spot list of the Rgel. This information is used to form the data base and finally to analyze the gel images.

## RESULTS

Cerebral EC grown in M 199 medium supplemented with 10% fetal calf serum (FCS), heparin (100 μg/mL), and ECGF (4 ng/mL) (medium A) showed a cobblestone-like phenotype (type I cEC). Replacing medium A by M 199 medium with 10% FCS only (medium B) resulted in a spindle-shaped, elongated phenotype (type II cEC) (Fig. 1). Immunofluorescence studies have revealed that both cell types stain positive by the specific endothelial cell marker *Bandeiraea simplicifolia* lectin (BSL) (Laitinen, 1987; Tontsch and Bauer, 1989). Expression of smooth-muscle α-actin (sm α-actin) mRNA (Fig. 2) and protein (Fig. 3) was taken as a marker for type II cells (Fig. 1). Only cEC that were cultivated in medium B expressed sm α-actin protein. As soon as medium B was changed to medium A, sm α-actin expression decreased, resulting in a total loss of α-actin after 2–3 d (Fig. 1). Switching of cells to either phenotype was shown to be reversible without cell passage (Fig. 1).

In a further study an attempt was made to find a possible interdependence of ECGF and heparin with respect to the phenotypic determination

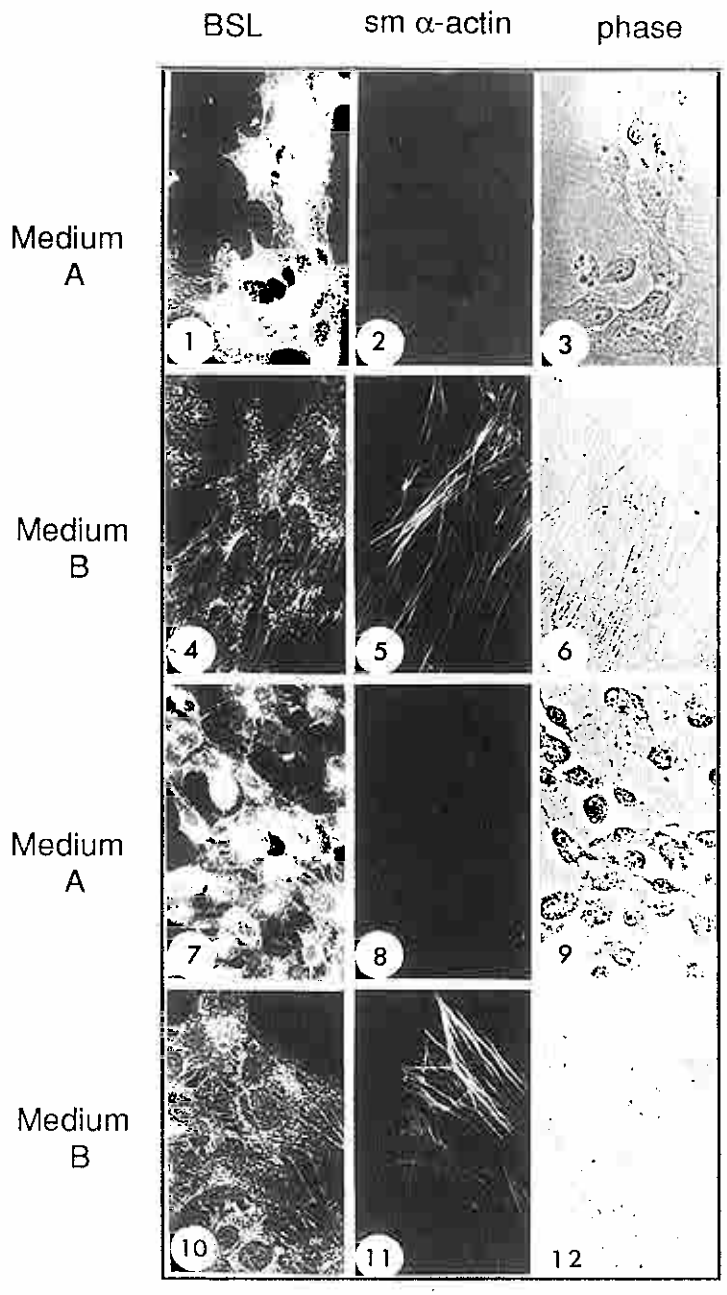


Fig. 1. Double-stain immunofluorescence of cEC grown in culture dishes for 3 d. Medium was then changed as indicated (Med. A—Med. B—Med. A—Med. B) and cultivated for another 3 d. In parallel cultures, medium was removed and cells were fixed. BSL: *Bandeiraea simplicifolia* lectin; sm α-actin: smooth-muscle α-actin. Medium A: Medium B supplemented with heparin and ECGF. Medium B: M 199 medium and 10% FCS.

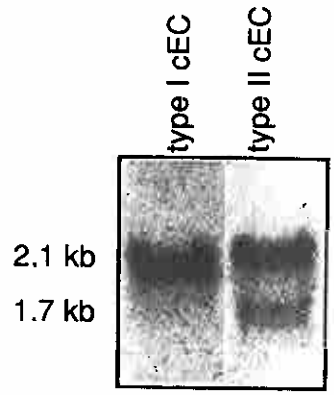


Fig. 2. Northern blot of RNA from cEC. The 2.1-kb bands show the cytoplasmic actin mRNA; a 1.7-kb band shows the sm α-actin specific mRNA.

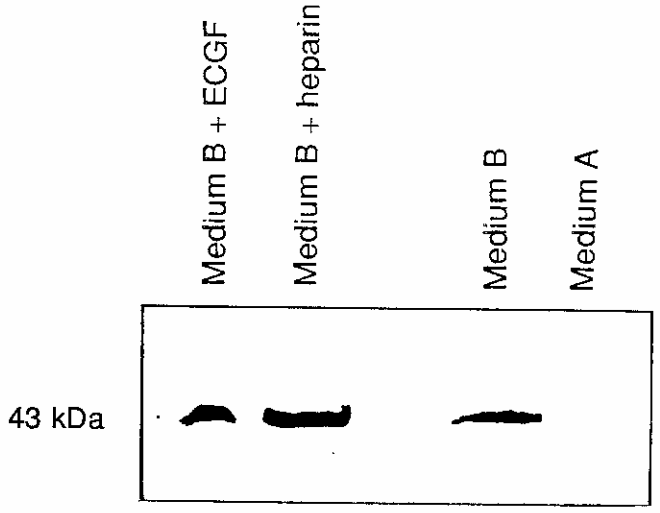


Fig. 3. Smooth-muscle α-actin expression in cEC cultivated in different media (*see* legend to Fig. 1).

of cloned cEC. Type II cEC grown in parallel were treated either with ECGF (2 ng/mL) or with heparin (100 μg/mL). Our data indicate that neither ECGF nor heparin alone suppressed α-actin expression as shown by immunoblotting (Fig. 3).

Cells of the two phenotypes also differed in their protein expression pattern as detected by 2-D gel analysis and the GELLAB II system. Two proteins (35 kDa pI 5.7, and 90 kDa pI 5.1) were found to be expressed only in type I cells (Fig. 4). However, we could not detect any difference in the expression of proteins known to be involved in actin modulation or polymerization.

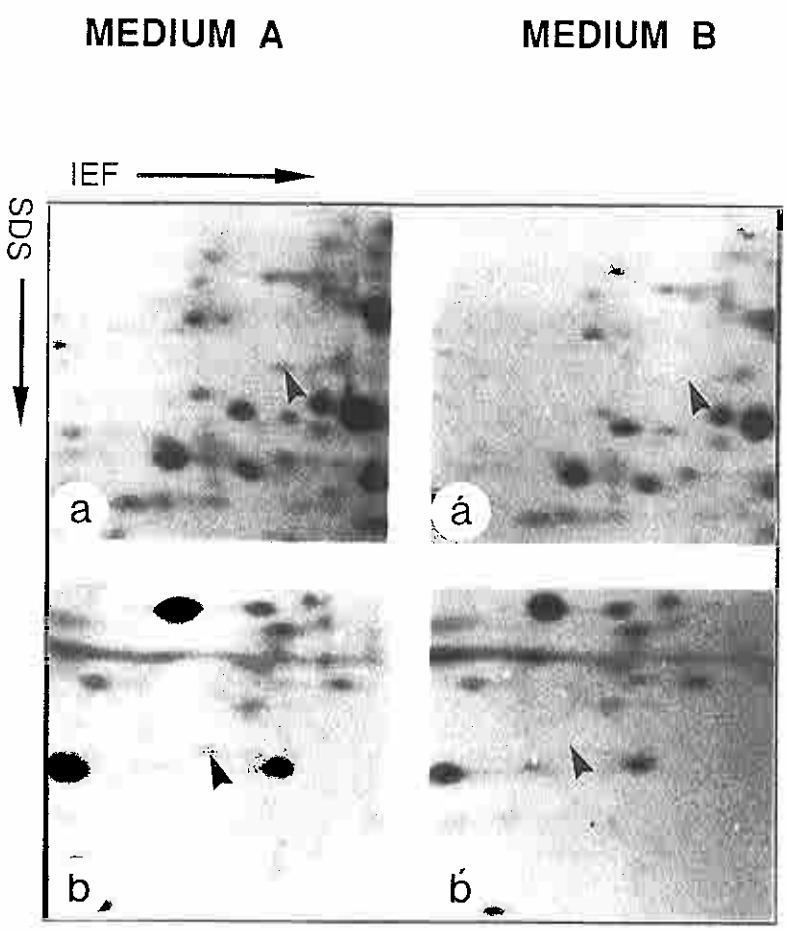


Fig. 4. Sector of two-dimensional gels showing the two proteins that are expressed only in type I cEC. IEF: Isoelectric focusing; SDS: SDS-polyacrylamide gel-electrophoresis. a/a′ arrows indicate the presence or absence of a 90-kDa protein, pI at 5.1. b/b′ arrows indicate the presence or absence of a 35-kDa protein, pI at 5.7.

## DISCUSSION

The goal of the present study was to elucidate the role of ECGF and heparin in the phenotypic determination of capillary endothelial cells in vitro. A data base was created by use of the GELLAB II system that provided a statistical analysis of the 2-D gel images and a subsequent determination of characteristic protein expression patterns of the cells.

Phenotypic diversity of capillary endothelial cells in vivo, as well as in vitro, is well-documented (Rupnick et al., 1988; Howard et al., 1991; Lipton

et al., 1991). It has also been suggested that different morphologies of EC are likely to originate from different microanatomical locations within the endothelium (Rupnik et al., 1988). Determination of cell morphology and function by endogenous and exogenous basic fibroblast growth factor (bFGF) has been shown by Tsuboi et al. (1990) using cloned adrenal capillary EC. Cells containing high levels of bFGF had an elongated appearance and migrated well in culture, in contrast to cells containing low levels of bFGF. The later showed a cobblestone-like morpology and did not migrate or invade under normal conditions. Cultivation of cerebral endothelial cells without cloning inevitably leads to "contamination" of the endothelial cell culture with other neural cells, particularly pericytes. The latter are intimately attached to the vessel wall in vivo, and, in contrast to endothelial cells, contain $\alpha$-actin under normal conditions and appear in primary cultures of EC. Since cloning of cells is the only way to get the most defined culture conditions possible, our in vitro system is based on endothelial cell populations derived from single clones.

We have demonstrated earlier that cloned cEC derived from porcine and murine brain cortices differentiate into two distinct phenotypes dependent on culture conditions (Tontsch and Bauer, 1989). Based on our data, there is evidence that neither heparin nor ECGF alone is able to induce the phenotypic switch in cEC. The polypeptide mitogen ECGF, which is often referred to as acidic FGF (Schwartz et al., 1990), is a member of the HBGF (heparin binding growth factor) family and has been shown to increase the replicative life span of endothelial cells in culture (Thornton et al., 1983). Its mitogenic activity on this cell type is known to be potentiated by the presence of heparin. However, the exact mechanism of growth factor activation by the glycosaminoglycan is not well understood. Our findings demonstrate that besides mitogenic cell responses, other major changes in morphology and protein expression also occur, but again, the molecular basis of these is unknown. The synergistic action of heparin and HBGF has been reported by other authors as well (for review, *see* Burgess and Maciag, 1989). In this respect, heparin has been shown to protect bFGF from denaturation and enzymic degradation (Gospodarowicz, 1990). Moreover, binding of bFGF to its receptor is known to require prior binding to heparin or heparan sulfate side chains of distinct proteoglycans (Yayon et al., 1991).

We have also shown that in contrast to type I cEC, the spindle-shaped type II cells grown in a characteristic hill-and-valley pattern, thus strongly resembling smooth-muscle cells. In addition, we have demonstrated that only the latter cell type expresses smooth-muscle specific $\alpha$-actin protein and mRNA as revealed by Western and Northern analysis (Amberger et al., 1991). Since it is generally known that the actin-isoforms appear in different grades of polymerization in the cell, the detection of proteins that are tightly related to actin polymerization and filament-modulation (such as the cytoplasmatic proteins gelsolin, profilin, or actinin) is of great

interest with respect to the phenotypic switch of cloned cEC. Unexpectedly, cells of the two phenotypes did not show any difference in their expression of the proteins mentioned above (Celis, 1990).

However, there were two proteins (90 kDa, pI 5.1, and 35 kDa, pI 5.7) found to be exclusively expressed in type I cEC, but the physical data of these proteins did not correlate to any protein known to be involved in actin polymerization or modulation. In order to elucidate the cellular localization or a function of these proteins, the generation of specific antibodies is desirable. By using new methods of immunization, it should be feasible to develop these antibodies and to use them for immunofluorescent studies and Western blot analysis.

The correlation of morphologic diversity with different functional properties of cloned endothelial cells was also demonstrated by our recent cocultivation studies mimicking BBB induction in brain capillary EC in vitro (Tontsch and Bauer, 1991). Only the cobblestone-like type I cells responded to contact with glial and neuronal plasma membranes in a manner characteristic for BBB-endothelium. Type II cells wer left more or less unaffected by this kind of neural stimulation (Bauer et al., 1990). Therefore, we believe that type I cEC possibly correspond to the BBB-endothelium in vivo, whereas type II cells may occur during capillary formation in the central nervous tissue. This suggestion was confirmed by our observation that type II cells show a significantly higher migration rate in vitro compared to type I cEC (Amberger, unpublished).

The complex mechanisms involved in the establishment and maintenance of the blood–brain barrier still leave many questions open. This situation results in part from the poor accessibility of the CNS in vivo. We believe that our in vitro system provides a useful tool for the detailed study of processes underlying growth, differentiation, and function of brain capillary cells under normal as well as pathological conditions.

## ACKNOWLEDGMENTS

The authors would like to thank R. Fuchs for expert technical assistance, T. Hale for helping us with the GELLAB II analysis, and H. Bauer for many useful discussions and critical readings of the manuscript. This work was supported by the Austrian FWF, Project number 7654.

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