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Reversible expression of sm α-actin protein and sm α-actin mRNA in cloned cerebral endothelial cells

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The expression of smooth muscle (sm) α-actin was studied in cloned capillary cerebral endothelial cells of two phenotypes. Type I cells were cultured in medium containing 10% FCS, heparin and ECGS (or α-ECGF) and stained positive for a specific endothelial cell marker (*Bandeiraea simplicifolia*). Depletion of heparin and ECGS resulted in a smooth muscle-like appearance after 2–3 days. Cells of this phenotype, (type II) stained positive for the endothelial cell marker and for sm α-actin. In contrast to type I cells, type II cells expressed sm α-actin protein and mRNA as evidenced by Immunoblots and Northern blots. This phenotypic switch was shown to be reversible and so was the expression of sm α-actin.

Cerebral endothelial cell; Phenotypic switch; Smooth muscle α-actin

1. INTRODUCTION

Some polypeptide mitogens, including FGFs, ECGFs and the beta isoforms of TGF, have been reported to exert multilateral effects on growth and cytodifferentiation of mesoderm-derived cells, such as fibroblasts, vascular endothelial cells and smooth muscle cells [1–9]. The mitogenic activities of FGFs, and their acidic form, ECGFs, are known to be potentiated by human thrombin or heparin binding [10–14].

We have previously demonstrated that cloned cerebral endothelial cells (cEC) reversibly developed into two distinct phenotyped, depending on whether or not ECGS, extracted from bovine hypothalamus, and heparin were added to the culture medium [12]. ECGS/heparin treatment led to an endothelial phenotype of cobblestone-like appearance (type I) which exhibited functional blood–brain barrier (bbb) characteristics in vitro. For example, cells of this phenotype proliferated at a high rate and showed a significant increase of bbb-associated transport enzyme activities after glial contact [13,14]. Removal of ECGS/heparin from the culture medium resulted in an elongated phenotype, in appearance similar to smooth muscle cells with a much lower proliferation rate and little or no response to glial stimulation. Cells of this phenotype were therefore suggested to be involved in capillary formation [14].

Abbreviations: ECGF, Endothelial cell growth factor; ECGS, endothelial cell growth supplement; FGF, fibroblast growth factor; TGF, transforming growth factor

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In this study we have shown, that purified α-ECGF [15,16] elicited similar effects on cultured cEC as did the ECGS. Morever, we present evidence that cEC of the two phenotypes differ not only in morphological and functional aspects but also at their genomic levels. Northern hybridization and Immunoblot analysis revealed that deprivation of mitogenic factors led to the transient expression of smooth muscle (sm) α-actin protein and sm α-actin mRNA in type II cEC.

2. MATERIALS AND METHODS

2.1. *Cell culture and immunofluorescence*

Cloned cerebral endothelial cells (cEC) were prepared and established from pure capillaries derived from porcine brain as described by Tontsch and Bauer [12]. Two distinct cEC phenotypes were obtained by either cultivation of cEC in medium 199 supplemented with 10% FCS, endothelial cell growth supplement (ECGS) or α-ECGF and heparin (type I), or by cultivation in medium without ECGS and heparin (type II). Immunofluorescence was carried out as described by Tontsch and Bauer [12]. The antibodies used are indicated in the legends to the figures.

2.2. *Northern blot*

Total RNA was extracted according to the method of Chomzcynski and Sacchi [17]. 10 μg RNA per lane was denatured with formaldehyde, electrophoresed in a 1% agarose gel and transferred overnight onto a nylon membrane as described by the manufacturer (Nytran, Schleicher and Schuell). The filters were baked under vacuum at 80°C for 2 h, prehybridized at 65°C for 2.5 h and hybridized at 65°C with 5×10^6 cpm/filter of a ^{35}S-labeled probe pRAo αC [18] for 17 h. The filters were then washed and exposed to Kodak XAO films at −80°C.

2.3. *Immunoblot*

50 μl of an extraction buffer (50 mM Tris-HCl, pH = 7.4; 0.154 M NaCl; 1 mM PMSF; 1 mM EDTA; 0.5% Triton X-100 and 0.05% SDS) was added to cEC cultures and the cells were scraped off the Petri dish with a rubber policeman and electrophoresed in a 12%

PAGE. Immunoblots were carried out on PVDF membranes according to the recommendation of the manufacturer (Millipore, USA). Antibodies used: anti smooth muscle α-actin (sm 1) from Biomakor (Israel) (1:500 diluted); alkaline phosphatase conjugated rabbit anti mouse from Dakopatts (Denmark) (1:500 diluted).

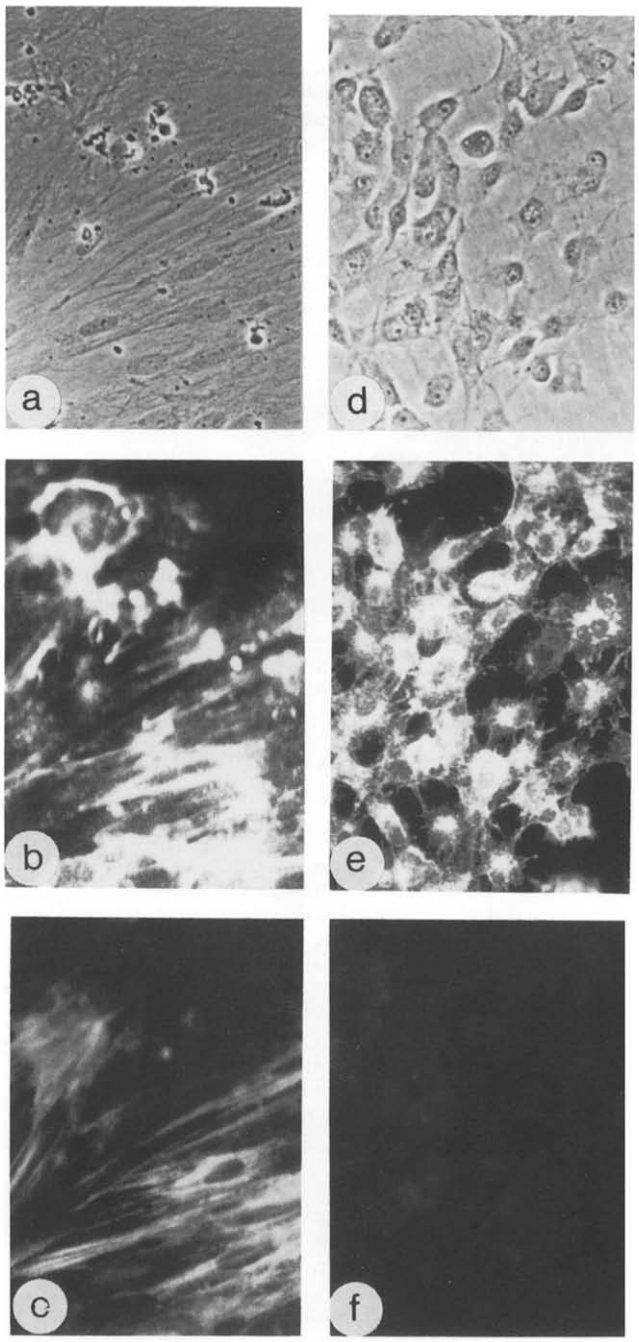

Fig. 1. Double stain immunofluorescence of cEC grown on coverslips for 1 day, fixed with ethanol and glacial acetic acid. (a) cEC type II phase photo; (b) cEC type II labeled with *Bandeiraea simplicifolia* lectin (TRITC conjugated; 1:40 diluted); (c) cEC type II labeled with sm-1 antibody (1:400 diluted), second antibody: FITC conjugated rabbit anti mouse; (d) cEC type I phase photo; (e) cEC type I labeled with *Bandeira simplicifolia* lectin as described in (b); (f) cEC type I labeled with sm-1 antibody as described in (c).

3. RESULTS

Cloned cerebral endothelial cells (cEC) are shown in Fig. 1 without (a) and with (d) addition of heparin and α-ECGF. Cells growth with these factors (type I cEC) exhibited a cobblestone-like appearance and grew in monolayers. Type II cEC, cultured in medium only supplemented with FCS were spindle-shaped and tended to form hill-and-valley structures. Cells of both types stained positive for the lectin *Bandeiraea simplicifolia* (Fig. 1b,e). Type II cells stained positive for sm α-actin whereas type I cells did not. Depletion of factors in type I cell cultures led to a phenotypic switch and moreover to α-actin antibody recognition. Similarly, addition of factors to type II cultures resulted in the loss of α-actin antibody binding. These findings were further confirmed by Immunoblot analysis. In Fig. 2, sm α-actin antibody binding to transferred cEC protein bands is demonstrated. Only type II cells of two cEC clones tested (A 9/B12, A 12) showed a strong band at 43 kDa.

In order to answer the question of whether sm α-actin mRNA expression is also induced, we used Northern blot analysis. Again, only in type II cells was a signal for α-actin (1.7 kb) induced. Figure 3 shows the time dependence of the reversible sm α-actin mRNA expression in cloned cEC. It took about 24 h for α-actin mRNA expression in cells deprived of heparin and ECGS. After 3 days a maximum of the 1.7 kb signal was reached. Addition of factors resulted in a disappearance of the signal 12 h after incubation with heparin and ECGS, the 1.7 kb band was only weakly present and disappeared the following day. The 2.1 kb signal, reflecting cytoplasmic actin isoform mRNA expression remained unchanged in both cells.

4. DISCUSSION

Smooth muscle (sm) α-actin is the major isoform of

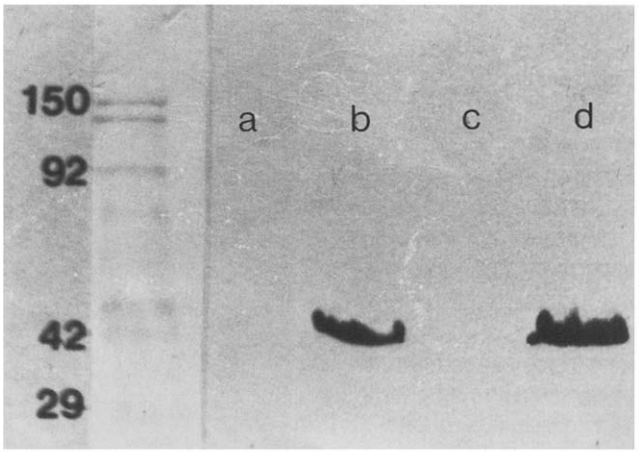

Fig. 2. sm α-actin expression in 2 different cEC clones detected by immunoblot technique. Clone A9/B12: (a) cEC type I, (b) cEC type II. Clone A12: (c) cEC type I, (d) cEC type II.

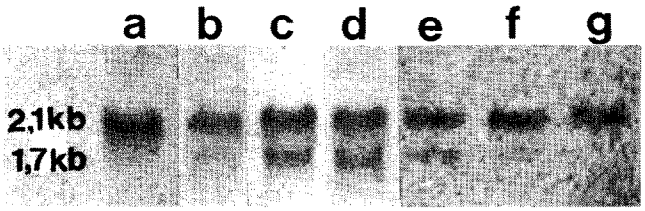

Fig. 3. Northern blot of total RNA extracted from cEC; probe: segment from the coding region of the sm α-actin gene from rat; [^{35}S]-methionin labeled. 2.1 kb bands show the cytoplasmic actin mRNAs; 1.7 kb bands show the sm α-specific actin mRNAs. (a) cEC type I, 3 days in culture; (b, c, d) cEC type II, 2, 4, 6 days in culture, respectively. (e) cEC type II, washed and incubated with medium 199, ECGS and heparin for 12 h; (f) same as (e) incubation time 24 h; (g) RNA from C6 glioma cells.

actin proteins found in smooth muscle cells and pericytes [6,18,19,20]. Endothelial cells, on the other hand, have been reported to contain exclusively the cytoplasmic β- and γ-actin isoforms under normal conditions [21]. In our study we have shown, that smooth muscle specific α-actin appeared in cloned cerebral endothelial cells (cEC) as a response to the removal of mitogenic factors from the culture medium. De novo synthesis of sm α-actin mRNA occurred within 1 day of ECGS/heparin deprivation (Fig. 3). Moreover, cells displayed a characteristic, spindle-shaped phenotype (type II) (Fig. 1). Addition of ECGS/heparin to the culture medium caused a phenotypic switch, resulting in a cobblestone-like appearance (type I) of the cells (Fig. 1). cEC of this phenotype neither expressed sm α-actin protein nor did we detect any induction of sm α-actin mRNA synthesis (Figs. 2,3).

In a previous study we demonstrated, that proliferation of type II cEC was significantly reduced compared to type I cells [12]. A similar phenomenon, i.e. the negative correlation of sm α-actin expression and cell-replicative activity, has only been reported for large vessel-derived smooth muscle cells during normal and experimentally induced vascular intimal thickening, obviously processes where enhanced migration of vascular tissue is involved [18,22]. Expression of sm α-actin in capillary endothelial cells from rat epididymal fat pads has recently been reported by Kocher and Madri [6]. Experimental conditions included TGF β1 treatment in a 2-dimensional culture system. Since pericytes have been shown to express smooth muscle specific isoactins [23], the possibility of a relationship between endothelial cells and pericytes has been discussed [22].

In our study we have demonstrated that cloned cEC, independent of their ability to express smooth muscle α-actin retained positive immunohistochemical staining for *Bandeiraea simplicifolia* [24] whereas pericytes were shown to be negative for this specific endothelial marker. However, the occurrence of an endothelial phenotype which displays smooth muscle cell characteristics gives rise to the speculation that endothelial cells, as well as smooth muscle cells and pericytes possibly originate from a common precursor cell.

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