IMMORTALIZED RAT BRAIN MICROVESSEL ENDOTHELIAL CELLS : II-PHARMACOLOGICAL CHARACTERIZATION

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INTRODUCTION

Vascular endothelial cells are known to display a variety of biological functions, including regulation of vascular tone through secretion of vasoactive factors and control of nutrient and cellular trafficking between the blood and the underlying tissue (1,2). Most of the available data concerning these functions have been collected in vitro from studies on macrovascular peripheral endothelial cells. Given the remarkable heterogeneity of endothelia in terms of morphology and function, we intended to assess the ability of brain microvessel endothelial cells, which constitute the blood-brain barrier (BBB), to secrete endothelin (ET-1) and nitric oxide (NO), two potent vasoactive factors, under the control of hormonal stimuli and to express the MHC molecules that may contribute to the adhesion of immune cells to the endothelium. This study was performed with immortalized rat brain microvessel endothelial cells that were isolated after transfection of primary cultures with the E1A-Adenovirus encoding gene. These cells present a non-transformed phenotype and express the blood-brain barrier markers γ-glutamyl transferase and alkaline phosphatase during angiogenesis, as described in the accompanying paper (Roux et al.).

EXPRESSION OF HORMONE RECEPTORS

Isoproterenol-stimulated cAMP Synthesis

RBE4 cells were tested for their ability to produce the cyclic nucleotides cAMP and cGMP under extracellular signal regulation. Figure 1A shows that isoproterenol, a β-adrenergic agonist, stimulated the accumulation of cAMP within RBE4 cells; this stimulation was blocked by the antagonist propranolol (not shown), indicating the involvement of β-adrenergic receptors, positively coupled to the enzyme adenylyl cyclase.

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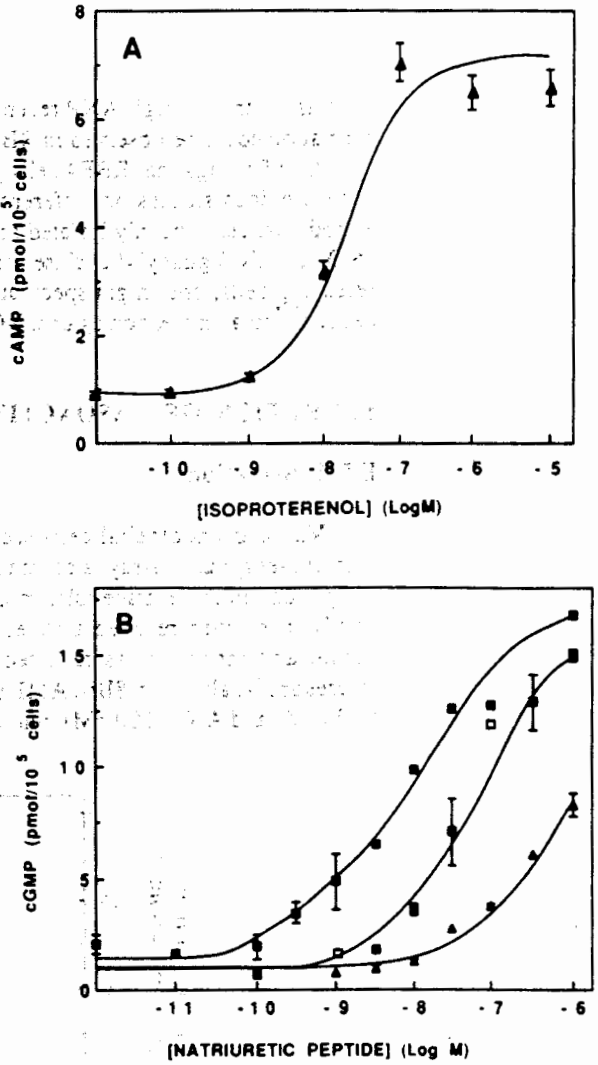

Figure 1. RBE4 cells were incubated for 10 min at 37°C in Hank's salt solution, 20 mM Hepes pH=7.4, 0.5 mM IBMX, with (A) increasing concentrations of (-)isoproterenol or (B) natriuretic peptides, ANP (■), BNP (□), CNP (▲). cAMP and cGMP accumulations were measured using Amersham kits.

Natriuretic Peptide-stimulated cGMP Synthesis

The synthesis of cGMP from GTP is catalyzed by different enzymes, the identity of which has been recently elucidated by molecular cloning: two related natriuretic peptide receptors present a guanylyl cyclase activity in their cytoplasmic domain with only limited homology to the NO-sensitive soluble enzyme (3). Evidence is presented in Figure 1B that the atrial natriuretic peptide (ANP) strongly stimulates the accumulation of cGMP within RBE4 cells. The related brain-derived peptide BNP was also found to activate the enzyme guanylyl cyclase, while the other natural analog CNP appeared much less active. This order of potency (ANP>BNP>CNP) suggests the expression of at least the A-subtype (3). In

contrast with this high ANP receptor / guanylyl cyclase activity, no NO-stimulated cGMP formation could be observed in RBE4 cells (not shown).

Our findings that RBE4 cells possess β-adrenergic and ANP receptors are in agreement with previous studies on different types of endothelial cells, including brain microvessel endothelial cells, freshly isolated or in short-term culture. Concerning the absence of soluble (NO-sensitive) guanylyl cyclase activity in these cells, which has been reported in other NO-releasing cells, one might speculate that the insensitivity to NO might constitute, for these cells, a natural protection against NO toxicity (2).

SECRETION OF VASOACTIVE FACTORS

ET-1 Secretion

Vascular endothelial cells secrete ET-1, a potent contracting factor (1). In a specific immuno-enzymatic assay, a constitutive secretion of ET-1 was observed: the accumulation of the peptide in the extracellular medium was linear over 6-8 hr (110±4 pg / 10^6 cells / hr, at confluence), then reached a plateau within about 16 hr. This secretion was stimulated by serum and thrombin, as reported previously with other endothelial cells (not shown); interestingly, although 8Br-cAMP (0.5 mM) strongly induced a release of ET-1, 8Br-cGMP (0.5 mM) and ANP (100 nM) inhibited the secretion (Figure 2).

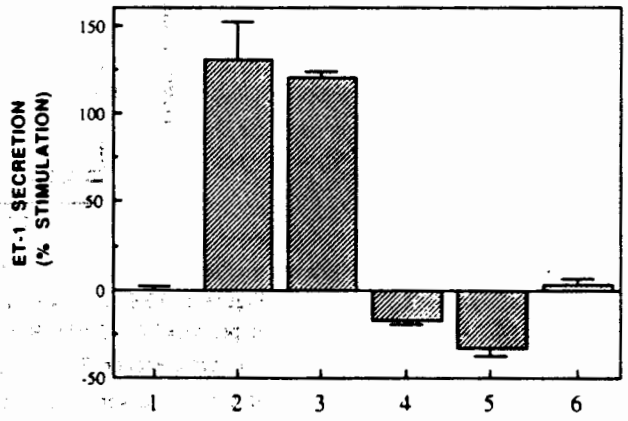

Figure 2. RBE4 cells, grown to confluence in 24-well dishes, were incubated, for 1 hr in serum free medium, supplemented with 0.15% BSA for 1 hr in the presence of the following agent: none(1), 0.5 mM 8Br-cAMP (2), 0.5 mM IBMX and 0.5 mM 8Br-cAMP (3), 0.5 mM 8Br-cGMP (4), 100 nM ANP (5), or 0.5 mM IBMX alone (6). After treatment, supernatants were assayed for ET-1 immunoreactivity. Results are expressed as means ± SE of 8 to 16 determinations; $p < 0.01$ for lanes 2 - 6.

NO Secretion

The endothelium-derived relaxing factor, which has been identified as NO or a closely related compound, is synthesized from L-arginine by at least two different NO synthases (2): one is calcium- and calmodulin-dependent and is constitutively expressed in a few cell types, including some neurons. The other one is inducible by cytokines and was first identified in macrophages. Both enzymes are potentially active in endothelium.

Table 1. Regulation of inducible NO synthase activity in RBE4 cells

Treatment	Nitrite Accumulation (μM)
None	<0.5
IFN	3.1±0.1
TNF	<0.5
TNF + IFN	10.0±0.1
TNF + IFN + NMA (100 μM)	1.7±0.1
TNF + IFN +NoA (10 μM)	7.6±0.4
TNF + IFN + CHX (0.5 μg/ml)	1.2±0.2
IFN + BrcAMP (500 μM)	3.8±0.1
TNF + IFN + BrcAMP (500 μM)	15.0±0.4

Cells grown at confluence in 24-well dishes were treated for 46 hr with 100 U/ml of rat interferon γ (IFN) and/or 50 U/ml of human tumor necrosis factor α (TNF), with or without N-methylarginine (NMA), nitroarginine (NoA), cycloheximide (CHX) or 8-bromo-cAMP (BrcAMP). Results are from a typical experiment performed in triplicate (±S.D.). Similar results were obtained in three other experiments.

Release of NO by RBE4 cells was monitored by a colorimetric determination of NO-derived nitrites. Only the inducible type of NO synthase is detectable in RBE4 cells (Table 1). Nitrite production by RBE4 was induced after treatment with IFN-γ and TNFα potentiated this effect. This activity was blocked by N-methyl-arginine and to a lesser extent by nitro-arginine, and required protein synthesis, as shown by its inhibition by cycloheximide. These properties are quite similar to that of the cytokine-activated NO synthase in macrophages. Our data are in good agreement with a recent study on murine brain endothelial cells in short-term culture (4), suggesting that lack of expression of the constitutive NO synthase might be a special feature of the BBB endothelial cells.

The cell-permeable analog of cAMP, 8Br-cAMP, although exhibiting no effect alone (not shown), was seen to potentiate the inductive effect of cytokines (Table 1).

Prostaglandin Secretion

RBE4 cells were found to constitutively secrete high amounts of PGE_2 (> 1000 pg / ml) but almost no 6-keto-PGF1α, the stable derivative of PGI_2 (< 50 pg / ml). These data are in agreement with a previously reported statement that a high PGE_2 / PGI_2 reflects the microvascular origin of endothelial cells (5).

EXPRESSION OF MHC MOLECULES

A number of disorders that affect the central nervous system, such as the demyelinating

diseases multiple sclerosis and its animal model experimental allergic encephalomyelitis (EAE), are characterized by infiltration of immune cells, through the BBB, into the brain parenchyma. During induction of EAE in susceptible animals by transfer of T lymphocytes from animals immunized with myelin basic protein, early steps in the pathogenesis probably involve interactions between cerebral endothelial cells and sensitized lymphocytes (6). For this reason, we investigated MHC class I and class II expression on RBE4 cells. By flow cytometry analysis under basal conditions, RBE4 cells were shown to constitutively express MHC class I, but no class II molecules. Analysis after IFN-γ treatment revealed that MHC class I expression was enhanced and class II expression dramatically induced (Figure 3): maximal class I expression was observed after 16 hr and remained stable for several hours, while 24 hr of treatment were required for optimal class II expression. Further experiments will have to assess the ability of these cells to efficiently present antigens to MHC class I- or class II-restricted T lymphocytes.

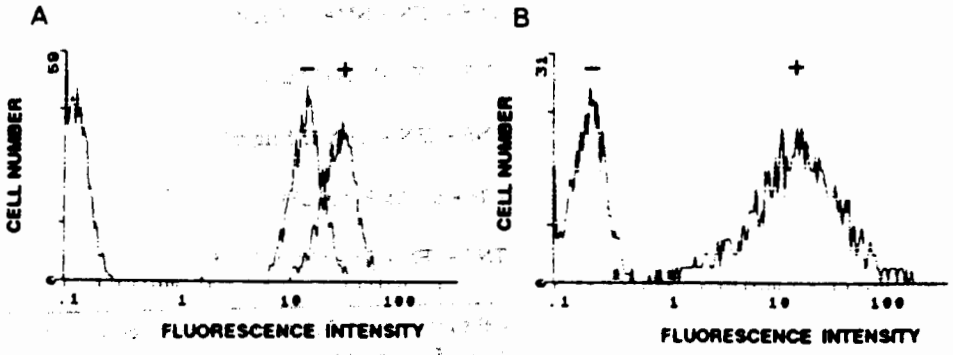

Figure 3. RBE4 cells were either not treated (-) or treated (+) with IFN-γ (50 U/ml) and stained for MHC class I, after 16 hr of treatment (A) or MHC class II, after 48 hr of treatment (B). The cells were then analyzed by flow cytometry.

CONCLUSION

In conclusion, it is shown in this study that the immortalized rat brain microvessel endothelial cells RBE4 have retained the ability to release a number of vasoactive factors, including NO and ET-1, two agents generally considered as local mediators for which astrocytes recently appeared as potential targets. Since astrocytes stimulate the expression, by brain microvessel endothelial cells in vivo and RBE4 cells in vitro, of a BBB-specific phenotype, and since cytokines regulate endothelial function, it is conceivable, from our results, that astrocytes, brain microvessel endothelial cells and leukocytes communicate, at the level of the BBB, through a network of pharmacological as well as immune interactions.

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