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ACTION OF ENDOTHELIN-1 ON RAT ASTROCYTES THROUGH THE ET_B RECEPTOR

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Summary: We investigated the effect of ET-1 on the state of rat cerebral astrocytes (AC) differentiation. AC ceased to proliferate and changed into its differentiated state by treatment with dibutyryl cyclic AMP (DBcAMP). The cell growth activity in DBcAMP-treated AC was stimulated by ET-1 in a dose-dependent manner. Over similar dose ranges, ET-1 suppressed the glutamine synthetase activity in DBcAMP-treated AC. The molar potency of ET-1 in this action was at least 3 orders of magnitude higher than that in mitogenic action in AC under the proliferative state previously reported. Northern blot analysis revealed that ET_B receptor mRNA level in DBcAMP-treated AC was markedly higher than that in AC untreated with DBcAMP. Consistently, binding studies showed that the B_{max} value for [^{125}I]ET-1 in DBcAMP-treated AC was 16 times higher than that in AC untreated with DBcAMP. These results suggest that ET-1 potently induced a retraction of the differentiation state of AC from fully the specialized state and that the high responsiveness of differentiated AC to ET-1 was partly attributed to the high level expression of the ET_B receptor.

Endothelins (ETs; ET-1, ET-2 and ET-3) are a highly conserved 21 amino-acid peptide known to have strong vasoconstricting activity. The first member of the family, ET-1 was initially isolated from a conditioned medium of the cultured porcine aortic endothelial cells (1). Additional work revealed that ETs exist in various tissues each with a distinct pattern and that ETs exert a wide variety of pharmacological effects on various types of cells and tissues (2).

It has been demonstrated that ETs (ET-1 & ET-3) as well as ET receptors (ET_A & ET_B) are present in the central nervous system (CNS) (Ref. 3,4,5,6). Furthermore, it has been suggested that ETs act as a neurotransmitter or neuromodulator (4,7).

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Several investigators reported that ET-1 has mitogenic activity in astrocytes (AC) and C6 glioma cells through an activation of phospholipase C (8). However, in these studies, the effect of ET-1 on the differentiated AC was not estimated. As for physiological conditions, AC are well-differentiated, and its morphology and metabolic activity are clearly shown. Proliferation of AC is exclusively observed after traumatic injury occurs, such as brain damage or brain ischemia (9). Thus, if ET-1 exerts a role in glial proliferation under pathological conditions of CNS, such as traumatic injury, cerebrovascular disease and brain tumor, it seems important to determine the effect of ET-1 on AC in the differentiated state. Dibutyryl cyclic AMP (DBcAMP) induces cyto-differentiation of AC as follows: 1) termination of cell proliferation; 2) morphological change from flat and polygonal shape to stellate shape; 3) and augmentation of glutamine synthetase (GS) activity (10). Therefore, in this study, we investigated the effect of ET-1 on the morphological change, cell growth activity and GS activity in AC under the DBcAMP-induced differentiated state. The results are discussed in the relation to the expression of the ET receptor.

Material and Methods

Cell culture: AC were obtained from the cerebrum of newborn Wistar rats (0-day-old) by using the slightly modified procedures of Lim et al (11) and Hertz et al (12). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, fungizone, and $NaHCO_3$, (1.2 g/l). The cells identified by glial fibrillary acidic protein (GFAP) were used for the experiments after the third passage (21~30 days old).

Cell growth assay and glutamine synthetase assay: AC in 96-well plates ($1.6X10^4$ cells/well) were cultured in the medium containing 20% FBS for 24 h, and the concentration of FBS was reduced to 10%. After 24 h, the medium was exchanged to serum-free medium and incubated for 24 h. Then, DBcAMP was added to the medium to be finally 0.5 mM, and the cells were incubated for 24 h. Various concentrations of ET-1 were also added to the medium. For the measurement of cell growth, 3-(4, 5-dimethyl-thiazoyl-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) assay was performed as previously described by Green et al (13). The GS activity was measured by the slightly modified method of Loret et al.(14).

Northern blot analysis: AC were subcultured in 10 cm dishes 3 days before, and allowed to grow into subconfluent monolayers. At the time of cell harvest, the medium was thoroughly aspirated and cells were immediately lysed by the direct addition of 3 ml of 3 M LiCl / 6 M urea to the dishes. Cell layers were scraped off with a rubber policeman, transferred to a microcentrifuge tube and incubated at 4 °C for 8 h. Precipitated RNA was collected by centrifugation at 12,000 g for 20 min., and the pellets were resuspended in 400 µl of 10 mM Tris-HCl (pH 8.0) / 1 mM EDTA / 0.5% SDS / 200 mg ml^{-1} proteinase K. After incubation at 37 °C for 4 h, RNA was purified by a phenol / chloroform extraction and an ethanol precipitation. Total RNA (10 µg) was separated by formaldehyde/1.1% agarose gel electrophoresis, and transferred to a HyBond-N^+ membrane (Amersham). The insert (2 kb) of prETR-7, which includes the entire coding region of ET_B receptor (6) was labelled to a specific activity of $8x10^8$ c.p.m./µg with [α-^{32}P]dCTP (3,000 Ci/mmol, New England Nuclear) by the random priming method, and hybridization was conducted at 42 °C in 1 M NaCl / 50% formamide / 1% SDS / 250 mg ml^{-1} salmon sperm DNA. The membranes were washed finally in 0.1 x SSC / 0.1% SDS at 50 °C, and autoradiographed with intensifying screens at -80 °C for 10 h. The same blots were re-hybridized by cDNA probe for β-actin as an internal control.

Binding study: AC in 24-well plates ($3X10^4$ cells/well) were washed three times with binding buffer (140 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 11 mM

glucose, 5 mM HEPES, 0.2% bovine serum albumin; pH 7.4). Then, the cells were incubated in the binding buffer containing various concentrations of [^{125}I]ET-1 (3-[^{125}I]iodotyrosyl^{13}-ET-1; 2,000 Ci/mmol, Amersham) for 2 h at 22 °C. After washing the cells two times with the binding buffer, the cells were collected with 1 N NaOH and the cell-bound radioactivity was counted with a γ-ray counter (Aloka). Specific binding was determined as total binding minus nonspecific binding. Nonspecific binding was determined in the presence of 10^{-6} M unlabeled ET-1.

Results

Effect of ET-1 on morphology of differentiated AC: We observed the morphological changes of AC. As shown in Fig. 1a, AC in a proliferative state, which cultured in the presence of FBS, demonstrated a flat and polygonal shape. By treatment with DBcAMP (0.5 mM) for 24 h, the morphology of AC was changed into a stellate shape which closely resembled the differentiated

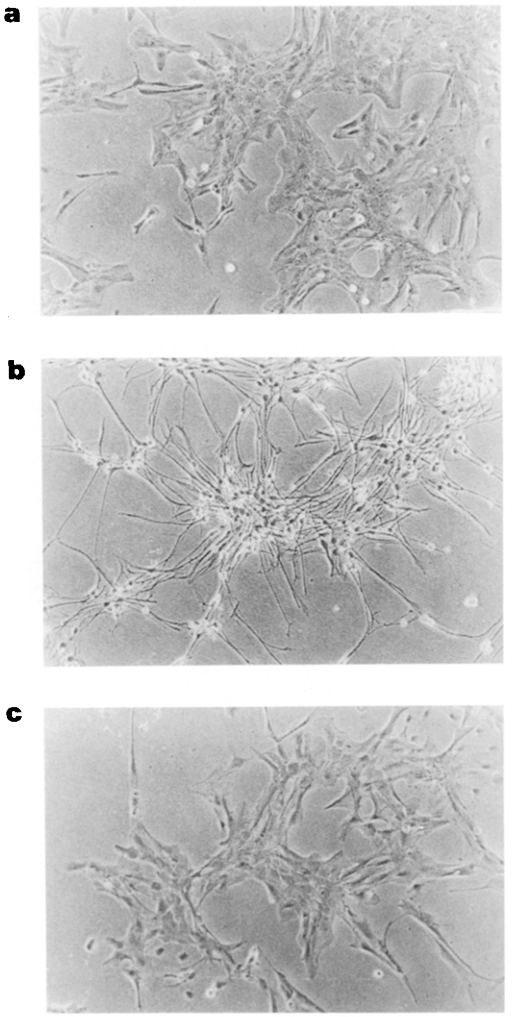

Fig. 1. Morphology of AC cultured in DMEM (a); with 10% FBS (b); with 0.5 mM DBcAMP for 24 h (c); after adding of 1 nM ET-1 to (b) for 3 h.

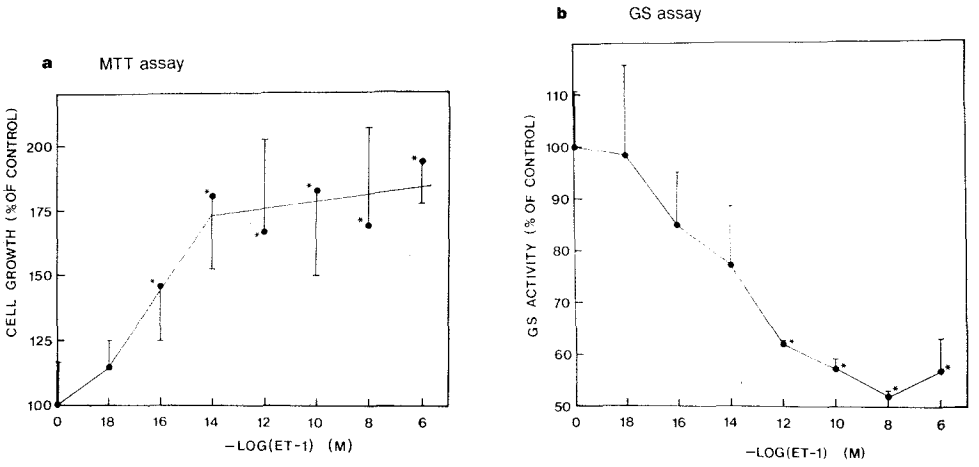

Fig.2. a) Effects of ET-1 on cell growth activity in differentiated astrocytes. The data was expressed as percentages of control. Each point represents mean ± S.D.(n=24).(b) Effects of ET-1 on glutamine synthetase activity in differentiated astrocytes. The data was expressed as percentages of control. Each point represents mean ± S.D.(n=12). *Significantly different with $P < 0.05$ (Student's *t* test for unpaired values).

cell morphology in the CNS (Fig. 1b). By the addition of ET-1 (1 nM) to the medium, most of the AC began the morphological change within 15 minutes and reverted back to the flat shape after 3 h (Fig. 1c).

Effect of ET-1 on cell growth activity and glutamine synthetase activity in differentiated AC: As shown in Fig. 2a, ET-1 stimulated the cell growth activity in DBcAMP-treated AC in a dose-dependent manner. The maximum response to ET-1 was 193.8±16.7% of the control. The minimum effective dose for ET-1 was 10^{-16} M. As shown in Fig. 2b, ET-1 suppressed the GS activity in DBcAMP-treated AC in a dose-dependent manner. The minimum effective dose and EC50 value (95% confidence limits) for ET-1 were 10^{-12} M and 4.3 x 10^{-10} M (6.0 x10^{-11} ~ 3.1 x 10^{-9} M), respectively. The maximum response to ET-1 was 54.3±1.0% of the control.

Northern blot analysis of ET receptor mRNA level: By northern blot analysis, the expression of ET receptor mRNA in AC under various conditions were investigated. As shown in Fig. 3, the ET_B receptor mRNA level in DBcAMP-treated AC was markedly higher than (lane 3) that in AC under a proliferative state (lane 1 & 2; subconfluent culture in the presence and absence of 10% of FBS, respectively). The expression level of ET_B receptor once up-regulated by the treatment with DBcAMP was decreased by the removal of DBcAMP (lane 4) and the addition of FBS (lane 5), respectively. Under the same experimental condition, the ET_A receptor mRNA was not detectable (*data not shown*).

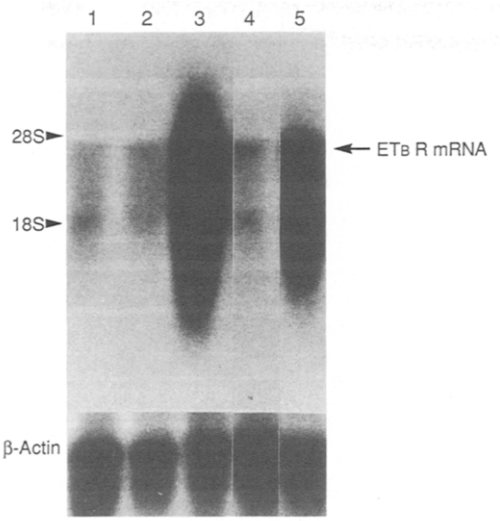

Fig.3. Expression of ET_B receptor mRNA in astrocytes under various conditions as measured by northern blot analysis. Each condition as follows: (lane 1); cultured in DMEM with 10% FBS, (lane 2); cultured in DMEM without FBS, (lane 3); cultured in DMEM with 0.5 mM DBcAMP, (lane 4); removed DBcAMP from the condition of lane 3, and (lane 5); added 10% FBS to the condition of lane 3.

Binding studies: To characterize the change of specific binding sites for ET-1 on AC, a radioligand binding assay was performed using [^{125}I]ET-1. Scatchard analysis showed that the dissociation constant (K_D) and the number of the binding sites (B_{max}) of [^{125}I]ET-1 were 162 fM and 37 x 10^5 sites per cell, respectively, on DBcAMP-treated AC. On AC untreated with DBcAMP (cultured in the presence of 10% FBS), K_D and B_{max} were 8.5 fM and 2.3 x 10^5 sites per cell (Fig. 4).

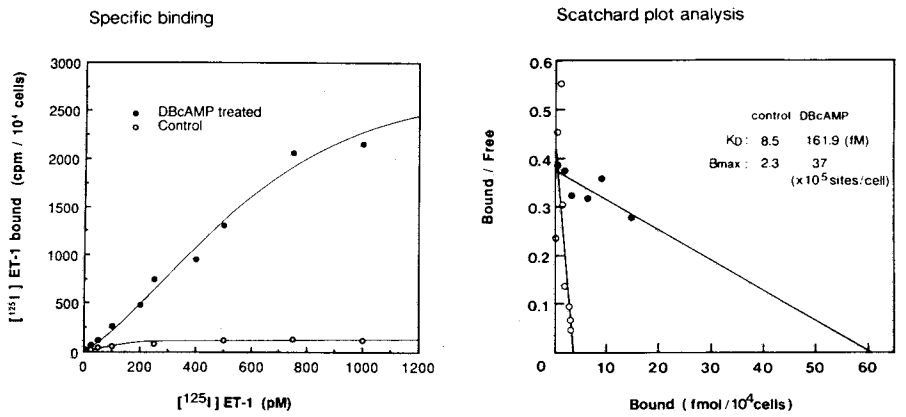

Fig.4. Binding studies of [^{125}I]ET-1 to astrocytes. Saturation of isotherm (left panel). Scatchard plot analysis (right panel). Astrocytes were cultured in DMEM with 10% FBS (○) or with 0.5 mM DBcAMP(●).

Discussion

This investigation demonstrated that ET-1 can potently modulate the differentiated state of AC. In the differentiated AC, ET-1 stimulated the cell growth activity and suppressed the GS activity in accordance with morphological change. Therefore between the two activities induced by ET-1, a reciprocal correlation was observed. These results suggested that ET-1 can liberate AC from the differentiated state to a proliferative state.

Previous studies demonstrated that ET-1 exerted mitogenic action in primary cultured rat AC in a proliferative state (8). In those studies, ET-1-induced mitogenic action might have been mediated through the activation of the ET_B receptor, as judged from the potency orders of ETs. Thus, the fact that ET-1 induced a retraction of the differentiated state of AC, and that only ET_B receptor was detectable by northern blot analysis, satisfied our expectation. However the action in this case, of the molar potency of ET-1, appeared to be surprisingly large (effective dose range: $>10^{-14}$ M). This might be ascribed to the fact that northern blot analysis and binding studies clearly revealed the high level expression of ET_B receptor in the differentiated AC. The number of ET_B receptors on the differentiated AC were estimated at about 3,700,000 sites / cells, which was 16 times that of AC in a proliferative state, though the affinity of receptor for ET-1 on the differentiated AC was rather lower than that on AC under a proliferative state.

It was previously reported that the up-regulation of ET receptors was induced by the pathophysiological conditions in renal and cardiac cell membrane, respectively (15,16). However, the ratio of up-regulation in these cases was about 2 times that of the control at the most. Such a marked up-regulation as in the present study has never been reported. The question arose as to how the expression of ET_B receptor was regulated. Did the accumulation of cytosolic cAMP level induce the up-regulation of the ET_B receptor ? This was unlikely because ET receptor in C6 glioma cells, which probably exhibited ET_B (17), was down-regulated by the accumulation of cytosolic cAMP level (18). Furthermore, lysophosphatidylserine which induced morphological change of AC, as in the case of DBcAMP, also induced the up-regulation of the ET_B receptor, but independently of the change in cAMP level (data not shown). This evidence suggested that the differentiation itself was more likely to induce the up-regulation of ET_B receptor. In other words, the expression level of the ET_B receptor in AC may have been dependent on the state of their differentiation. In this regard, it was tempting to speculate that the ET_B receptors were well expressed in the AC in the adult CNS, which were at a preparatory state for proliferation of AC

after traumatic injury such as brain damage or brain ischemia, and that ET-1 which possibly derived from endothelial cells of brain capillary, neural cells or plasma may have played some important roles in the recovery process. Previous reports indicated that ET-1 was expressed in postnatal rat brain and cerebellar AC but not in brain capillary endothelial cells (19,8). We therefore examined the expression of ET-1 in AC in not only mRNA level by northern blot but also in peptide level by enzyme-linked immunoassay (EIA), respectively. Furthermore, we investigated the existence of ET-1 in the conditioned medium of the cultured neural cells from rat brain by using EIA. However, all these results were negative (data not shown). Thus, the plasma-derived ET-1 from damaged vessels in the brain appeared to have been a plausible source. If the ET_B receptors were highly expressed in the CNS, ET-1 at a normal concentration level in the plasma could stimulate the proliferation of AC.

In conclusion, ET-1 potently modulated the differentiated state of AC through ET_B receptor. The high responsiveness of the differentiated AC to ET-1 may have been due to the extremely high level expression of ET_B receptor.

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