Journal of Food and Drug Analysis

Volume 5 | Issue 1 Article 8

1997

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Cheng, H.-F.; Yang, S.-Y.; and Lin, Y.-H. (1997) "Dibutyryl cAMP down-regulates gene expression of endothelin receptor A subtype in rat astrocytoma C6 cells," *Journal of Food and Drug Analysis*: Vol. 5 : Iss. 1 , Article 8.
Available at: https://doi.org/10.38212/2224-6614.2959

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Journal of Food and Drug Analysis
1997. 5(1) : 43-50

EJ087199700041

Dibutyryl cAMP Down-regulates Gene Expression of Endothelin Receptor A Subtype in Rat Astrocytoma C6 Cells

HWEI-FANG CHENG[1]*, SHIU-YAN YANG[2] AND YEN-HUEI LIN[1]

[1.] *National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, Taipei, Taiwan, R.O.C.*
[2.] *Deh Yu Junion College of Nursing, Taipei, Taiwan, R.O.C.*

ABSTRACT

In this study, we have investigated the gene expression of endothelin receptors in rat astrocytoma C6 cells regulated by the increase of intracellular cAMP. Following treatment with 1 mM dibutyryl cAMP, the specific binding of [^{125}I]-endothelin-1 to the C6 cells was down-regulated to about 40 % of the original level. Radioligand binding assay and Northern blot analysis indicated that endothelin receptors on C6 cells, which were either treated or untreated with 1 mM dibutyryl cAMP, belong to subtype A. Analysis of RNA by Northern blotting and slot hybridization demonstrated that the presence of dibutyryl cAMP induced the down regulation of ET_A endothelin receptor gene in C6 cells. The level of ET_A mRNA was rapidly reduced to about 10 % of its initial level after 4 hour treatment with dibutyryl cAMP and then was slightly elevated to about 40 % after one day treatment. All the findings suggest that the down regulation of ET_A receptor in C6 cells by the treatment of dibutyryl cAMP occurs not only at the receptor binding level but also at the mRNA level.

Key words: **Endothelin receptor gene, astrocytoma C6 cell, radioligand binding assay, Northern blot analysis, slot hybridization, dibutyryl cAMP, down regulation.**

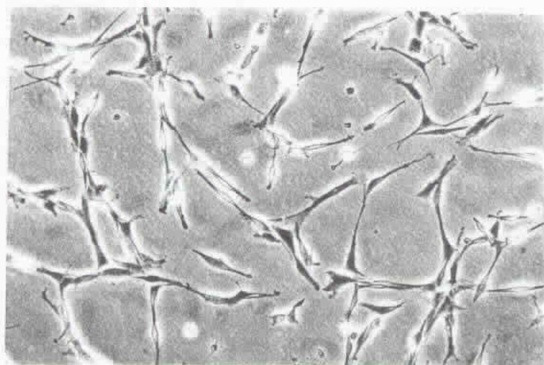
INTRODUCTION

Endothelins (ETs), a vasoactive 21-amino-acid peptide family consisting of three peptides, ET-1, ET-2, and ET-3(1,2), have been shown to exert their physiological actions through specific cell surface receptors(3). Two subtypes of endothelin receptor (ET_A and ET_B) have been identified by their distinct affinities toward the endothelin isopeptides(4,5). The ET_A selectively binds, in a decreasing order of affinity, to ET-1, ET-2, and ET-3, while the nonselective type ET_B binds all three ETs with the same affinity. The cDNA of both endothelin receptor subtypes has been isolated and the deduced amino acid sequences have demonstrated that each receptor contains seven transmembrane domains, suggesting they belong to the superfamily of G-protein

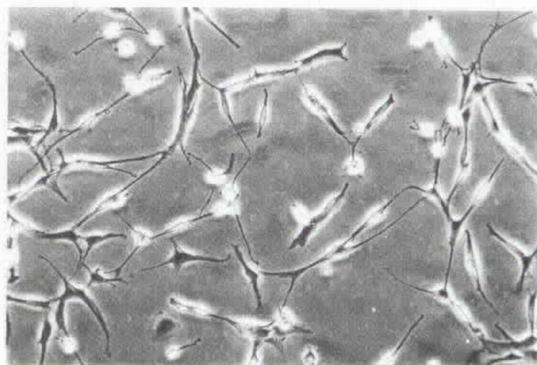
Correspondence to: Hwei-Fang Cheng
Accepted for Publication: Sep. 21, 1996

4 Hours

Control



db-cAMP



1 Day

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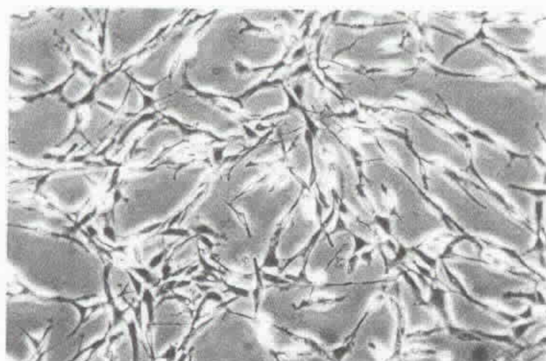

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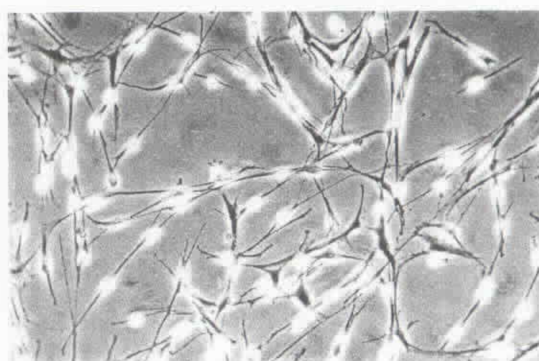
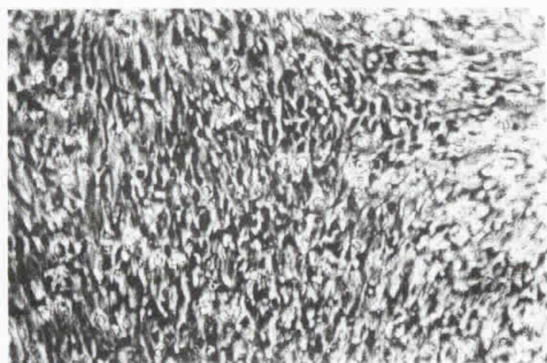


2 Days

Control



db-cAMP

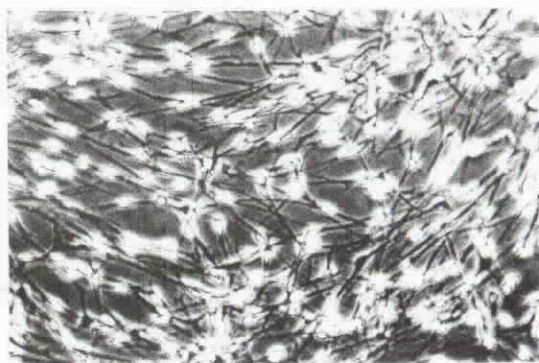


Figure 1. The morphological changes during the db-cAMP treatment. The C6 cells were treated with 1 mM db-cAMP for 4 hours,1 day, and 2 days.

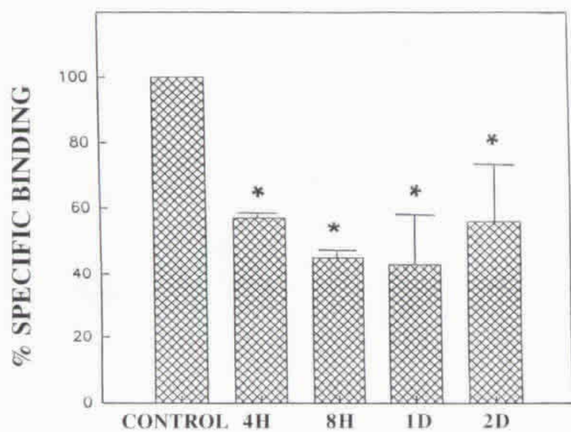
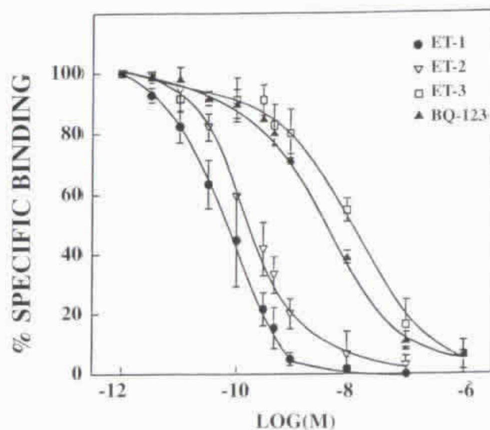


Figure 2. Effect of db-cAMP treatment on the specific binding of [^{125}I]-ET-1 to C6 cells at various time course. 4H: 4 hours; 8H: 8 hours; 1D: 1 day; 2D: 2days. Data were expressed as percentage of control (100 %), which representing C6 cells untreated with db-cAMP. P value < 0.05.



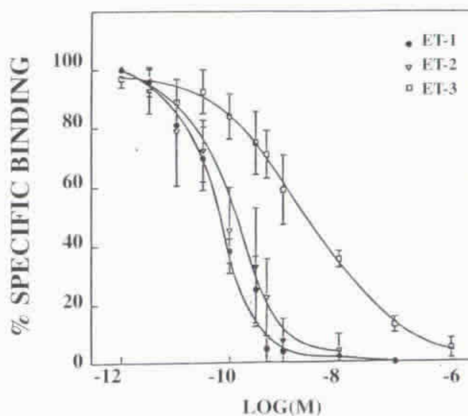


Figure 3. Competition between [^{125}I]-ET-1 and increasing concentrations of ET-1, ET-2, ET-3, and BQ-123 on cell membrane from C6 cells untreated (A) or treated with db-cAMP for 2 days (B). About 8 pM of [^{125}I]-ET-1 was incubated with C6 cell membrane (25 μg/ml) and the designated concentrations of ET-1, ET-2, ET-3, and BQ-123 at 25°C for 2 hours. Results are expressed as percentage of the maximal specific [^{125}I]ET-1 binding. Each point represents the mean of three separate experiments, each done in triplicate.

coupled receptor.

Endothelins are also neuropeptides found in mammalian brain(6). It has been suggested that endothelins in brain may play a fundamental role in regulating nervous system function. Recently, we have identified and characterized the endothelin receptor in rat cerebellum(7) and an alternative ET_B cDNA was isolated from rat brain(8). It has also been reported that endothelin-1 can increase the PI-turnover in rat astrocytoma C6 cells through a single high affinity endothelin receptor (9,10). However, incomplete down-regulation of the endothelin receptor was found by the increase of intracellular cAMP(11). The question was raised whether it was possible that a different population of endothelin receptors was present on C6 cells or the increase of intracelluar cAMP could induce another subtype of endothelin receptor. Therefore, in the present study, we investigated the feasibility of using radioligand binding assay and mRNA analysis to identify the receptor subtype on C6 cells with and without treatment with a membrane permeable cAMP analogue, dibutyryl cAMP. The results demonstrate that the endothelin receptors on C6 cells belong to A subtype, and the treatment of the

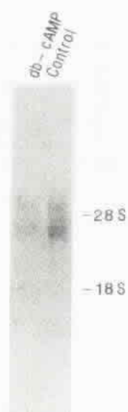


Figure 4. Northern blot analysisof ET_A mRNA in C6 cells untreated or treated with 1 mM dibutyryl cAMP. About 8 μg of poly $(A)^+$ RNA from C6 cells untreated (control) or treated with 1 mM dibutyryl cAMP (db-cAMP) for 2 days was analyzed. Northern membrane was blotted with the specific probe of ET_A receptor. The position of 28 S and 18 S rRNA are shown on the right.

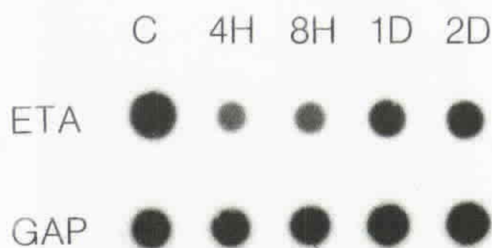


Figure 5. Slot hybridization analysis for ET_A mRNA level at different time course during dibutyryl cAMP treatment. About 5 μg of poly $(A)^+$ RNA from C6 cells untreated (control) or treated with 1 mM dibutyryl cAMP for 4 hours (4H), 8 hour (8H), one day (1D), and 2 days (2D) was analyzed. The membrane was hybridized with the specific probe for the ET_A receptor (ETA) or GAPDH (GAP) .

dibutyryl cAMP does not alter the specificity of receptor subtype. Moreover, the addition of dibutyryl cAMP can cause the down regulation of endothelin receptors not only at the receptor binding level but also at the mRNA level.

MATERIALS AND METHODS

I. *Cell Culture and Dibutyryl cAMP Treatment*

The rat astrocytoma C6 cells were grown in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in a humidified incubator of 10% CO_2 at 37°C. One day following subculture, C6 cells were incubated in the medium containing 1 mM dibutyryl-cAMP (db-cAMP), and harvested at the indicated time. In parallel experiments, C6 cells cultured in the medium without containing dibutyryl cAMP were used as controls.

II. *Membrane Preparation*

Subsequent to harvest, C6 cells were resuspended in 10 volumes of hypotonic buffer (5 mM Tris-HCl pH 7.7, 0.1 mM PMSF, 50 μg/ml soybean trypsin inhibitor (STI), 20 μg/ml leupeptin, 5 mM EDTA, 5 mM EGTA) and homogenized by a polytron at 12,000 rpm for 20 seconds. The homogenate was then centrifuged at 19,000 x g for 30 minutes. After removing the supernatant, the pellet was resuspended in binding buffer (50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 3 mM EDTA, 1 mM EGTA, 20 μg/ml bacitracin, 20 μg/ml leupeptin, 50 μg/ml STI, 10 mM PMSF and 0.1% BSA) and stored at -20°C until use. The membrane protein concentration was determined by Protein Assay Reagent (BioRad).

III. *Radioligand Binding Assay*

For direct receptor binding assay, C6 cells were harvested and respended in DMEM containing 0.1% (w/v) BSA and 5 mM $MgCl_2$ to a final concentration of 2.5×10^6 cells/ml. Approximately 400 μl of cell suspension was incubated with 14-18 pM of $[^{125}I]$endothelin-1 (specific activity 2,000 Ci/mmol, Amersham) at 25°C for 2 hours. The nonspecific binding was determined in the presence of 1 μM nonradioactive ET-1. After incubation, the cells were filtered through GF/C glass fiber filter (Whatman) presoaked with 0.3 % BSA and washed in 5 ml of

ice-cold phosphate buffer saline (PBS) containing 0.1% BSA. For competition binding assay, membrane proteins from C6 cell either untreated (25 μg/ml) or treated (50 μg/ml) with 1 mM dibutyryl cAMP for 2 days was incubated with 8 pM of [^{125}I]-ET-1, and the designated concentrations of ET-1, ET-2, ET-3, and BQ-123[12] at 25°C for 2 hours. The receptor-[^{125}I]ET-1 complex was separated from [^{125}I]-ET-1 by filtration through a Whatman GF/C glass filter. The filters were washed twice with 10 ml cold buffer. The radioactivity of each filter was counted with a gammar counter.

IV. *Preparation and Analysis of RNA*

Total RNA was extracted from C6 cell treated or untreated with 1 mM dibutyryl cAMP for the indicated time. After further purification by oligo (dT)-cellulose column chromatography, poly (A)$^+$ RNA was analyzed by Northern blotting or slot hybridization. For Northern blot analysis, 8 μg poly (A)$^+$ RNA from C6 cells untreated or treated with 1 mM dibutyryl cAMP for 2 days was separated by 1% formaldehyde/agarose gel electrophoresis, and transferred to a nylon membrane (NEN/DuPont). The membrane was hybridized with the specific probe for ET_A or ET_B. The ET_A probe was prepared from a cDNA fragment of ET_A (nucleotide +353 to +1122) which was amplified by PCR using the first-strand cDNA from C6-cells as template. The amplified DNA fragment was subcloned into pTZ19 plasmid and the nucleotide sequence was further determined. On the other hand, a specific probe for ET_B was prepared from ET_B cDNA (nucleotide +78 to +225), which was isolated from rat brain cDNA library in our laboratory[8]. After hybridization, the membrane was washed twice for 5 min in 2X SSC (1X SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) at 25°C, twice for 30 min in 2X SSC / 1.0% SDS at 65°C, and finally twice for 30 min in 0.1X SSC at 25°C. The hybridized membrane was autoradiographed at -80°C for 2 weeks. For slot hybridization analysis, 5 μg of poly(A)$^+$ RNA was directly applied onto the SSC-rinsed nylon membrane. After cross-linking by UV, the slot membrane was hybridized with the specific probe of the ET_A receptor as described above. The washing protocol was the same as described above for Northern blot analysis. The Northern membrane or slot membrane was reprobed with the GAPDH cDNA as an internal control.

RESULTS

I. *The Effect of Dibutyryl cAMP on C6 Cell Morphology*

It was found that the membrane permeable cAMP analogue, dibutyryl cAMP, could markedly reduce the cell division and induce the morphological differentiation of C6 cells at lower cell density better compared to higher cell density (data not shown). As shown in Fig. 1, the change in the shape of C6 cells acquiring threadlike processes occurred as early as 4 hours after the treatment with 1 mM db-cAMP and continued up to 2 days of treatment .

II. *Effect of Dibutyryl cAMP Prétreatment on the Specific^{125}I-Endothelin-1 Binding to C6 Cells*

The effect of dibutyryl cAMP treatment on the specific binding of [^{125}I]ET-1 to C6 cells was examined by conducting the radioligand binding assay on C6 cells harvested at various time course during treatment. As shown in Fig. 2, the [^{125}I]ET-1 specific binding was found to be rapidly decreased to about 60 % of the original level as early as 4 hour treatment with dibutyryl cAMP, and about 40 % original level following 8 hours of treatment, and maintained the same level up to one day treatment.

III. *Competition Binding Analysis*

To examine the subtype of endothelin receptors on C6 cells, ET-1, ET-2, and ET-3 were used as competitors to displace the specific [^{125}I]ET-1 binding from C6 cell membrane. As shown in Fig. 3a, the order of displacement of the [^{125}I]ET-1 specific binding from C6 cell membrane is ET-1>ET-2>>ET-3. Judging from the affinity toward the endothelin isopeptides suggests that the endothelin receptors on C6 cells belong to sub-

type A. A similar pattern of the competition curves was observed for the membrane from C6 cells treated with db-cAMP for 2 days (Fig.3b), indicating that the endothelin receptor subtype on C6 cells is not affected by the treatment of db-cAMP. The inhibition of [^{125}I]ET-1 specific binding to C6 cell membrane by BQ-123, a specific antagonist of ET_A receptor(12), at the micromolar range (Fig.3a), further confirmed the presence of ET_A receptor on C6 cells.

IV. *Effect of Dibutyryl cAMP Treatment on the ET_A mRNA of C6 Cells*

The subtype and the level of the mRNA encoding the endothelin receptor expressed on C6 cell before and after db-cAMP treatment were examined by Northern blot analysis. When the Northern membrane was hybridized with the specific probe of the ET_B receptor, no significant band was detected (data not shown). However, when the membrane was blotted with the specific ET_A probe, as shown in Fig. 4, two sizes of ET_A mRNA could be detected. One is about 5.6 kb and the other is 3.9 kb, which were thought to be generated by using different polyadenylation signals. In agreement with radioligand binding assay, the result from Northern blotting analysis further confirmed that only ET_A mRNA, but not ET_B mRNA, could be expressed in C6 cells, which were either treated or untreated with dibutyryl cAMP. Furthermore, the ET_A mRNA level in C6 cells after 2 day treatment with db-cAMP was found to be much less than that of untreated C6 cells. The application of equal amounts of mRNA in each lane was confirmed by reprobing the membrane with the GAPDH cDNA as the internal control (data not shown). This result suggested that the treatment of db-cAMP could reduce the gene expression of ET_A receptor in C6 cells.

To obtain more detailed down regulation of ET_A mRNA induced by db-cAMP, the mRNA of C6 cells treated with db-cAMP at various time intervals was further analyzed by slot hybridization. As shown in Fig.5, ET_A mRNA was rapidly decreased to 10 % as early as 4 hours following dibutyryl cAMP treatment. After one day, the ET_A mRNA level appeared slightly elevated, but still remained about 40% of its original level, even up to 2 days of treatment. The application of equal amounts of mRNA in each slot was confirmed by reprobing the membrane with the GAPDH cDNA as the internal control. Both mRNA analysis by Northern blotting and slot hybridization indicated that db-cAMP could down-regulate the gene expression of ET_A receptor in C6 cells.

DISCUSSION

Endothelin receptors have been demonstrated in C6 astrocytoma cells, which were involved in the IP_3 production(9,10) induced by endothelin. However, in these previous studies, the subtype species of ET receptors on C6 cells were not clearly identified by biochemical methods. In the present communication, the ET receptors on C6 cells are suggested to be only ET_A receptors either by radioligand binding assays or by Northern blot analysis. Thus, the endothlin-induced pharmacological responses in C6 cells may be mediated through ET_A receptor subtype.

The incomplete down-regulation of the ET receptors on C6 cells induced by the increase of intracellular cAMP has been previously reported (11) and the incompletely down regulation was postulated to be involved with a different receptor subtype. However, our results demonstrate that only ET_A receptors exist on C6 cells when untreated or treated with 1 mM db-cAMP and that the receptor subtype is remaining unaffected. In addition, the presence of db-cAMP could induce the down regulation of endothelin receptors on C6 cells not only at the receptor binding level but also at the mRNA level. The reduced level of ET_A mRNA in C6 cells showed coincidence with that of [^{125}I]ET-1 specific binding to C6 cells after 1 day treatment with dibutyryl cAMP. Therefore, the incomplete down-regulation of endothelin receptor by the treatment of dibutyryl cAMP was not due to the presence of different population of receptor; instead, it may

be effected by the incomplete down-regulation of ET_A mRNA. Detailed machanisms of how db-cAMP down-regulates the ET_A gene expression warrants further investigation.

Footnotes: ET, endothelin; ET_A, endothelin receptor subtype A; ET_B, endothelin receptor subtype B; $[^{125}I]$ET-1, $[^{125}I]$endothelin-1; db-cAMP, dibutyryl cAMP.

ACKNOWLEDGMENTS

This work was supported partially by research grants from Department of Health (DOH 83-FD-015) and National Science Council (NSC 84-2331B-043A-001), Taipei, Taiwan, R. O. C.

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以雙丁烷基環狀腺嘌呤單磷核苷酸抑制鼠神經膠質瘤細胞—C6上內皮素受體A亞型之基因表現

陳惠芳[1]* 楊續研[2] 林彥輝[1]

[1] 行政院衛生署藥物食品檢驗局
[2] 德育護理專科學校

摘 要

在本篇論文中，我們探討鼠神經膠質瘤細胞C6內之環狀腺嘌呤單磷核苷酸濃度之增加，對於該細胞內之內皮素受體基因表現之調控。當C6細胞以1mM之雙丁烷基環狀腺嘌呤單磷核苷酸處理一天後，發現其細胞上之碘125標誌之內皮素特異性結合，只剩為原來未處理前之約百分之四十。以放射性配位體結合試驗與北方墨點分析方法，證明了C6細胞上之內皮素受體屬於A亞型受體，不管是在有無以環狀腺嘌呤單磷核苷酸處理C6細胞情形下皆然。進而以北方墨點分析方法與細縫雜交反應法進行分析C6細胞之RNA量，發現雙丁烷基環狀腺嘌呤單磷核苷酸可抑制C6細胞之內皮素A亞型受體之基因表現。當環狀腺嘌呤單磷核苷酸處理C6細胞後4小時內，便可測得其內皮素A亞型受體之RNA量只剩為原先之百分之十，繼續處理一天後，其RNA量會緩慢回升至原先量之百分四十。本研究結果證明了雙丁烷基環狀腺嘌呤單磷核苷酸處理C6細胞後，對於其細胞上內皮素A亞型受體之向下調控作用，不僅會減少其受體之結合量，亦會降低其mRNA量。

關鍵詞：鼠神經膠質瘤細胞C6，內皮素受體，雙丁烷基環狀腺嘌呤單磷核苷酸，放射性配位體結合試驗，北方墨點分析方法，細縫雜交反應法。