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Induction of glutathione *S*-transferase, placental type in T9 glioma cells by dibutyryladenosine 3′,5′-cyclic monophosphate and modification of its expression by naturally occurring isothiocyanates

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Summary. The effect of dibutyryladenosine 3′,5′-cyclic monophosphate (dibutyryl cAMP) on the expression of glutathione *S*-transferase placental type (GST-P) was examined in rat glioma cell line using an immunohistochemical technique. Cultured T9 glioma cells were negative for GST-P activity under normal conditions. However, treatment with 1 mM dibutyryl cAMP produced GST-P expression in about 50% of the cells, as well as some morphological changes. The expression of GST-P was increased with addition of dibutyryl cAMP together with 1 μg/ml allyl isothiocyanate (AITC) or 0.1 μg/ml benzyl isothiocyanate (BITC). With these combinated treatments, almost all cultured cells showed a strong positive reaction for GST-P, although AITC or BITC alone elicited GST-P in only 5% of the cultured cells. The results of the present study indicate that dibutyryl cAMP causes functional as well as morphological differentiation of T9 glioma cells.

Key words: Glutathione *S*-transferase placental type – T9 glioma cells – Dibutyryladenosine 3′,5′-cyclic monophosphate – Allyl isothiocyanate – Benzyl isothiocyanate

Adenosine 3′,5′-cyclic monophosphate (cAMP) and its dibutyryl derivative are known to induce morphological differentiation and inhibition of cell growth in various glioma cell lines [9, 11, 14, 16, 22, 28] and play a significant role in benign transformation of the malignant glioma cells.

Glutathione *S*-transferases (GST) have an important function as detoxifying enzymes which catalyze the conjugation of a wide variety of hydrophobic electrophilic substances with glutathione [4, 13, 19]. GST has several isozymes which have been extensively studied in rats. Its placental form (GST-P) is used as a marker for preneoplastic or neoplastic lesions in rat liver, since normal hepatocytes of rats lack the activity of this isozymes [23, 24]. GST-P is, on the other hand, well recognized in normal rat astrocytes, and is regarded as a major form of isozyme expressed in rat brain [29]. However, no study of the stimulation of expression of GST-P by dibutyryl cAMP has yet been conducted in any organ or cell line.

Benzyl isothiocyanate (BITC) is a naturally occurring compound, found in some plants such as Indian cress [15] and garden cress [10]. When these plants are included in the diet, BITC is released by enzymatic hydrolysis. Recently, BITC has been shown to elevate GST activities in many organs [2, 26, 30] and inhibit carcinogen-induced tumorigenesis in some animal models [31, 32].

Allyl isothiocyanate (AITC) is related to BITC and is a major component of the volatile oil of mustard. It is also found in various plants such as cabbage, broccoli, kale, cauliflower, and horseradish [5, 20]. AITC is reported to be mutagenic and carcinogenic [7, 8, 12].

The present study has been conducted to investigate the possibility stimulation of expression of GST-P by dibutyryl cAMP and its modification by these isothiocyanates in T9 glioma cells. Our preliminary study has revealed that the T9 glioma cell line is negative for GST-P activity (unpublished data).

Material and methods

Chemicals

Dibutyryl cAMP was purchased from Sigma Chemical Co. (St. Louis, MO, USA). AITC and BITC were supplied by Nakarai Chemical Co., Osaka, Japan.

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Table 1. The effect of dibutyryl cAMP, AITC and BITC on the expression of GST-P in T9 cells

	Percentage of positive staining cell numbers (%)	Immunostaining response	Morphology
1. control	0	−[a]	U[b]
2. d-cAMP	50	+~++	D
3. AITC	5	+	U
4. AITC + d-cAMP	>95	++	D
5. BITC	5	+	U
6. BITC + d-cAMP	>95	++	D

[a] −: Negative; +: positive; ++: strongly positive
[b] U: Undifferentiated; D: differentiated
d-cAMP: Dibutyryladenosine 3′,5′-cyclic monophosphate; AITC: allyl isothiocyanate; BITC: benzyl isothiocyanate; GST-P: placental form of glutathione S-transferases

Cell cultures

Rat brain T9 glioma cells, which were derived from a glioma induced by N-methyl N-nitrosourea in a CD Fisher rat [1, 6], were used. T9 cells were seeded at a density of $1 \times 10^4 - 2 \times 10^4$/ml in Lab-Tek tissue culture chamber/slides containing RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% calf serum, 0.3 mg/ml L-glutamine and 100 μg/ml Streptomycin. Each test chemical, such as dibutyryl cAMP, AITC or BITC, was added to medium 2 days after seeding. A total of six chamber/slides (no. 1–6) were prepared: no. 1 was control; nos. 2–6 were exposed to chemicals as follows: (2) 1 mM dibutyryl cAMP; (3) 1 μg/ml AITC; (4) 1 mM dibutyryl cAMP and 1 μg/ml AITC; (5) 0.1 μg/ml BITC; (6) 1 mM dibutyryl cAMP and 0.1 μg/ml BITC. Mediums containing each chemical were changed every other days and cultures were maintained for 7 days in an atmosphere of 95% air/5% CO_2 at 37°C.

Immunohistochemical methods

Anti-GST-P antibody was raised as described previously [23, 24]. The peroxidase-antiperoxidase (PAP) technique [27] using Dako PAP kit (Dako, Santa Barbara, Calif) was adopted. Immunohistochemical study was performed 7 days after seeding of cells. Each specimen was fixed in acetone for 3 min, washed in phosphate-buffered saline solution (PBS) and then treated sequentially with normal swine serum, rabbit anti-GST-P antibody (1:800), swine anti-rabbit antibody and PAP. The specimens were carefully rinsed in PBS after each step. The peroxidase binding sites were detected by staining with 0.05% 3,3′-diaminobenzidine tetrahydrochloride in PBS for 5 min and rinsed quickly in distilled water. They were counterstained with Mayer's hematoxylin. Negative control stainings were prepared by absorption of immune serum with purified GST-P and by replacement of the primary antibody with non-immunized rabbit serum. All specimens were stained simultaneously.

Positive immunoreactivity of GST-P was graded semiquantitatively as follows: negative (−), positive (+) and strongly positive (++). The ratio of the cells which showed positive or strongly positive response was calculated. The morphological changes were divided into two pattern, i.e., undifferentiated and differentiated.

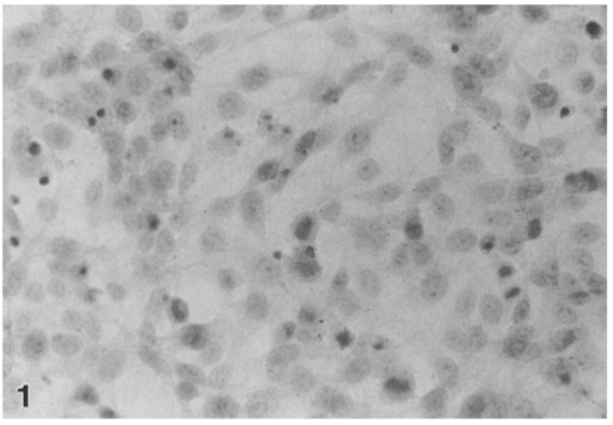
Results

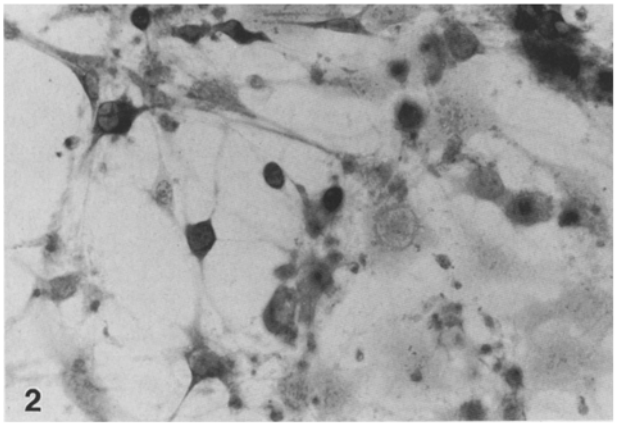
The results were summarized in Table 1. Non-treated T9 cells from chamber/slide no. 1 did not show any positive reaction for GST-P. The cells were spindle shaped, lacked cell processes and were sometimes piled up (Fig. 1). T9 cells treated with 1 mM dibutyryl cAMP, no. 2, showed weak to strong reactivity for GST-P in about 50% of the cells. They were elongated and separated from each other with fine cell processes forming a loose network. The appearance of the cells resembled normal astrocytes (Fig. 2). On the other hand, T9 cells from no. 3, cultured with AITC alone, did not change their morphology. However, about 5% showed a positive reaction for GST-P, especially around the cell nuclei (Fig. 3). Most of the cells from no. 4, treated with dibutyryl cAMP and AITC, showed a strongly positive response for GST-P. The staining reaction for GST-P was diffusely recognized in the cytoplasm including cell processes. The morphology of the cells was also similar to that of normal astrocytes (Fig. 4). Cells cultured with BITC, no. 5, gave a result similar to that with AITC (Fig. 5). Treatment with BITC and dibutyryl cAMP (no. 6) gave almost the same effect as no. 4 (Fig. 6).

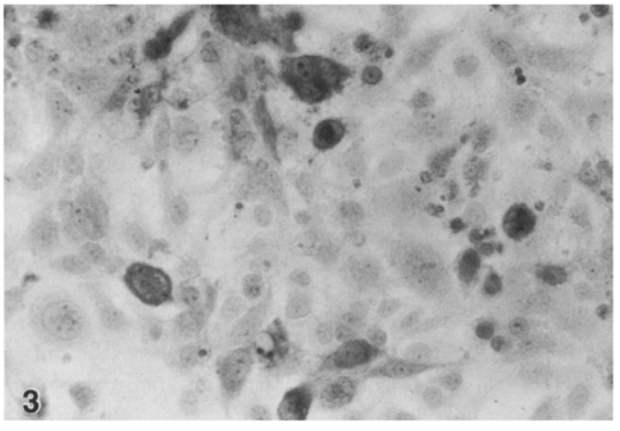
Discussion

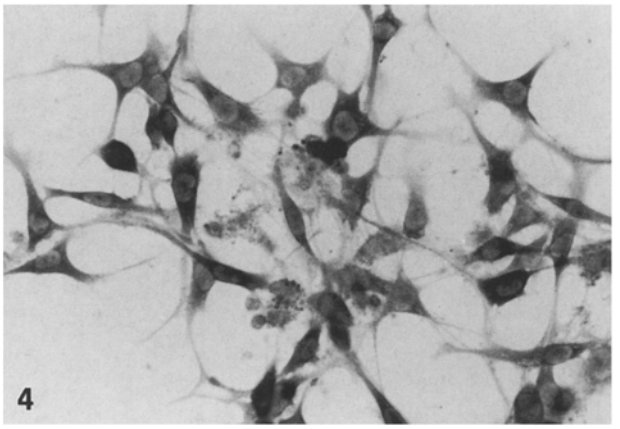
In the present study, T9 glioma cells which had been negative for GST-P activity were found to acquire a positive reaction for the anti-GST-P antibody by treatment with dibutyryl cAMP. The reaction was further increased by addition of AITC or BITC. These results indicate that dibutyryl cAMP generates enzymatic or functional changes as well as morphological alteration and that naturally occurring isothiocyanates enhance the expression of GST-P induced by dibutyryl cAMP.

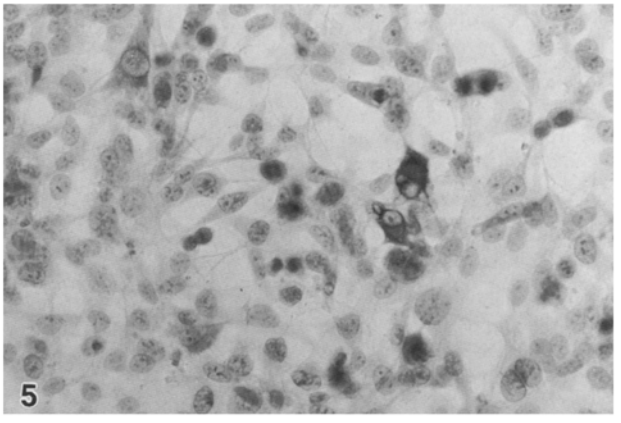
Glioma cells are generally considered to change their morphology to that of normal astrocytes when exposed to dibutyryl cAMP in the medium, such changes being reversible. However, repeated administration of dibutyryl cAMP to the cells is considered to cause irreversible morphological alterations and a decrease in cell proliferation [9, 11, 14, 16, 22]. The effect of dibutyryl cAMP on the expression of S-100 protein associated with the morphological differentiation in glioma cells has been reported [28]. S-100 protein [21] has calcium-binding activity and is regarded as a member of calcium-binding proteins [3, 17, 18], but its physiological activities and functions are unknown. In this study, we demonstrated that T9 glioma cells, originally negative for GST-P, showed expression of GST-P under the present conditions. Almost all T9 cells resembled normal astrocytes in











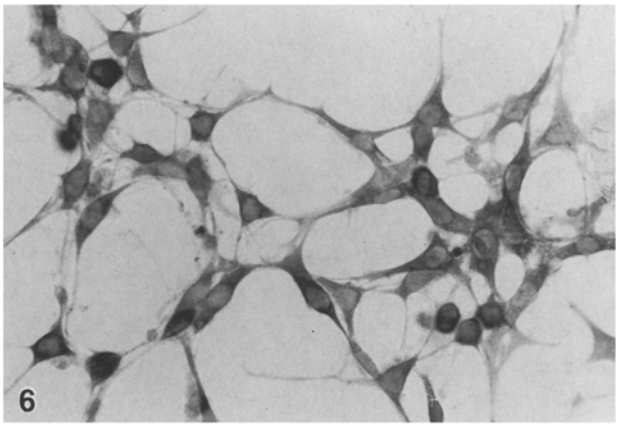


Fig. 1. Control T9 glioma cells did not show any positive reaction for the placental form of glutathione *S*-transferase (GST-P) and morphological changes; anti-GST-P and counterstaining with Mayer's hematoxylin, ×150

Fig. 2. T9 glioma cells treated with 1 mM dibutyryl cyclic adenosine mono-phosphate (cAMP) showed weak to strong reactivity of GST-P and the appearance of the cells resembled normal astrocytes; anti-GST-P and counterstaining with Mayer's hematoxylin, ×150

Fig. 3. T9 glioma cells cultured with allyl isothiocyanate (AITC) alone did not change their morphology. However, about 5% of them showed positive reaction for GST-P; anti-GST-P and counterstaining with Mayer's hematoxylin, ×150

Fig. 4. Most of the cells treated with dibutyryl cAMP and AITC showed strongly positive response for GST-P and the morphology of the cells was similar to that of normal astrocytes; anti-GST-P and counterstaining with Mayer's hematoxylin, ×150

Fig. 5. Cells cultured with benzyl isothiocyanate (BITC) alone showed almost similar result to that with AITC alone; anti-GST-P and counterstaining with Mayer's hematoxylin, ×150

Fig. 6. Treatment with BITC and dibutyryl cAMP resulted in almost the same effect as that seen in Fig. 4; anti-GST-P and counterstaining with Mayer's hematoxylin, ×150

appearance, and about 50% of the cells expressed weak to strong reactivity for GST-P in the presence of dibutyryl cAMP. Furthermore, the expression of GST-P with cAMP was strongly enhanced by the addition of AITC or BITC. These findings are of interest because when either AITC or BITC was added alone, only about 5% of the cells were weakly positive for GST-P without morphological changes. GST catalyze the conjugation of a wide variety of hydrophobic electrophilic substances with glutathione. AITC and BITC are hydrophobic substances. They may act as substrates of GST-P and are considered to induce the enzyme. It is considered that morphologically differentiated T9 cells on treatment with dibutyryl cAMP were also differentiated functionally, and that the enzyme expression was further increased by the stimulation of AITC or BITC. AITC is reported to have a carcinogenic activity in the urinary bladders of rats [7, 8, 12]. On the other hand, BITC is described as a compound capable of elevating GST activities in many organs [2, 26, 30]. Moreover, this chemical has an inhibitory effect on carcinogen-induced tumorigenesis [30, 31]. These two compounds which possess different physiological activities have a toxic effect on T9 cells above a certain concentration. The concentrations of AITC or BITC used in the present study were one tenth of the toxic concentrations, revealed by our preliminary study, and these concentration of AITC or BITC did not influence the cell proliferation.

In rat liver, it is considered that the actual role and function of the expression of GST-P in the preneoplastic lesion involve resistant mechanisms to cytotoxic agents including hepatocarcinogens [23, 24]. However, in normal rat brain tissue, GST-P is a major form of the representative isozymes [29] and GST in rat brain was demonstrated to be localized in the astroglial cells of white and gray matters, but not in neurons [25]. It may be considered that GST-P plays a part in the blood-brain barrier because of its detoxifying function. Therefore, "re-expression" of GST-P in glioma cells exposed to dibutyryl cAMP, as shown here, is considered to be a functional differentiation. Moreover, certain substances such as AITC or BITC (which may be substrates of GST-P) stimulate the expression of GST-P in differentiated T9 cells treated with dibutyryl cAMP.

Further studies are warranted to elucidate the mechanism of "re-expression" of GST-P in glioma cells induced by dibutyryl cAMP and the modifying effect of these naturally occurring isothiocyanates.

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