Differentiative and Proliferative Effects of (But)₂cAMP, *n*-Butyric Acid and Prednisolone on the Malignant Melanoma Cell Line (TM-1) in Vitro and in Vivo

MICHIO KIMURA, TETSURO NISHIHIRA, MORIO KASAI and
HARUO SATO*

*The Second Department of Surgery, Tohoku University
School of Medicine, and *Department of Oncology, the Research
Institute for Tuberculosis and Cancer, Tohoku University,
Sendai 980*

KIMURA, M., NISHIHIRA, T., KASAI, M. and SATO, H. *Differentiative and Proliferative Effects of (But)₂cAMP, n-Butyric Acid and Prednisolone on the Malignant Melanoma Cell Line (TM-1) in Vitro and in Vivo.* Tohoku J. exp. Med., 1980, 131 (1), 29-35 — Both (But)₂cAMP and *n*-butyric acid suppressed the proliferation of TM-1 cells and induced the formation and elongation of the cellular processes in vitro. Prednisolone stimulated the proliferation of TM-1 cells and increased the number of polygonal cells in vitro. These results indicated that (But)₂cAMP and *n*-butyric acid induce the differentiation of TM-1 cells, whereas prednisolone brings the cells in an undifferentiated state. The growth of TM-1 cell tumor transplanted into nude mice was suppressed by 5 mg of (But)₂cAMP and 1 mg of theophylline. Remarkable histological changes were seen in the tumors treated with these agents as compared with untreated tumors. This suggests that (But)₂cAMP suppresses the proliferation of TM-1 cells in vivo. — malignant melanoma cell line; differentiation; (But)₂cAMP; *n*-butyric acid, prednisolone

In recent years, cyclic AMP has been verified to participate in cell differentiation and has been widely used in the study on the differentiation of normal cells. On the human malignant neoplastic cells, it has been reported that (But)₂cAMP induces characteristic changes in both morphology and enzyme activity (Hsie and Puck 1971). Furthermore, it is known that *n*-butyric acid, 4-carbon fatty acid, has an effect similar to that of (But)₂cAMP upon neuroblastoma cells (Waymire et al. 1972; Schneider 1976). But there is also a report that *n*-butyric acid does not cause any morphological changes in malignant melanoma cells (Helson et al. 1974).

Recently, we have established a human malignant melanoma cell line (TM-1) (Nishihira et al. 1977), which made various experiments with it possible. This time we studied the effects of the above-mentioned agents on the morphology of TM-1 cells in vitro. In addition, we performed in vivo experiments using nude mice and obtained some interesting results.

A great deal of research on glucocorticoid receptors in the cytosol has been done recently, and it has been found that such corticoids are necessary for the growth of certain malignant tumors with these receptors (Shyamala 1974). We also investigated the effect of glucocorticoids on the TM-1 cells in vitro.

MATERIALS AND METHODS

Tumor cells. The human malignant melanoma cell line (TM-1) utilized in this study was established from a metastatic axillary lymphnode of a melanoma originated on the abdominal skin of a 67-year-old woman. The cells used in these experiments had been cultivated for about 1.5 years, being roughly in their 30th generation, and having a doubling time of 72 hr. Their dopa reaction was strongly positive at the beginning of the cell culture, but gradually weakened and the reaction is almost negative at present.

In vitro experiments. TM-1 cells were prepared to the concentration of 1×10^{-5} cells/ml in RPMI 1640 with 20% fetal calf serum, and then they were dispersed into Falcon 3002 plastic dishes (a diameter of dishes is 55 mm). Various agents were added to these dishes and they were then cultured at 37°C in an atmosphere of 5% CO₂ and 95% air. The morphological changes were periodically observed. Final concentrations of the agents were as follows: (a) adrenocorticotropin (Acther-Gel, Tokyo Tanabe Pharmaceuticals, Tokyo), 5×10^{-5} to 5×10^{-2} U/ml, (b) dibutyladenosine 3':5'-cyclic monophosphate ((But)₂cAMP) (Boehringer-Manheim, Germany), 1 mM, (c) *n*-butyric acid (Wako Chemicals, Tokyo), 0.5 mM, and (d) prednisolone (Predonine, Shionogi Pharmaceuticals, Tokyo), 0.5 mg/ml. As a control, a saline was used.

In vivo experiments. 1×10^7 TM-1 cells were injected subcutaneously in nude mice and tumors of about 1 cm in size were obtained after 2 months. Then, 5 mg of (But)₂cAMP and 1 mg of theophylline were injected into the peritoneal cavity of the tumor-bearing mice every 3 days up to a total of 10 times. At the end of this treatment the tumor was excised, fixed in 10% formalin for hematoxylin-eosin staining. The histological changes were studied in comparison with control tissues.

RESULTS

In vitro experiments

The control culture into which a saline solution had been added showed a cobblestone-like arrangement of cells at 48 hr. The great majority of cells were multipolar with 2 to 4 poles, but the process projecting from each pole was rather short (Fig. 1a).

At the concentrations used in our experiments (5×10^{-5} – 5×10^{-2} U/ml), ACTH caused no morphological changes in the TM-1 cells in comparison with the control culture.

When (But)₂cAMP was added to the culture at a final concentration of 1 mM, distinct morphological differences from the control were found. Namely, after 48 hr, the cell size had decreased slightly and the cellular processes had greatly elongated. Finally, the tips of some processes became divided to make a net-like pattern as a whole (Fig. 1b). At this time, almost no cell proliferation was seen and dopa reaction was slightly stronger than in the control.

No obvious morphological changes could be seen at 48 hr in TM-1 cells cultivated with 0.5 mM of *n*-butyric acid, though the proliferation was suppressed slightly. However, at 96 hr each cellular process was elongated and net-like

Fig. 1. Effects of various agents on TM-1 cells in culture.

a) Control culture for 47 hr, adding saline solution, $\times 32$. b) Treated with $(\text{But})_2\text{cAMP}$, 1 mM, for 48 hr, $\times 80$. c) Treated with *n*-butyric acid, 0.5 mM, for 96 hr, $\times 80$. d) Treated with prednisolone, 0.5 mg/ml, for 96 hr, $\times 80$.

pattern similar to that seen with $(\text{But})_2\text{cAMP}$ was found (Fig. 1c). At the same time dopa reaction of the cells became slightly stronger than in the control. These results indicate that *n*-butyric acid has an effect similar to that of $(\text{But})_2\text{cAMP}$ on the differentiation of TM-1 cells in vitro.

In the TM-1 cells in the medium with prednisolone at a concentration of 0.5 mg/ml, obvious morphological changes were not seen at 48 hr, though they showed a slight tendency to proliferate. At 96 hr, the tendency to proliferate became more obvious with the cell count reaching 1.3 times that of the control (Fig. 2). A lack of uniformity in cell size and an increase in polygonal cells were also seen (Fig. 1d). From these results, it was suggested that prednisolone stimulates the growth of TM-1 cells *in vitro*.

Fig. 2. Growth curve of TM-1 cells treated with prednisolone.
○—○, control; ●—●, prednisolone, 0.5 mg/ml.

As the morphological changes described above were diminished after the culture medium was replaced by that without these agents, we considered that the changes caused by (But)₂cAMP, *n*-butyric acid and prednisolone were reversible.

In vivo experiments

As a control, transplanted TM-1 tumors into nude mice were used without any treatment. The tumors were composed of spindle-shaped cells and polygonal cells, with occasional giant cells. Degeneration or necrosis was not evident in them. The tumor cells proliferated as a mass with almost no supporting tissue, resembling amelanotic melanoma (Fig. 3a).

By administration of (But)₂cAMP and theophylline, a cyclic AMP phosphodiesterase inhibitor, intraperitoneally, the tumor growth was suppressed and blackened regions of the tumors were seen macroscopically. Microscopically, as compared with the control, the number of spindle-shaped cells had increased and there was a tendency to align themselves in one direction. Cells in vacuolar degeneration (Fig. 3b) and necrotic tissues with an infiltration of macrophages (Fig. 3c) were often seen around the spindle-shaped cell groups. The microscopically blackened regions of the tumors were composed of necrotic TM-1 cells among which macrophages were dominant.

Since the level of cyclic AMP in the tissue was not measured, it was not concluded that these pathological changes were due to cell differentiation. But it was suggested that such cell destruction was caused by the increase of intracellular concentration of cyclic AMP resulting from the administration of (But)₂cAMP and theophylline.

Fig. 3. Histopathological findings of transplanted TM-1 tumors into nude mice. a) Control tumor without any treatment, $\times 80$. b) Tumor of a mouse given a total of 10 injections of 5 mg of $(\text{But})_2\text{cAMP}$ and 1 mg of theophylline, $\times 32$. c) A high power view of the tumor similar to b), $\times 80$.

DISCUSSION

The action of melanocyte-stimulating hormone (MSH) on malignant melanoma cells was investigated in vitro with Cloudman S 91 melanoma cells (Wong et al.

1974; Wong and Pawelek 1975) and in vivo with B-16 melanoma cells (Lee et al. 1972). In either type of tissue, MSH administration caused a marked increase in tyrosinase activity and melanogenesis. Most of amino acid sequences of ACTH are known to be identical with those of MSH, and ACTH is also known to have weak MSH activity. In our experiments, ACTH was applied in vitro to TM-1 cells, expecting some MSH activity of ACTH, but no changes in the cells were seen. There is a report that MSH at a low concentration ($40\text{ }\mu\text{g/ml}$) with $2\times 10^{-3}\text{ M}$ theophylline caused morphological changes in human melanocytes in vitro, while MSH without theophylline, caused no such changes (Kitano 1976). We also examined the effect of ACTH with $2\times 10^{-3}\text{ M}$ theophylline on the TM-1 cell line, but no changes were seen. This result cannot be clearly explained at present; either the TM-1 cells do not have MSH receptors, or ACTH used in this experiment was unsuitable as an MSH analog.

(But)₂cAMP is known to accelerate the development of long narrow processes of neurons in the rat brain (Shapiro 1973) and mouse neuroblastoma cells (Prasad and Hsie 1971). Similar effect of (But)₂cAMP was noted on cultivated Greene melanoma cells (DiPasquale et al. 1976). Even in the human malignant cells (TM-1) used here, a concentration of 0.1–1 mM of (But)₂cAMP caused marked morphological changes in vitro.

It is said that *n*-butyric acid increases adenylyl cyclase activity in the cell membrane, which in turn causes an increase in the intracellular cyclic AMP concentration. Prasad and Hsie (1971) and Helson et al. (1974) have reported that at a concentration of 0.5 mM of *n*-butyric acid, the proliferation of mouse neuroblastoma cells and human melanoma cells is suppressed, but that their morphological differentiation is not induced. On the other hand, Schneider (1976) has found that *n*-butyric acid at the same concentration results in morphological changes of mouse C-1300 neuroblastoma cells. We also found morphological changes at a concentration of 0.5 mM of *n*-butyric acid in TM-1 cells. The changes caused by (But)₂cAMP and *n*-butyric acid are thought to be due to increased intracellular concentrations of cyclic AMP following the addition of these agents. Hereafter, it is necessary to measure intracellular cyclic AMP directly.

It is widely known that glucocorticoids play an important role in cell differentiation. But, in certain types of mouse mammary tumors, glucocorticoids are also needed for tumor proliferation, and specific cytoplasmic glucocorticoid receptors were demonstrated (Shyamala 1974). In TM-1 cells, as well, prednisolone clearly accelerates cell proliferation in vitro, which suggests that TM-1 cells possess specific glucocorticoid receptors. The relationship between TM-1 cells and prednisolone may be useful as a model for the study of cell differentiation.

On the other hand, there is a report of Chandra et al. (1972) concerning in vivo effects of (But)₂cAMP. They believed that there are two factors involved in the tumor-suppressive effect of cyclic AMP with regard to transplanted NKL-lymphosarcomas: 1) the route of (But)₂cAMP administration, and 2) the period of treatment. Only by means of an intratumoral injection (But)₂cAMP exerted the

tumor-suppressive effect and the effect was quickly lost when injections were halted. This is perhaps due to the rapid decomposition of cyclic AMP. In our experiments, intraperitoneal injections of (But)₂cAMP with theophylline suppressed the proliferation of TM-1 cells transplanted to nude mice.

So far, we are going to study tumor suppression caused by combination of cyclic AMP phosphodiesterase inhibitor and various other agents which directly increase intracellular production of cyclic AMP. And we are also planning to test their clinical application.

References

- 1) Chandra, P., Gericke, D. & Becker, B. (1972) Effects of some adenosine 3':5'-monophosphate analogues on cell proliferation, tumor growth and macromolecular synthesis in various organs of the mouse. *Hoppe-Seyler's Z. physiol. Chem.*, **353-2**, 1506-1507.
- 2) DiPasquale, A.M., McGuire, J., Moellmann, G. & Wassermann, S.J. (1976) Microtubule assembly in cultivated Greene melanoma cells is stimulated by dibutyl adenosine 3':5'-cyclic monophosphate or Cholera toxin. *J. Cell Biol.*, **71**, 735-748.
- 3) Helson, L., Lai, K. & Young, C.W. (1974) Papaverine-induced changes in cultured human melanoma cells. *Biochem. Pharmacol.*, **23**, 2917-2920.
- 4) Hsie, A.W. & Puck, T.T. (1971) Morphological transformation of Chinese hamster cells by dibutyl adenosine cyclic 3':5'-monophosphate and testosterone. *Proc. nat. Acad. Sci. USA*, **68**, 358-361.
- 5) Kitano, Y. (1976) Effects of melanocyte stimulating hormone and theophylline on human melanocytes in vitro. *Arch. Derm. Res.*, **255**, 163-168.
- 6) Lee, T.H., Lee, M.S. & Lu, M. (1972) Effects of α -MSH on melanogenesis and tyrosinase of B-16 melanoma. *Endocrinology*, **91**, 1180-1188.
- 7) Nishihira, T., Kuriya, Y., Hayashi, Y., Ishii, H., Kimura, M., Tan, M., Sasaki, T., Kasai, M., Kato, T., Matsutani, Y. & Yamane, I. (1977) Some properties of new continuous cell lines (TM-1) derived from a patient of malignant melanoma. *Tissue Culture*, **3**, 73-81. (Japanese)
- 8) Prasad, K.N. & Hsie, A. (1971) Morphologic differentiation of mouse neuroblastoma cells induced in vitro by dibutyl adenosine 3':5'-monophosphate. *Nature New Biol.*, **233**, 141-142.
- 9) Schneider, F.H. (1976) Effects of sodium butyrate on mouse neuroblastoma cells in culture. *Biochem. Pharmacol.*, **25**, 2309-2317.
- 10) Shapiro, D. (1973) Morphological and biological alterations in fetal rat brain cells cultured in presence of mono-butyl cyclic AMP. *Nature (Lond.)*, **241**, 203-204.
- 11) Shyamala, G. (1974) Glucocorticoid receptors in mouse mammary tumors. *J. biol. Chem.*, **249**, 2160-2163.
- 12) Waymire, J.C., Weiner, N. & Prasad, K.N. (1972) Regulation of tyrosine hydroxylase activity in cultured mouse neuroblastoma cells: Elevation induced by analogs of adenosine 3':5'-cyclic monophosphate. *Proc. nat. Acad. Sci. USA*, **69**, 2241-2245.
- 13) Wong, G. & Pawelek, J. (1975) Melanocyte-stimulating hormone promotes activation of pre-existing tyrosinase molecules in Cloudman S-91 melanoma cells. *Nature (Lond.)*, **255**, 644-646.
- 14) Wong, G., Pawelek, J., Sansone, M. & Morowitz, J. (1974) Response of mouse melanoma cells to melanocyte stimulating hormone. *Nature (Lond.)*, **248**, 351-354.