Jpn. J. Cancer Res. 83, 851–858, August 1992

Non-transformed, but not *ras/myc*-transformed, Serum-free Mouse Embryo Cells Recover from Growth Suppression by Azatyrosine

Takahiro Nomura,[1,4] Kazuo Ryoyama,[2] Gensaku Okada,[1] Sadaya Matano,[3] Shinobu Nakamura[3] and Tadanori Kameyama[1]

[1]*Department of Molecular Biology and* [2]*Experimental Therapeutics, Cancer Research Institute,* [3]*Third Internal Medicine, School of Medicine, Kanazawa University, 13-1, Takaramachi, Kanazawa 920*

The anti-proliferative effect of azatyrosine, a newly discovered antibiotic from *Streptomyces*, was examined in Balb/c-originated serum-free mouse embryo (SFME) cells and transformed *ras/myc* SFME cells which have activated human c-Ha-*ras* genes. Azatyrosine suppressed their growth in a concentration-dependent manner. Growth suppression in both cells was detectable within 2 days after culture with 250 μg/ml azatyrosine. Non-transformed SFME cells, however, regained rapid growth after 6 days even in the presence of azatyrosine, whereas *ras/myc* SFME cells did not recover from the suppression. Despite the growth inhibition of *ras/myc* SFME cells, expression of human *ras* in the cells was not inhibited by azatyrosine. Meanwhile, SFME cells have the ability to express glial fibrillary acidic protein (GFAP). This expression is induced by serum-supplemented medium, though the serum inhibits the growth of SFME cells. Azatyrosine did not induce GFAP in *ras/myc* SFME cells, but inhibited growth. Furthermore, azatyrosine did not induce GFAP in SFME cells, and had no effect upon the expression of GFAP induced by serum in these cells. These results suggest that azatyrosine inhibited the growth of *ras/myc* SFME cells through a mechanism independent of those involved in growth inhibition and induction of GFAP expression by serum in SFME cells.

Key words: Azatyrosine — SFME cell — *ras/myc* SFME cell

Serum-free mouse embryo (SFME)[5] cells were established by Loo *et al.*[1] under serum-free culture conditions. The growth of SFME cells depends on an exogenous supply of epidermal growth factor (EGF)[2] and they are relatively stable. That is, they do not show any sign of crisis, they do not acquire tumorigenicity, and their karyotype remains diploid after more than 100 passages. The *ras/myc* SFME cell line, which has been co-transfected with human c-Ha-*ras* and mouse c-*myc* genes, is independent of exogenous EGF and is tumorigenic in syngeneic hosts, Balb/c mice.[3] The introduction of cloned mouse c-*myc* into the SFME cells immortalizes them and effectively increases their transformation by human c-Ha-*ras*, though the c-*myc* gene does not transform the SFME cells.[3,4] Parental SFME cells tend to cease growth and express glial fibrillary acidic protein (GFAP), an intermediate filament protein characteristic of astrocytes, after supplementation with fetal calf serum (FCS).[5] The effect of FCS upon SFME cells is reversible, so that the treated cells can grow again if transferred to a serum-free culture condition.[6]

Azatyrosine [L-beta-(5-hydroxy-2-pyridyl)alanine] is a newly discovered antibiotic purified from *Streptomyces*. This product inhibits growth of NIH 3T3 cells transformed with cloned human oncogenes such as *ras*, *raf* and *neu*, and of various tumor cells bearing activated *ras* genes.[7] Although the exact mechanism is still unclear, the inhibitory effect on *ras*-transformed NIH 3T3 cells is irreversible, and the resultant cells exhibit a flat revertant morphology and reduced tumorigenic activity.

We have examined the effects of azatyrosine on SFME and *ras/myc* SFME cells from two perspectives, namely whether azatyrosine selectively inhibits the growth of *ras/myc* SFME but not SFME cells, and if so, whether it induces the expression of GFAP in the cytoplasm of treated *ras/myc* SFME cells.

MATERIALS AND METHODS

Cells Balb/c mouse embryo-derived SFME and *ras/myc* SFME cells were provided by S. Shirahata of Kyushyu University courtesy of D. Barnes, Oregon State University. Both cell lines were plated in plastic culture ware pre-coated with either fibronectin or poly-D-lysine and fibronectin, cultured at 37°C in a humidified CO_2 (5%) atmosphere and passaged for 6 days. The culture medium mainly used in the present study was F/D medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12) supplemented with 10 n*M* sodium selenite, 10 μg/ml insulin (bovine), 10 μg/ml transferrin (bovine), 10 μg/ml high-density lipoprotein (human) and 50 ng/ml EGF (mouse submaxillary) [serum-free culture medium].[1,4] The culture medium

[4] To whom correspondence should be sent.

[5] Abbreviations: SFME, serum-free mouse embryo; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; FCS, fetal calf serum.

supplemented with FCS instead of growth factors was F/D with sodium selenite (10 n*M*). Media and supplements were prepared in pyrogen-free ultra-pure water (NANO pure, Barnstead, IA).

Chemicals and reagents Azatyrosine was kindly provided by O. Makabe (Meiji Seika Co. Ltd., Tokyo) and S. Nishimura (National Cancer Center, Tokyo). Medium and growth factors were purchased from Sigma (St. Louis, MO), FCS from Flow Laboratories (Irvine, KS), rabbit anti-cow glial fibrillary acidic protein (Lot No. 119) from Dako (Denmark), peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Lot No. 33235) from Cappel (West Chester, PA), and normal rabbit serum from Wako Pure Chemicals Co. (Osaka). The human C-Ha-*ras* plasmid pUCEJ6.6 (from EJ bladder carcinoma) was obtained from ATCC (American Type Culture Collection); restriction enzymes and the random-labeling kit reagents were from Takara (Tokyo), and other chemicals and reagents were from Wako Pure Chemicals Co.

Estimation of cell numbers Cells were cultured in the absence or presence of azatyrosine at 37°C in a humidified CO_2 (5%) atmosphere at appropriate intervals. The numbers of cells detached with trypsin were counted with a particle counter (model ZB1, Coulter Electronic, FL). Instrument settings were as follows: amplification, 1/2; aperture current, 1/2; lower threshold, 15; upper threshold, 100; gain trim, 5; and aperture tube 100 μm in diameter. When cells were cultured in 96-well microplates (Falcon 3072), the cell numbers were estimated in terms of reduction of 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).[8] Briefly, after addition of MTT to each well at the end of the culture period, plates were further incubated for 4 h at 37°C. The amount of resulting blue formazan, after solubilization with dimethyl sulfoxide, was measured at 540 nm with a microplate reader (Immuno reader NJ-200, Inter Med.).

RNA isolation and Northern blotting Total cellular RNA from cells cultured with or without azatyrosine was extracted using quanidium thiocyanate-phenol-chloroform.[9] To prepare the poly(A)$^+$ RNA fraction, 100 μg of cellular RNA was applied to an oligo(dT)-cellulose column.[10] Cellular RNA (15 μg) or poly(A)$^+$ RNA (5 μg) was heat-denatured at 65°C for 7 min in a sample buffer solution (6% formaldehyde, 50% formamide, 20 m*M* 3-(N-morpholino)propanesulfonic acid pH 7, 5 m*M* sodium acetate, and 1 m*M* EDTA), then fractionated by electrophoresis with ethidium bromide in 1.5% agarose containing 6% formaldehyde. RNA was then transferred to a Hybond N membrane (Amersham) by means of the capillary method.[10] The membrane was dried, UV-irradiated and pre-hybridized for 4 h at 42°C in 10 ml of 25 m*M* sodium phosphate, pH 6.5, containing 0.1% SDS, 5×SSC, 1×Denhart's solution and 200 μg/ml salmon sperm DNA. The membrane was then hybridized for 12 h at 42°C, in 5 ml of the hybridization solution, containing a randomly labeled (^{32}P) 1304 bp exon 4 fragment with the restriction sites *Bgl* I and *Sac* I of pUCEJ6.6, as the probe.[11, 12] Blotted membranes were washed then exposed to X-ray film using intensifying screens and autoradiographed at −70°C for 24 h.[10]

Expression of GFAP Cells cultured with or without FCS and/or azatyrosine in 96-well microplates were fixed with 75% ethanol and treated with Triton X-100. GFAP levels were measured using an enzyme-linked immunosorbent assay.[5, 13] Rabbit anti-cow GFAP was used as the first antibody, horse-radish peroxidase-conjugated goat anti-rabbit IgG as the second, and orthophenylenediamine as the colorimetric reagent. In the control, properly diluted normal rabbit serum was used as the first reagent. Oxidized phenylenediamine, which indicates the amount of GFAP, was determined by measuring absorption at 490 nm. Net GFAP absorption values were obtained after correction for the controls. To avoid non-specific absorption by the cells, we estimated the levels of expression in the cells using the GFAP index, A_{490}/A_{540} where A_{490} is the net absorption value at 490 nm and A_{540}

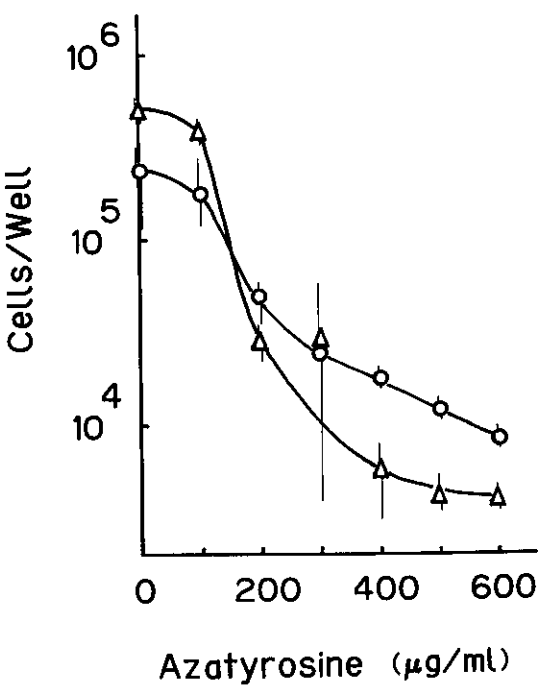

Fig. 1. Effect of azatyrosine on the growth of non-transformed and *ras/myc*-transformed SFME cells. Both cell lines (1×10^4/2 ml/well) were cultured in the serum-free culture medium with or without azatyrosine. Cell numbers were determined 6 days after culture was initiated. ○, *ras/myc* SFME cells; △, SFME cells.

is the absorption value at 540 nm of MTT, which represents the cell number. The MTT and the GFAP assays were performed in separate wells. The correlation between cell number per well and A_{540} for a standard was determined independently (not shown).

Statistical analysis Student's t test was used to evaluate the statistical significance of differences between groups.

RESULTS

Effect of azatyrosine on cell growth The anti-proliferative activity of azatyrosine was examined in SFME and *ras/myc* SFME cells. Cells were plated at a density of 1×10^4 cells in a total volume of 2 ml/well of serum-free culture medium in 24-well culture plates one day before azatyrosine was added. Six days later, the cells were dissociated with trypsin and counted. Azatyrosine inhibited the growth of both cell types in a concentration-dependent manner (Fig. 1). Fifty percent growth inhibition of both SFME and *ras/myc* SFME cells was achieved with about 200 μg/ml azatyrosine, and complete inhibition at 500 μg/ml.

Next, the kinetics of growth inhibition of both cell lines in the presence of 250 μg/ml azatyrosine, were investigated under the serum-free culture conditions. Cells were plated at 5×10^3 cells/1 ml/well, and azatyrosine was added on the next day (day 0). The cultures were maintained for 12 days with fresh media changes at days 6 and 9 with or without azatyrosine. The cells were counted every second day. SFME cells without azatyrosine proliferated fairly well up to day 8, then their growth slowed (Fig. 2). Azatyrosine significantly retarded the growth of SFME cells for the first 6 days, but thereafter the cells regained their rapid growth activity. In addition, the degree of the first growth suppression with azatyrosine was variable. Contrary to the results obtained from the SFME cells, *ras/myc* SFME cells did not regain their growth activity in the media with azatyrosine after the first growth reduction (Fig. 3). Similar

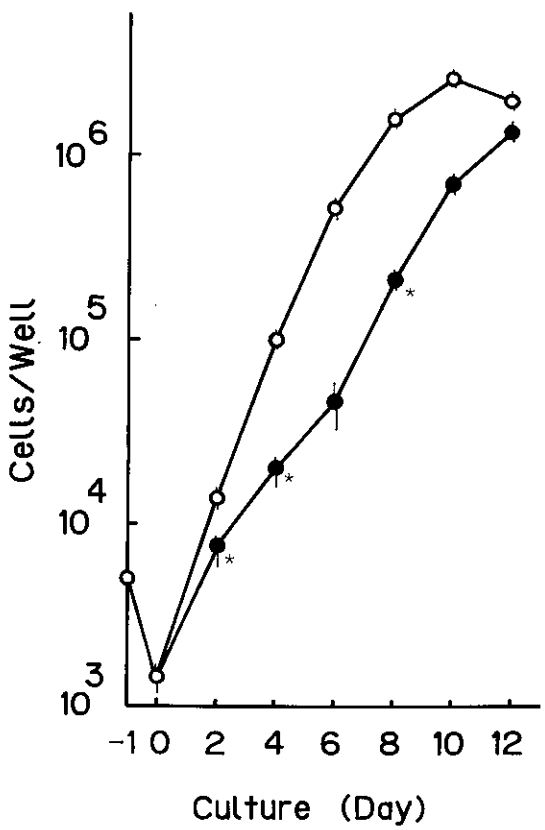

Fig. 2. Growth kinetics of SFME cells with or without azatyrosine. Cells (5×10^3/2 ml/well) were plated at day -1 in the serum-free culture medium. Azatyrosine (250 μg/ml) was added at day 0, and the media were changed at day 6 and 9. ○, without azatyrosine; ●, with azatyrosine. *, $P<0.001$, day 2 vs. day 4; day 4 vs. day 6; and day 6 vs. day 8.

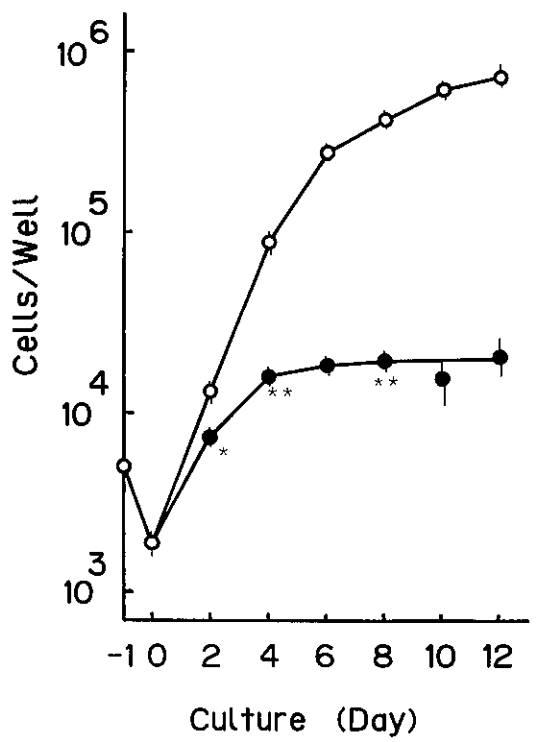

Fig. 3. Growth kinetics of *ras/myc* SFME cells with or without azatyrosine. Details are presented in Fig. 2. ○, without azatyrosine; ●, with azatyrosine. *, $P<0.001$, day 2 vs. day 4; **, not significant, day 4 vs. day 6; and day 6 vs. day 8.

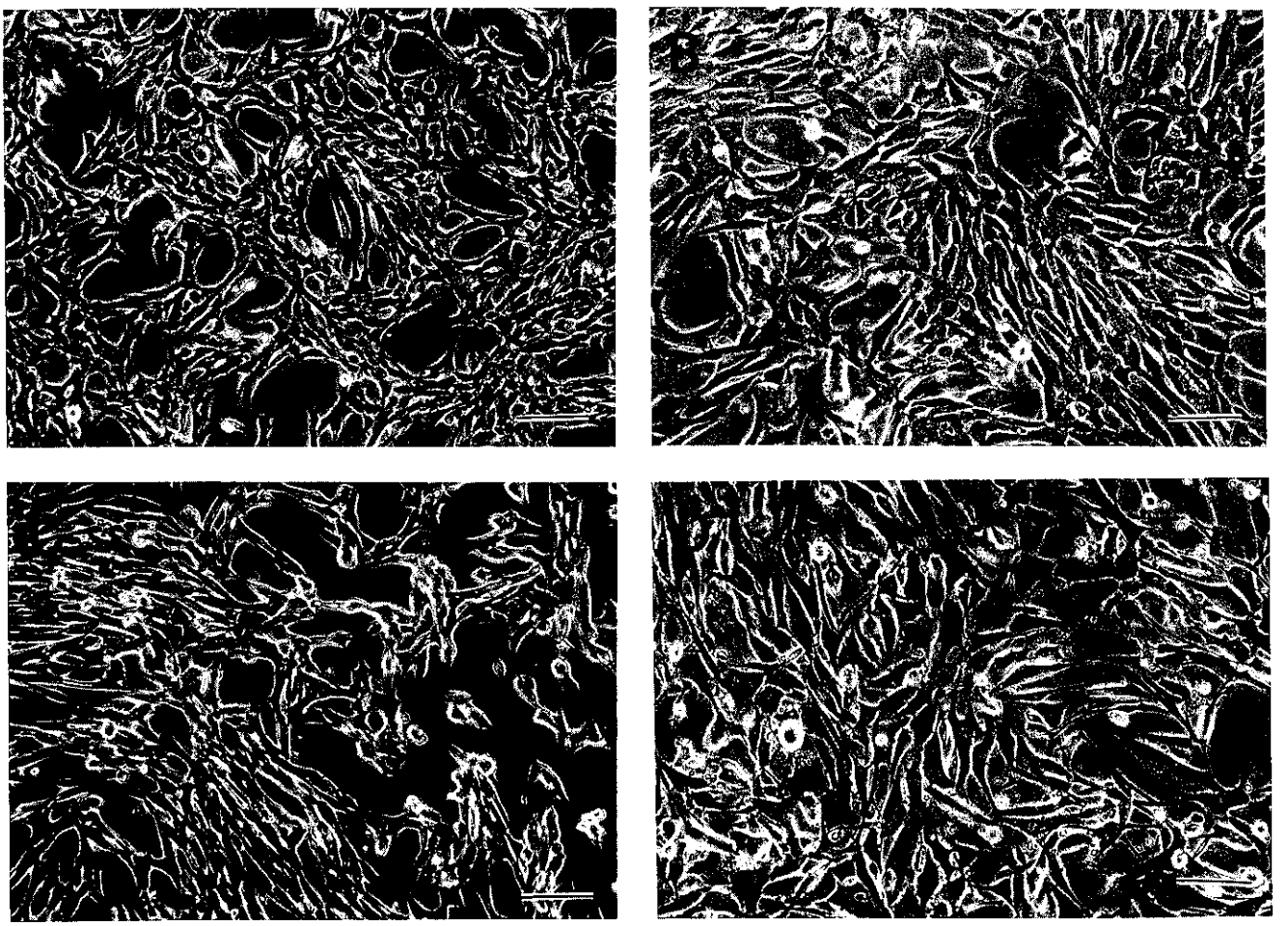

Fig. 4. Effect of azatyrosine on cell morphology. Cells (5×10^4/3 ml/well) were cultured with or without azatyrosine (300 μg/ml) in the serum-free culture medium, and their morphology was assayed 6 days after the start of the culture. A, SFME cells without azatyrosine; B, *ras/myc* SFME cells without azatyrosine; C, SFME cells with azatyrosine; D, *ras/myc* SFME cells with azatyrosine. Bars indicate 100 μm.

results were also obtained in the media supplemented with 10% FCS instead of the serum-free culture medium (data not shown).

Further, we examined whether or not azatyrosine causes morphological alterations in these cell lines. When *ras/myc* SFME cells were plated at 5×10^4 cells/3 ml/well in 6-well plates (Costar) and cultured with azatyrosine (300 μg/ml) under the serum-free conditions for 6 days, morphologically atypical cells appeared (Fig. 4). The *ras/myc* SFME cells, but not the parental non-transformed SFME cells, cultured with more than 200 μg/ml of azatyrosine exhibited more or less atypical morphological alteration. The proportion of these atypical cells in *ras/myc* SFME cell culture increased with the period of azatyrosine treatment. However, visibly damaged cells appeared in culture with azatyrosine maintained with medium changes at 7-day intervals (Fig. 5), and finally all of the viable cells disappeared by one month.

Effect of azatyrosine on *ras* expression The steady-state level of *ras* expression by *ras/myc* SFME cells was examined using the exon 4 fragment of human c-Ha-*ras* DNA from pUCEJ6.6 (human EJ bladder carcinoma) as the probe (Fig. 6). *Ras/myc* SFME cells at the logarithmic growth stage expressed moderate amounts of human c-Ha-*ras* RNA, whereas the endogenous mouse *ras* RNA level in SFME cells was so low as to be detectable only in the poly(A)$^+$ RNA fraction. Twenty-four hours after plating of *ras/myc* SFME cells, 250 μg/ml azatyrosine was added to the medium and the cells were cultured for a further 3 or 6 days under the serum-free conditions.

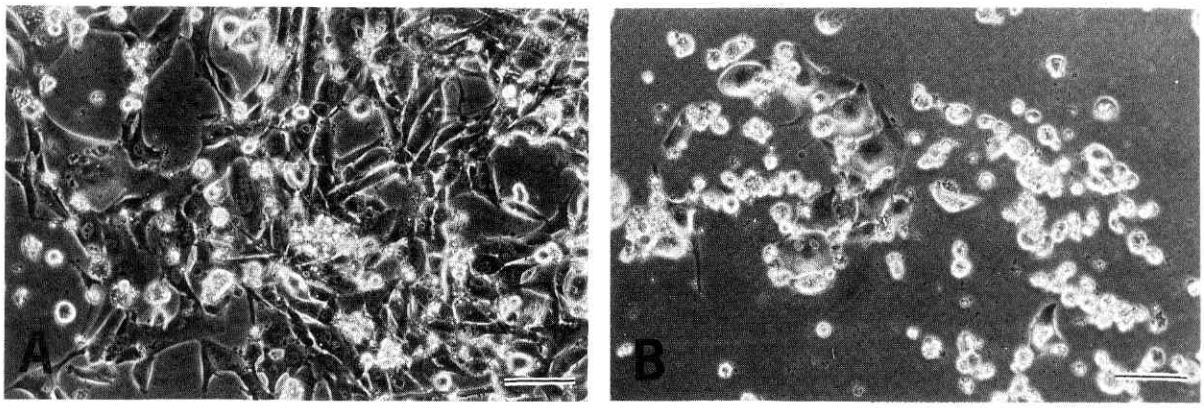

Fig. 5. Cell morphology after long-term treatment with azatyrosine. *Ras/myc* SFME cells (5×10^3/1 ml/well with 24 well plate) were cultured in the serum-free medium with azatyrosine (300 μg/ml) for 12 days (A) or 22 days (B). Bars indicate 100 μm.

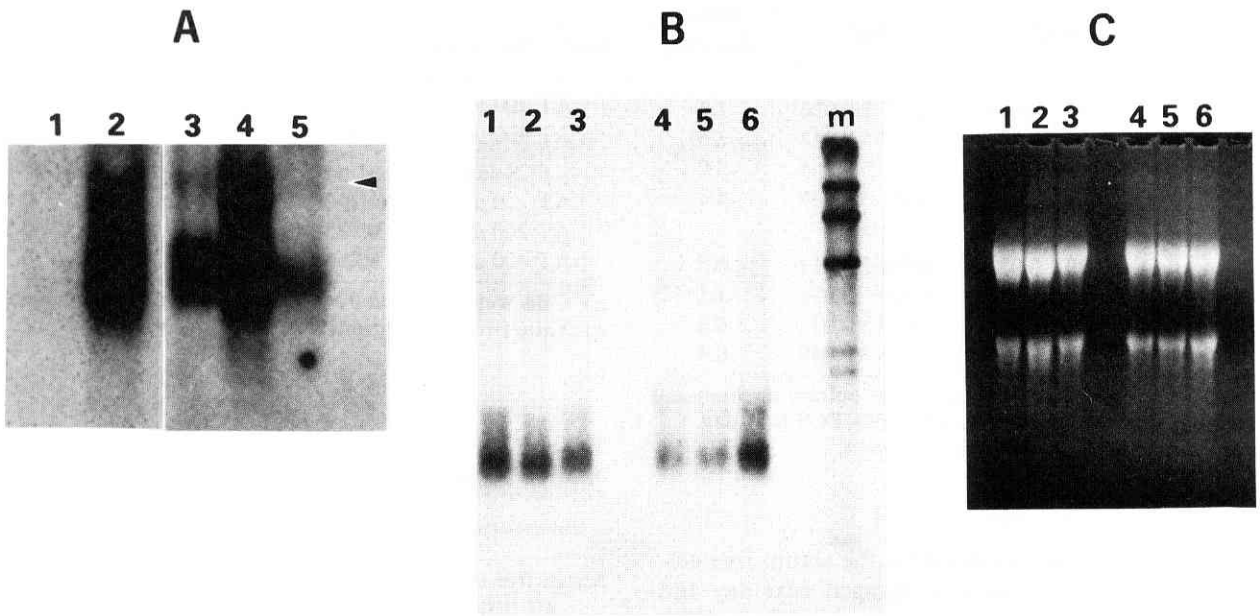

Fig. 6. Northern blot analysis of *ras* expression in cells cultured in the absence (A) or the presence (B, C) of 300 μg/ml azatyrosine. Human c-Ha-*ras* exon 4 fragment was used as the probe. The methodology is described under "Materials and Methods." A: 1, total RNA from SFME cells; 2, total RNA from *ras/myc* SFME cells; 3, poly(A)$^+$ RNA from SFME cells; 4, total RNA from *ras/myc* SFME cells; 5, poly(A)$^+$ RNA from HeLa cells. ◀ indicates 18 S r-RNA position. B: Total cytoplasmic RNA was extracted from *ras/myc* SFME cells grown without (1, 2 and 3) or with (4, 5 and 6) azatyrosine for various periods: 1 and 4, 12 h; 2 and 5, 3 days; 3 and 6, 6 days. m, *Hind* III-digested λ phage DNA, 23.5 kb, 9.8 kb, 6.7 kb, 4.5 kb, 2.4 kb, 2.0 kb, 0.6 kb from the top. C: Ethidium bromide-stained RNA banding in agarose gel electrophoresis of (B).

Figure 6 shows that azatyrosine did not inhibit the *ras* gene expression of *ras/myc* SFME cells even when growth of the cells was completely inhibited at day 6 (see Fig. 3).

Effect of azatyrosine on the expression of GFAP An enzyme-linked immunosorbent assay was used to detect GFAP in both SFME and *ras/myc* SFME cells. First, SFME cells were plated at a density of 5×10^3 cells/0.2

Table I. Appearance of GFAP in SFME Cells after 3 Days in the Presence of FCS[a]

FCS (%)[b]	Cell growth			GFAP		
	A_{540}[c]	P	Cells/well ($\times 10^3$)	A_{490}[c]	P	Index A_{490}/A_{540}
0	0.241±0.045		9.2	0.441±0.058		1.8
1	0.094±0.009	<0.001	1.3	0.748±0.081	<0.001	8.0
5	0.114±0.014	<0.005	1.9	1.804±0.090	<0.001	15.8
10	0.159±0.013	<0.025	3.5	1.859±0.102	<0.001	11.7

a) Cells were plated at 5×10^3/well with the serum-free culture medium and cultured for 3 days after the media were changed on the next day to the experimental one.
b) The medium without FCS has growth factors whereas ones with FCS have no factors.
c) Values represent means±SDs of 4 wells, and *P* values are with respect to 0% FCS.

Table II. Appearance of GFAP in SFME or *ras/myc* SFME Cells Cultured in 10% FCS-supplemented Medium[a]

Seeding cells/well ($\times 10^3$)	Cells after 3 days in culture		
	A_{540}[b]	A_{490}[b]	Index A_{490}/A_{540}
SFME cells			
0.5	0.033±0.006	0.077±0.026	2.3
1.0	0.056±0.006	0.139±0.042	2.5
15.6	0.460±0.089	2.288±0.065	5.0
125.0	0.470±0.014	2.247±0.036	4.8
ras/myc SFME cells			
0.4	0.089±0.027	0.066±0.015	0.7
1.6	0.310±0.023	0.329±0.056	1.1
25.0	0.873±0.125	0.409±0.017	0.5
100.0	1.017±0.134	0.388±0.040	0.4

a) Cells were plated with the serum-free culture medium and the medium was changed to one with 10% FCS next day.
b) Values represent means±SDs of 4 wells.

Table III. Effect of Azatyrosine (AT) on GFAP Expression of *ras/myc* SFME Cells[a]

Culture conditions	A_{540}[b]	A_{490}[b]	Index A_{490}/A_{540}
Serum-free medium			
+AT 0 μg/ml	0.945±0.041	0.172±0.080	0.2
+AT 200 μg/ml	0.742±0.026	0.327±0.072	0.4
+AT 400 μg/ml	0.404±0.029	0.217±0.019	0.5
10% FCS-supplemented medium			
+AT 0 μg/ml	1.192±0.223	0.592±0.041	0.5
+AT 200 μg/ml	0.575±0.101	0.277±0.044	0.5
+AT 400 μg/ml	0.266±0.086	0.173±0.020	0.7

a) Cells were plated at 1×10^4/well and cultured for 6 days.
b) Values represent means±SDs of 4 wells.

Table IV. Effect of Azatyrosine (AT) on GFAP Expression of SFME Cells[a]

Culture conditions	A_{540}[b]	A_{490}[b]	Index A_{490}/A_{540}
Serum-free medium			
+AT 0 μg/ml	0.380±0.018	0.709±0.140	1.9
+AT 250 μg/ml	0.283±0.007	0.531±0.065	1.9
10% FCS-supplemented medium			
+AT 0 μg/ml	0.354±0.007	2.042±0.042	5.8
+AT 250 μg/ml	0.378±0.019	1.948±0.016	5.5

a) Cells were plated at 1.6×10^4/well and cultured for 4 days.
b) Values represent means±SDs of 4 wells.

ml/well in 96-well microplates with the serum-free culture medium. The medium was changed next day and cells were cultured for 3 days with or without FCS, where growth factors were added to the medium without FCS (Table I). Since SFME cells are very sensitive to growth factor depletion, more than half of the cells was lost in the culture with 1% FCS. The GFAP index reached the maximum at 5% FCS, and the index was always lower than 2.0 when cells were cultured in the serum-free culture medium. Therefore, we adopted a GFAP index of over 2.0 as the criterion of positive GFAP expression. Depending on FCS concentration, the growth of SFME cells was inhibited, but the GFAP levels in the cells increased. On the contrary, FCS (10%) neither inhibited the growth of *ras/myc* SFME cells nor increased the GFAP indices above 2.0, irrespective of the cell density (Table II). We then examined the effect of azatyrosine on GFAP expression in the *ras/myc* SFME cell line. The cells were incubated with various concentrations of azatyrosine for 6 days in the serum-free culture medium or that containing 10% FCS (Table III). Under the present culture conditions, azatyrosine did not

increase the GFAP index of *ras/myc* SFME cells above 2.0, despite its anti-proliferative activity. In addition, azatyrosine neither inhibited GFAP induction by FCS nor induced GFAP in SFME cells (Table IV).

DISCUSSION

Shindo-Okada *et al.* reported that azatyrosine significantly inhibits growth of *ras*-transformed but not the parental non-transformed NIH 3T3 cells.[7] This effect also occurs in other cells transformed with cloned-human oncogenes such as *ras, raf* and *neu,* and in various tumor cells harboring activated *ras* genes.[7] They also reported that another feature of the azatyrosine effect on *ras*-transformed NIH 3T3 cells is the induction of so-called revertant cells. These revertants appear after 6 days in culture with azatyrosine and are morphologically different from both the transformed NIH 3T3 cells and the parent cells. They are flat, giant and have clear nuclei.

The present study showed a slight but significantly different effect of azatyrosine on the growth of our transformed and non-transformed SFME cells. The growth of both kinds of SFME cells was significantly inhibited by azatyrosine for the first few days. Parental non-transformed SFME cells, however, regained their rapid growth activity thereafter, whereas *ras/myc*-transformed SFME cells did not. Furthermore, no apparent revertant cells were detectable in *ras/myc* SFME cells cultured in the presence of azatyrosine, since their growth recovered after the medium changed to 10% FCS-supplemented medium without azatyrosine, in which non-transformed SFME cells cannot grow (data not shown). Morphologically atypical cells were observable in *ras/myc* SFME cell culture after treatment with azatyrosine. However, these cells failed to grow in the presence of azatyrosine. It is noteworthy that all of the cells were irreversibly damaged and no cells recovered after 1 month in culture with medium changes at 7-day intervals. The different responses to azatyrosine of the *ras/myc* SFME and *ras*-transformed NIH 3T3 cells might result from the presence of the c-*myc* gene in the *ras/myc* SFME cells. But the co-transfected mouse c-*myc* gene reportedly only increases the efficiency of transformation of cells containing the c-Ha-*ras* gene.[4] In our preliminary experiment, *ras*/SFME cells, which have no exogenous c-*myc* gene but have human c-Ha-*ras,* showed a similar result on treatment with 250 μg/ml azatyrosine (data not shown). Besides, the cell we used here is an astrocyte precursor which is quasi-terminally differentiated, as discussed below, so its biological character should be different from that of NIH 3T3 cells. This needs further investigation.

Expression of GFAP, which may have important roles in the course of the ontogenic differentiation of brain cells, is a useful marker of the cytological condition of astrocytes.[14,15] Non-transformed SFME cells express GFAP coinciding with the suppression of proliferation when they are cultured in medium with serum, and so they are distinguished as astrocyte precursors.[5] In many cases, introduction of activated oncogenes promotes cell proliferation but depresses the differentiation.[16–18] The ability of SFME cells to express GFAP is also depressed by *ras* transfection.[3] Since azatyrosine reverts *ras*-transformed NIH 3T3 cells to apparently normal cells,[7] there may be a possibility that the suppression of proliferation by azatyrosine accompanies the differentiation of *ras/myc* SFME cells. In spite of the inhibitory effect on cell proliferation, however, azatyrosine had no effect on the induction of GFAP expression by *ras/myc* SFME cells. In addition, azatyrosine did not substitute for serum in SFME cells, which expressed GFAP in the presence of serum even in a culture with sufficient azatyrosine. Therefore, it may be speculated that the action mechanisms of azatyrosine do not involve normalization and/or differentiation of *ras/myc* SFME cells and the process of GFAP expression.

The expression of the introduced human *ras* gene is reportedly not inhibited by azatyrosine in *ras*-transformed NIH 3T3 cells and their revertant cells. Further, azatyrosine reverts the NIH 3T3 cells transformed by activated *raf* and *neu* other than *ras,* so the product of *ras* may not be a direct target of azatyrosine.[7] Azatyrosine did not inhibit expression of the human *ras* gene in *ras/myc* SFME cells either. On the other hand, growth suppression of *ras/myc* SFME cells with azatyrosine was competitively inhibited by simultaneous addition of tyrosine in our preliminary experiments. Then, there may be another possibility, that the oncogene products are modified directly by the incorporated azatyrosine in our cells. Further investigation on this point may be required.

ACKNOWLEDGMENTS

We are grateful to Dr. Osamu Makabe (Central Research Laboratories, Meiji Seika Co. Ltd., Yokohama) and Dr. Susumu Nishimura (Chief, Biology Division, National Cancer Center Research Institute, Tokyo) for the gift of azatyrosine and useful suggestions. We thank Dr. Sanetaka Shirahata (Associate Professor, Graduate School, Genetic Resources Technology, Kyushyu University, Fukuoka) and Dr. David W. Barnes (Professor, Department of Biochemistry and Biophysics, Environmental Health Science Center, Oregon State University, Corvallis, OR, USA) for the gift of SFME and *ras/myc* SFME cells and lots of useful suggestions on the cells and their culture methods. This work was supported in part by a Grant-in-Aid (No. 03671181) from the Ministry of Education, Science and Culture of Japan.

(Received January 8, 1992/Accepted May 9, 1992)

REFERENCES

1) Loo, D. T., Fuquay, J. I., Rawson, C. L. and Barnes, D. W. Extended culture of mouse embryo cells without senescence: inhibition by serum. *Science,* **236,** 200–202 (1987).
2) Loo, D. T., Rawson, C. L., Helmrich, A. and Barnes, D. W. Serum-free mouse embryo cells: growth response *in vitro*. *J. Cell. Physiol.,* **139,** 484–491 (1989).
3) Shirahata, S., Rawson, C. L., Loo, D. T., Chang, Y. J. and Barnes, D. W. *Ras* and *neu* oncogenes reverse serum inhibition and epidermal growth factor dependence of serum-free mouse embryo cells. *J. Cell. Physiol.,* **144,** 69–76 (1990).
4) Rawson, C. L., Shirahata, S., Collodi, P., Natsuno, T. and Barnes, D. W. Oncogene transformation frequency of nonsenescent SFME cells is increased by c-*myc*. *Oncogene,* **6,** 487–489 (1991).
5) Sakai, Y., Rawson, C. L., Lindburg, K. and Barnes, D. W. Serum and transforming growth factor β regulate glial fibrillary acidic protein in serum-free-derived mouse embryo cells. *Proc. Natl. Acad. Sci. USA,* **87,** 8378–8382 (1990).
6) Rawson, C. L., Loo, D. T., Helmrich, A., Ernst, T., Natsuno, T., Merrill, G. and Barnes, D. W. Serum inhibition of proliferation of serum-free mouse embryo cells. *Exp. Cell Res.,* **192,** 271–277 (1991).
7) Shindo-Okada, N., Makabe, O., Nagahara, H. and Nishimura, S. Permanent conversion of mouse and human cells transformed by activated *ras* or *raf* genes to apparently normal cells by treatment with the antibiotic azatyrosine. *Mol. Carcinog.,* **2,** 159–167 (1989).
8) Carmichael, J., Degraff, W. G., Gazdar, A. F., Minna, J. D. and Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.,* **47,** 936–942 (1987).
9) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156–159 (1987).
10) Sambrook, J., Fritsch, E. F. and Maniatis, T. "Molecular Cloning, A Laboratory Manual," Second Edition, pp. 7.26–7.52 (1989). Cold Spring Harbor Laboratory Press, New York.
11) Feinberg, A. P. and Vogelstein, B. Addendum of "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity." *Anal. Biochem.,* **137,** 266–267 (1984).
12) Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. and Goeddel, D. V. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature,* **302,** 33–37 (1983).
13) Engvall, E. Enzyme immunoassay ELISA and EMIT. *Method Enzymol.,* **70,** 419–439 (1980).
14) Lewis, S. A., Balcarek, M. J., Krek, V., Shelanski, M. and Cowan, N. J. Sequence of a cDNA clone encoding mouse glial fibrillary acidic protein: structural conservation of intermediate filaments. *Proc. Natl. Acad. Sci. USA,* **81,** 2743–2746 (1984).
15) Morrison, R. S., De Vellis, J., Lee, Y. L., Bradshaw, R. A. and Eng, L. F. Hormones and growth factors induce the synthesis of glial fibrillary acidic protein in rat brain astrocytes. *J. Neurosci. Res.,* **14,** 167–176 (1985).
16) Weissman, B. E. and Aaronson, S. A. BALB and Kirsten murine sarcoma viruses alter growth and differentiation of EGF-dependent BALB/c mouse epidermal keratinocyte lines. *Cell,* **32,** 599–606 (1983).
17) Yuspa, S. H., Kilkenny, A. E., Stanley, J. and Licht, U. Keratinocytes blocked in phorbol ester-responsive early stage of terminal differentiation by sarcoma viruses. *Nature,* **314,** 459–462 (1985).
18) Yoakum, G. H., Lechner, J. F., Gabrielson, E. W., Korba, B. E., Malan-Shibley, L., Willey, J. C., Valerio, M. G., Shamsuddin, A. M., Trump, B. F. and Harris, C. C. Transformation of human bronchial epithelial cells transfected by Harvey *ras* oncogene. *Science,* **227,** 1174–1179 (1985).