Acta Medica Okayama

Volume 29, *Issue* 4 1975 *Article* 7

AUGUST 1975

Effect of anti-alpha-fetoprotein serum on growth and plating efficiency of alpha-fetoprotein-producing hepatoma cells in vitro

Kyoichiro Nishina[*] Toshiaki Tsutamune[†] Takayoshi Tokiwa[‡]
Masahiro Miyazaki[**] Jiro Sato[††] Akiharu Watanabe[‡‡]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,
[**]Okayama University,
[††]Okayama University,
[‡‡]Okayama University,

Effect of anti-alpha-fetoprotein serum on growth and plating efficiency of alpha-fetoprotein-producing hepatoma cells in vitro*

Kyoichiro Nishina, Toshiaki Tsutamune, Takayoshi Tokiwa, Masahiro Miyazaki, Jiro Sato, and Akiharu Watanabe

Abstract

The effect of a specific rabbit antiserum to rat alpha-fetoprotein (AFP) was examined on the growth and the plating efficiency of AFP-producing rat hepatoma cells (AH70Btc Clone 10-5) in cultures. The addition of anti-AFP serum to the culture medium inhibited cell growth moderately and inhibited plating efficiency markedly, although no inhibitory effect of complexes of AFP and antibody to AFP was observed on cell growth. Anti-AFP globulin in the immune serum was demonstrated on the cell surface by fluorescent antibody technique. Several clones producing low levels of AFP were obtained by long-term treatment of the original Clone 10-5 cells with anti-AFP serum. These treated clones showed characteristics that differed from the untreated original clone 10-5 cells: The relative plating efficiency of the treated clones on agar plates containing 5% anti-AFP serum was higher than the original Clone 10-15 cells and the amount of AFP secreted by the treated clones was lower.

*PMID: 55059 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 29, 319—326 (1975).

EFFECT OF ANTI-ALPHA-FETOPROTEIN SERUM ON GROWTH AND PLATINC EFFICIENCY OF ALPHA-FETOPROTEIN-PRODUCING HEPATOMA CELLS *IN VITRO*

Kyoichiro NISHINA, Toshiaki TSUTAMUNE, Takayoshi TOKIWA, Masahiro MIYAZAKI and Jiro SATO

Division of Pathology, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. J. Sato)

Akiharu WATANABE

Department of Internal Medicine, Okayama University Medical School, Okayama, Japan (Director: Prof. K. Kosaka)

Received for publication, March 19, 1975

Abstract: The effect of a specific rabbit antiserum to rat alpha-fetoprotein (AFP) was examined on the growth and the plating efficiency of AFP-producing rat hepatoma cells (AH70Btc Clone 10-5) in cultures. The addition of anti-AFP serum to the culture medium inhibited cell growth moderately and inhibited plating efficiency markedly, although no inhibitory effect of complexes of AFP and antibody to AFP was observed on cell growth. Anti-AFP globulin in the immune serum was demonstrated on the cell surface by fluorescent antibody technique. Several clones producing low levels of AFP were obtained by long-term treatment of the original Clone 10-5 cells with anti-AFP serum. These treated clones showed characteristics that differed from the untreated original Clone 10-5 cells: The relative plating efficiency of the treated clones on agar plates containing 5% anti-AFP serum was higher than the original Clone 10-5 cells and the amount of AFP secreted by the treated clones was lower.

Alpha-fetoprotein (AFP) is synthesized not only by embryonic liver cells but also by hepatoma cells *in vivo* (1). It has also been known that hepatoma cells secrete AFP *in vitro* (2). The inhibitory effect of heat-inactivated antiserum to mouse leukemia cell growth *in vitro* (3) and the cytotoxic effect of anti-AFP serum on cultured hepatoma cells (4) have been demonstrated, although the mechanisms are not fully understood.

The present study was carried out to examine the effects of rabbit antiserum to rat AFP on the growth and the plating efficiency of cloned rat hepatoma cells (AH70Btc Clone 10-5) established in our laboratory (5). Furthermore, the biological characteristics of cells treated with anti-AFP serum were examined.

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education

MATERIALS AND METHODS

Cells and culture: Cells used in this experiment were AH70Btc Clone 10-5 described in detail in the previous paper (5). The cells were maintained in Eagle's MEM (Chiba Pref. Serum Inst.) supplemented with 20% heat-inactivated bovine serum (the basal medium). The effect of anti-AFP serum on cell proliferation was examined by a simplified replicate tissue culture method (6). The cells were cultured in a medium containing 5% heat-inactivated rabbit anti-AFP serum or non-immune rabbit serum with 95% basal medium. Non-immune serum was prepared by absorbing normal rabbit serum with adult rat serum at a ratio of 1 : 1. The medium was renewed every two days. Cell viability was determined by a dye exclusion test with trypan blue. The plating efficiency was performed according to Kuroki's method using 0.5% special agar-Noble (Difco), in final concentration (7). An aliquot (0.1 ml) of cell suspension containing more than 95% single cells was added to the agar plate containing 5% anti-AFP serum or non-immune serum and spread evenly over the surface of plates. The plates were incubated at 37°C for two weeks in a CO_2-incubator. The number of colonies formed was counted under low-powered microscope.

Anti-AFP serum and detection of AFP: Pooled serum from ascites hepatoma (AH70B)-bearing Donryu rats was precipitated with 50% saturation of ammonium sulphate and dialyzed overnight. This preparation was used as the crude AFP for immunization. Rabbits were immunized with a mixture of the crude AFP and Freund's complete adjuvant (Difco) three times at intervals of seven days and bled on the seventh day after the last injection. The sensitized rabbit serum was absorbed with normal serum of adult Donryu rats. The immunological properties of monospecific anti-AFP serum have been reported previously (5). The concentrations of AFP in the culture medium were determined by the double antibody technique with ^{131}I- and ^{125}I-labeled AFP (8, 9). A solution containing complexes of AFP and antibody to AFP was prepared as follows: AFP secreted by Clone 10-5 cells was precipitated by the addition of anti-AFP serum, and the precipitate was washed thoroughly and suspended in the basal medium. Localization of anti-AFP globulin in cells treated with anti-AFP serum for 24 hr was examined by fluorescent microscopy following staining with goat anti-rabbit IgG serum conjugated fluorescent isothiocyanate (Behringerwerke) (10).

Chromosome preparation: The chromosome preparation was performed by the air-drying method of Moorhead *et al.* (11).

Tumorigenicity: The cultured cells were removed from the flasks and 5×10^6 cells were transplanted intraperitoneally into adult Donryu rats. The rats transplanted with cells were observed daily and the number of days of survival was determined.

RESULTS

AFP secretion and growth curve: Fig. 1 shows the correlation between the amount of AFP secreted per 24 hr and the cell proliferation of Clone 10-5

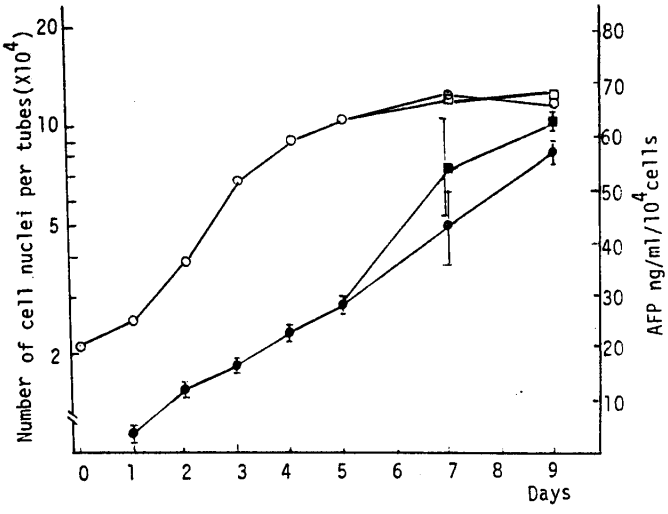

Fig. 1 AFP secretion and growth curve.

AFP in the culture medium (2 ml) was collected prior to cell count.

$$\text{AFP/ml/}10^4\text{ cells} = \frac{\text{AFP for 24 hr}}{\text{Average cell number}} \times 10^4$$

Each point indicates an average of three tubes. The upper and lower limits are shown on the AFP secretion values.
○ □, growth curve; ● ■, AFP secretion; □ ■, cells cultured in Eagle's MEM without serum.

cells. AFP was secreted into the medium at all cell growth phases. The AFP secretion at the logarithmic phase was less marked than at the stationary phase.

Effect of anti-AFP serum on Clone 10-5 cells: The effect of various concentrations of anti-AFP serum on the growth of Clone 10-5 cells is shown in Fig. 2. Anti-AFP serum at 5% of concentration in the basal medium provided near maximum inhibition of cell proliferation, but the addition of the same percentage of non-immune serum or complexes of AFP and antibody to AFP was ineffective (Fig. 2). Five percent anti-AFP serum in the basal medium and 5% non-immune serum as control were thus used in the following experiments.

The inhibition of cell proliferation by anti-AFP serum added at various growth phases is summarized in Fig. 3. Cell proliferation was inhibited by adding anti-AFP serum at all growth phases, although inhibition of cell proliferation with anti-AFP serum at a low inoculum dose was higher than that at a high inoculum dose. Moreover, the addition of anti-AFP serum to the

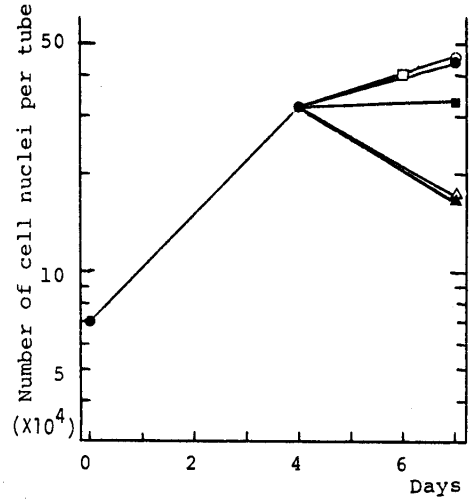

Fig. 2 Effect of various concentrations of anti-AFP serum on the growth of Clone 10-5 cells. The cells were cultured in the basal medium for four days and thereafter supplemented with 10% anti-AFP rabbit serum (△), 5% anti-AFP serum (▲), 1% anti-AFP serum (■), 5% non-immune rabbit serum (○), no supplement (●) and complexes of AFP and antibody to AFP (□), as described in Materials and Methods. Each point indicates the mean value of three tubes.

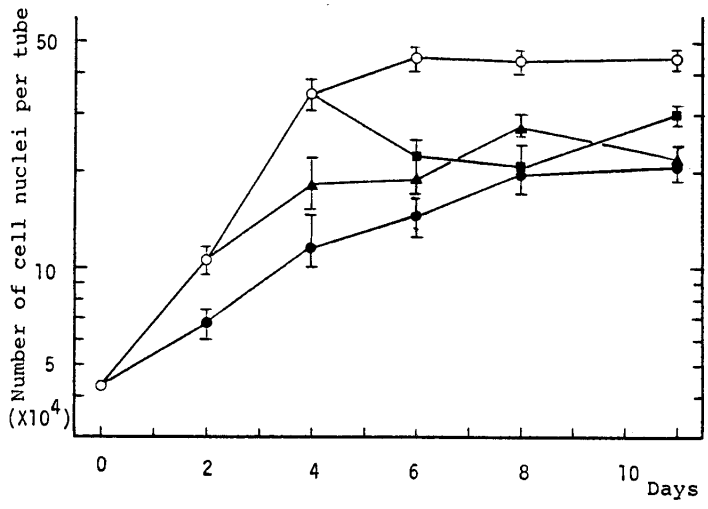

Fig. 3 Effect of anti-AFP serum on the growth of Clone 10-5 cells. The cells were cultured in the medium containing 5% anti-AFP serum or 5% non-immune serum. Control (○), addition of anti-AFP serum at 0 day (●), 2nd day (▲), and 4th day (■). Each point indicates the mean value of three tubes, and the upper and lower limits are shown.

medium led to a massive agglutination of cultured cells and the number of dead cells gradually increased after treatment by antiserum. The plating efficiency of the cells was reduced markedly by the addition of anti-AFP serum, and the size of the colony formed on agar plates containing anti-AFP serum was smaller than without antiserum (Table 1). Anti-AFP globulin

TABLE 1 EFFECT OF ANTI-AFP SERUM ON THE PLATING EFFICIENCY

Cell lines	Inoculum cell number/plate	P.E. on agar plate (%)[a] A	B	Relative[b] P.E. (%)
Wild[c]	1000	12.2±0.7	0.2±0.1	1.6
	2000	10.4±0.4	0.3±0.1	2.9
Clone 10-5[d]	1000	8.8±2.7	0.6±0.2	6.9
	2000	6.3±0.8	0.5±0.3	7.9
PC[e]	1000	6.2±0.9	0.9±0.1	14.5
	2000	—	—	

a) Plating efficiency (P.E.) indicates the percent of cells formed in colony per cells plated. Agar plates contain non-immune rabbit serum (A) or anti-AFP rabbit serum (B) as described in Materials and Methods. Mean±SD. $SD=\sqrt{\frac{\sum Xi^2-NM}{N}}$

b) Relative plating efficiency $=\frac{\text{P.E. on B}}{\text{P.E. on A}}\times 100$

c) AH70Btc cells.

d) Colonial cloned cells (the original cells of the treated clones).

e) Single cloned cells.

was detected on the cell surfaces of cells treated with anti-AFP serum by fluorescent antibody technique.

Long-term treatment of Clone 10-5 cells with anti-AFP serum and the biological characteristics of treated clones: Eight colonies as described in Table 1 were cultured for two weeks on agar plates containing anti-AFP serum. Each colony was picked up by pipettes and transferred into new flasks. Among these, four clones were continuously treated with anti-AFP serum for a further three months and thereafter, transferred into flasks with the basal medium without antiserum (designated as long-term treated Clones 1, 2, 3 and 4). The long-term treated clones were further cultured for one month to eliminate the anti-AFP serum that adhered on cells. Four other clones were immediately cultured back into the basal medium without antiserum (designated as short-term treated Clones 5, 6, 7 and 8). The amounts of AFP secreted by the treated clones were determined and the values are summarized in Table 2. AFP levels secreted by long-term treated clones were lower, but those of short-term treated clones were similar to Clone 10-5.

The biological characteristics of long-term treated clones were investigated. Morphological findings of the treated Clones 1 and 2 were almost

TABLE 2 AFP SECRETION OF TREATED CLONES

Cells		Culture days	AFP (ng/ml/10^4cells)*
Long-term treated clone	1	326	33.0
	2	327	16.7
	3	327	28.2
	4	326	11.6
Short-term treated clone	5	270	98.4
	6	270	77.0
	7	270	66.9
	8	270	70.7
Clone 10-5 (control)		265	75.3

AFP was measured in the medium of cells cultured for 48 hr. Long-term treated Clones 1 to 4 and short-term treated Clones 5 to 8 were obtained as described in the text.

*$\text{AFP} = \frac{\text{AFP for 48 hr}}{\text{Average cell number}} \times 10^4$

TABLE 3 DISTRIBUTION OF CHROMOSOME NUMBER OF LONG-TERM TREATED CLONES

Chromosome number	>60	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	100<
Clone 1								2						2	4	6	11	11	5	4	1	2	2
2		1			1	1				3	4	1	9	12	7	8	1	2					
3	1		1							1		4	7	17	16			1					2
4			2	1	2	3	7	13	5	5	3	1	1	4	1								
Clone 10-5															1	7	6	7	13	9	2		2

50 metaphases were counted in each clone.

TABLE 4 SURVIVAL PERIOD OF RATS TRANSPLANTED WITH LONG-TERM TREATED CLONES

Cells		Days of survival*
Long-term treated clone	1	22±5.3
	2	21±2.7
	3	35±6.7
	4	24±2.7
Clone 10-5 (control)		16±3.3

Each cell of long-term treated clones and Clone 10-5 was transplanted into three Donryu rats. *Average±SD.

similar to those of Clone 10-5 cells. Clone 3 was inclined to float and form aggregates and Clone 4 was composed of small pavement-like cells. The chromosome number of the long-term treated clones decreased compared with Clone 10-5, but significant differences in number and distribution were not seen between the long-term treated clones and Clone 10-5 cells (Table 3). The mean survival days of rats transplanted with the cells from long-term treated clones were a little longer than with Clone 10-5 (Table 4). The plating efficiency of the long-term treated clones was suppressed by anti-AFP serum. But the relative

plating efficiency of those clones was higher than Clone 10-5 (Table 5). The inhibitory effect of anti-AFP serum on the growth of long-term treated clones was similar to Clone 10-5.

TABLE 5 PLATING EFFICIENCY OF LONG-TERM TREATED CLONES

Cell lines	Inoculum cell number/plate	P.E. on agar plate (%)[a] A	B	Relative[b] P.E. (%)
Clone 1	1000	6.9±1.1	0.9±0.3	13.0
	2000	4.4±0.8	0.6±0.2	13.6
2	1000	13.0±3.7	4.2±0.5	32.3
	2000	11.7±3.6	3.8±0.3	32.5
3	1000	6.7±0.9	1.6±0.8	23.9
	2000	5.3±1.9	1.5±0.2	28.3
4	1000	10.6±0.9	0.8±0.1	7.5
	2000	8.1±0.7	0.9±0.2	11.1

a) Plating efficiency (P.E.) indicates the percent of cells formed in colony per cells plated. Agar plates contained non-immune rabbit serum (A) or anti-AFP rabbit serum (B) as described in Materials and Methods. Mean±SD.

b) Relative plating efficiency $= \frac{\text{P.E. on B}}{\text{P.E. on A}} \times 100$

DISCUSSION

It has been already demonstrated by Tsukada *et al.* (12) that low AFP-producing clones can be isolated from AH66 cells producing high levels of AFP. From this finding and the experiment of ascites hepatoma, it has been suggested that the hepatoma nodules formed in the liver of rats by a carcinogen might consist of many types of tumor cells (13). However, low AFP-producing clones could not be isolated from the original strain, AH70Btc, cells with high levels of AFP, as described in detail (5). In the present study, low AFP-producing clones could not be isolated by short-term treatment with anti-AFP serum. It remains unknown whether the appearance of low AFP-producing cells by long-term treatment of Clone 10-5 cells producing high levels of AFP with anti-AFP serum is due to the mutation of Clone 10-5 cells or to the selection of low AFP-producing cells appearing at cell divisions from Clone 10-5 cells. However, the present results strongly suggest the probability of cell mutation, since cloned cells were used.

Biological characteristics of long-term treated clones differ compared to Clone 10-5. The relative plating efficiency of long-term treated clones on agar plates containing anti-AFP serum is higher than that of Clone 10-5 and might indicate that anti-AFP serum was more sensitive to high AFP-producing cells than to low AFP-producing cells. This observation is consistent with the previous experiment of AH66 cells (4). The mean survival days of rats transplanted with long-term treated clones was a little longer than with Clone

10-5. However, the tumorigenicity of low AFP-producing cells is not always lower than that of high AFP-producing cells, because the tumorigenicity of cultured cells decreases during long-term cultivation of cells (14).

Acknowledgment: We express thanks to Dr. Kazuhisa Taketa, Department of Internal Medicine, Okayama University Medical School, for helpful suggestions and proofreading this manuscript.

REFERENCES

1. Abelev, G.I., Perova, S.D., Khramkova, N.I., Postnikova, Z.A. and Irlin, I.S.: Production of embryonic α-globulin by transplantable mouse hepatoma. *Transplantation* **1**, 174-180, 1963.
2. Irlin, I.S., Perova, S.D. and Abelev, G.I.: Changes in the biological and biochemical properties of mouse hepatoma during long-term cultivation *in vitro*. *Int. J. Cancer* **1**, 337-347, 1966.
3. Yang, T.J. and Vas, S.I.: Effect of antibodies on L5178Y mouse leukemia cells cultured *in vitro*. *Cancer Res.* **30**, 1231-1235, 1970.
4. Tsukada, Y., Mikuni, M., Watabe, H., Nishi, S. and Hirai, H.: Effect of anti-alpha-fetoprotein serum on some cultured tumor cells. *Int. J. Cancer* **13**, 187-195, 1974.
5. Nishina, K.: Alpha-fetoprotein producing clones derived from ascites hepatoma AH70B in culture. *Acta Med. Okayama* **29**, 103-109, 1975.
6. Katsuta, H., Takaoka, T., Oishi, Y., Baba, T. and Chang, K.G.: Cultivation of fibroblasts from chick embryo heart in simplified replicate tissue culture. *Japan J. Exp. Med.* **24**, 125-139, 1954.
7. Kuroki, T.: Colony formation of mammalian cells on agar plates and its application to Lederburg's replica plating. *Exp. Cell Res.* **80**, 55-62, 1973.
8. Hirai, H., Nishi, S. and Watabe, H.: Radioimmunoassay of α-fetoprotein. In *Protides Biological Fluids*, Vol. 20, ed. H. Peeters, Pergamon Press, New York, pp. 579-587, 1973.
9. Watanabe, A., Mori, O., Taketa, K. and Kosaka, K.: Purification and immunochemical characterization of alpha-fetoprotein from rat fetal serum and liver. *Acta Med. Okayama* (in press).
10. Coons, A.H. and Kaplan, M.H.: Localization of antigen in tissue cells. II. Improvement in a method for detection of antigen by means of fluorescent antibody. *J. Exp. Med.* **91**, 1-13, 1950.
11. Moorhead, P.S., Nowell, P.C., Mellman, W.J., Battips, D.M. and Hungerford, D.A.: Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell Res.* **20**, 613-616, 1960.
12. Tsukada, Y., Mikuni, M. and Hirai, H.: *In vitro* cloning of a rat ascites hepatoma cell line, AH66, with special reference to alpha-fetoprotein. *Int. J. Cancer* **13**, 196-202, 1974.
13. Watabe, H. and Hirai, H.: Production of α-fetoprotein by transplantable rat hepatoma. *Gann Monograph on Cancer Res.* **14**, 279-287, 1973.
14. Hsu, T.C. and Klatt, O.: Mammalian chromosomes *in vitro*. X. Heteroploid transformation in neoplastic cells. *J. Nat. Cancer Inst.* **22**, 313-339, 1959.