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Effects of Heterologous Antisera to Rat Alpha-Fetoprotein on Growth of Cultured Fetal Rat Hepatocytes

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SUMMARY

The effects of heterologous antisera on rat alpha-fetoprotein (AFP) on cell growth were examined on primary culture of fetal rat hepatocytes secreting AFP and albumin into the medium. Cell growth was determined by the number of viable hepatocytes after culture. When these cells were cultured in antiserum (5 to 20%) containing medium, cell growth of the hepatocytes was markedly inhibited by horse or rabbit antisera. The inhibitory effect of horse or rabbit antisera was dose dependent. Rabbit antiserum on rat albumin or goat anti-AFP serum showed little or no effect on cell growth.

INTRODUCTION

Alpha-fetoprotein (AFP), a fetal serum protein in numerous mammalian species (4, 17, 25), has been extensively investigated both as a fetal and tumor -associated protein since Bergstrand and Czar (3) described it as a new serum protein in human fetus. Various aspects of AFP to be used as a marker in tumor diagnosis and in obstetrics were recently reviewed (1, 6, 18, 19).

More recent interest in the studies on AFP focuses on whether the antiserum against AFP has any effect on cells which produce AFP in light of the possibility of immunotherapy of hepatoma patients. Results of our previous in vitro and in vivo studies (11, 16, 23), led us to adopt a simpler system to elucidate the effect of the antiserum in vitro. In this article we are reporting the effect of heterelogous antisera to AFP on the growth of cultured fetal rat hepatocytes which produce AFP and other serum proteins (21, 26).

MATERIAL AND METHODS

Reagents

Collagenase (Cat No. C-0130) and *l*-ornithine were obtained from Sigma

Abbreviation : AFP, alpha-fetoprotein

(Sigma Chemical Co., St. Louis, Mo.). Fetal calf serum and trypsin were purchased from Grand Island Biological Co., Grand Island, N. Y. and Difco Laboratories, Detroit, Mich., respectively. Amino acids and vitamins required for culture medium were purchased from Wako Pure Chemical Industries, LTD. (Osaka, Japan).

Cell culture

Timed pregnant rats (Charles River CDF, F344) weighing 200 to 250g were supplied by Charles River Japan Inc. (Atsugi, Japan) and fetuses were obtained under sterile conditions. Fetal hepatocytes were prepared according to the method of Leffert and Paul (10). Suspensions of 10^6 cells were plated in 35 mm Falcon plastic dishes in 2 m*l* of Dulbecco and Vogt's modification of Eagle's medium deficient in arginine and supplemented with *l*-ornithine (0.4 mM) and 60 μg per m*l* Kanamycin sulfate (Meiji Seika Kaisha, LTD., Tokyo, Japan).

Addition of normal or anti-AFP sera was done immediately after plating to make a final serum concentration of 5 to 20% in the medium. All sera used were decomplemented by heat-inactivation at 56°C for 30 min and dialyzed extensively against 0.15 M NaCl before use. All cultures were then incubated at 37°C in a 90% air/10% CO_2 atmosphere with high humidity without medium change.

Determination of cell number

In each experiment, the cultures were examined under a light microscope. The medium was then removed and the cells were washed twice with Tris-saline buffer (pH 7.4). Two m*l* of a 0.05% trypsin solution in Ca^{++} -and Mg^{++} -free Tris -saline buffer (pH 7.4) were added to the dish and incubated at 37°C for 30 min. After pipetting to produce a single cell suspension, it was diluted by an addition of 4 m*l* of Cellkit-7 solution and cell numbers were counted with a Microcellcounter (Toa Medical Electronics Co., LTD, Kobe, Japan, Model CC-108). Triplicate cultures were used for cell counting and the mean value was calculated. Measurement errors ranged between 5 to 10%.

Antisera

Rat AFP was purified by the immunochemical method as described previously (24) from the ascites fluid of rats bearing a AFP-producing ascites hepatoma (25) . Antisera to rat AFP were raised in rabbit, goat and horse as described in a previous report (17). Precipitation titers of each antiserum were adjusted at a 64 -fold dilution with 0.15 M NaCl solution.

Antiserum to rat albumin was prepared by immunization of rabbit with purified albumin. Purification of rat serum albumin was performed according to the method described by Korner and Debro (9). Rabbits were given subcutaneously four injections of an emulsion of albumin (0.2 mg) and Freund's complete adjuvant at an interval of 10 days. Ten days after the last injection, rabbits were bled and the

serum was then collected. Specificity of both antisera to rat AFP and albumin were identified by immunoelectrophoresis.

RESULTS

Treatment of the cultured hepatocytes with antisera to rat AFP from horse and rabbit resulted in a marked inhibition of cell growth as expressed by cell numbers. As shown in Figure 1, horse anti-AFP serum revealed a high inhibition on cell growth compared with both normal horse and fetal calf sera. When the inhibitory effect by the antiserum was expressed as follows:

$$\text{Percent inhibition} = \frac{\text{No. of cells (normal serum)} - \text{No. of cells (antiserum)}}{\text{No. of cells (normal serum)}} \times 100,$$

the inhibition by anti-AFP horse serum 5 days after plating was 83.3%. On this occasion, only the cells with clear cytoplasm were observed microscopically. These cells might correspond to the dedifferentiating hepatocytes (10), which did

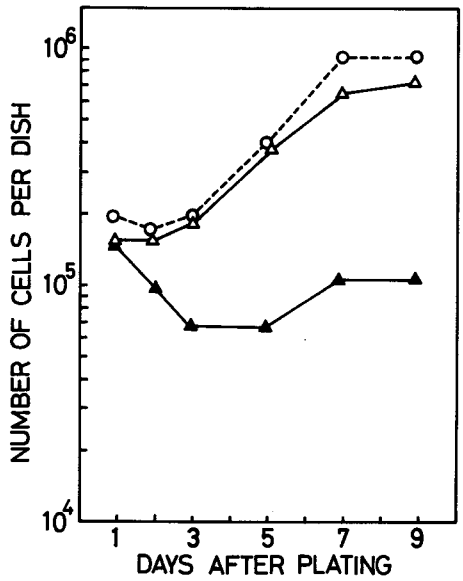

Fig. 1. Effect of horse sera on cell growth of cultured hepatocytes. Each serum was added to the cultures immediately after plating at a final concentration of 10%.
Fetal calf serum, ○·····○ ;
Normal horse serum, △——△ ;
Horse anti-AFP serum, ▲——▲

not synthesize AFP (26). Similarly rabbit anti-AFP serum manifested an inhibitory effect on hepatocyte growth (Fig. 2), although the degree of inhibition was different from that of horse antiserum. Normal rabbit serum has some effect on the attachment of cells to culture dishes when compared with fetal calf serum on day-1, nevertheless an obvious difference was observed on the growth curves between normal and antiserum treated cultures.

Goat anti-AFP serum treatment had little effect on the cultures even when the concentration of antiserum was raised up to 20% (Fig. 3). In addition, both antisera from rabbit and goat showed non-specific recovery efficiency on hepatocyte growth.

On the other hand, an inhibitory effect was observed even when the horse antiserum was added 2 days after plating. As shown in Figure 4, cell growth was inhibited in direct proportion to the concentration of antiserum added. On the contrary, enhancement of the cell growth was observed in cultures treated with

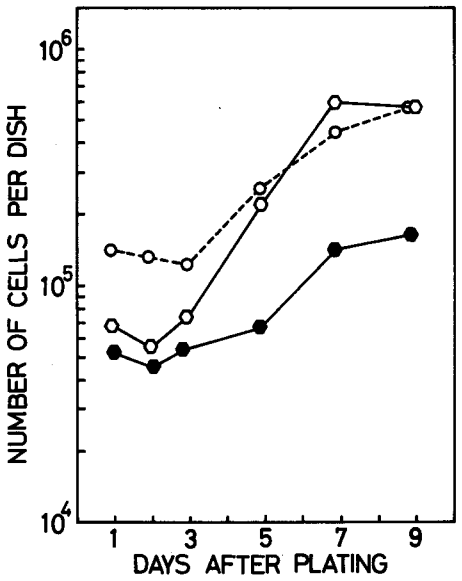

Fig. 2. Effect of rabbit sera on cell growth of cultured hepatocytes. Each serum was added to the cultures immediately after plating at a final concentration of 10%.
Fetal calf serum, ○·····○ :
Normal rabbit serum, ○——○ ;
Rabbit anti-AFP serum, ●——●.

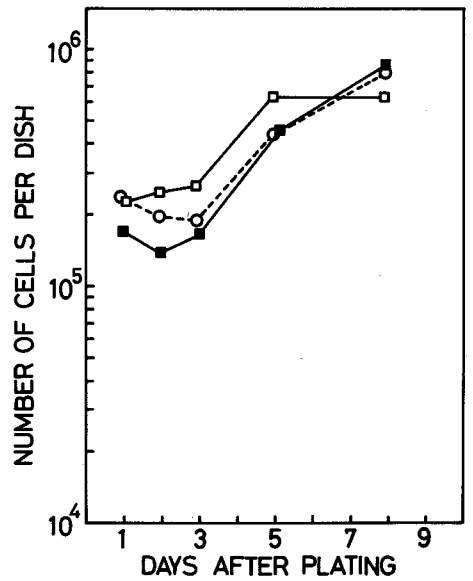

Fig. 3. Effect of goat sera on cell growth of cultured hepatocytes. Each serum was added to the cultures immediately after plating at a final concentration of 10%.
Fetal calf serum, ○·····○ ;
Normal goat serum, □——□ ;
Goat anti-AFP serum, ■——■

normal horse serum. The same results were obtained in the case of rabbit serum.

The effect of rabbit anti-albumin serum was also studied. As shown in Figure 5, anti-albumin serum did not show any inhibitory effect on the cell growth even when the concentration of antiserum was raised up to 20%.

Preliminary experiment (data not shown) of immunoperoxidase staining was undertaken on cells treated with anti-AFP horse serum on day-5 for 6 hr. Controls were performed using fetal calf or normal horse serum treatments. The anti-AFP treated cells showed: [1] faint staining of AFP and albumin, [2] irregularity of

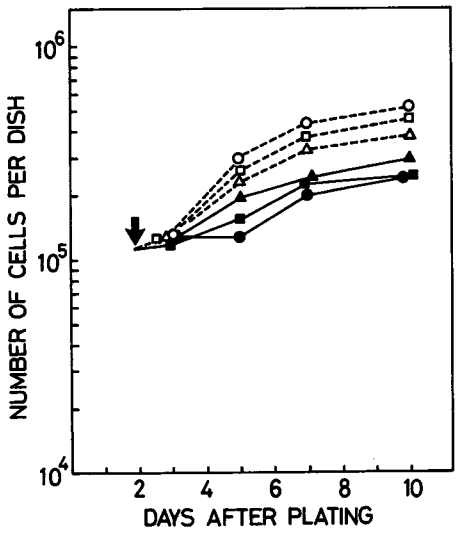

Fig. 4. Dose dependency of normal and anti-AFP horse sera on cell growth of cultured hepatocytes.

Cells were cultured for 2 days with a medium containing 5% of fetal calf serum and then each horse serum was added to the cultures as indicated by an arrow. Open and closed symbols indicate normal and anti-AFP horse serum respectively. Final concentration of sera added to the medium are:

△(▲), 5% ;
□(■), 10% ;
○(●), 20%.

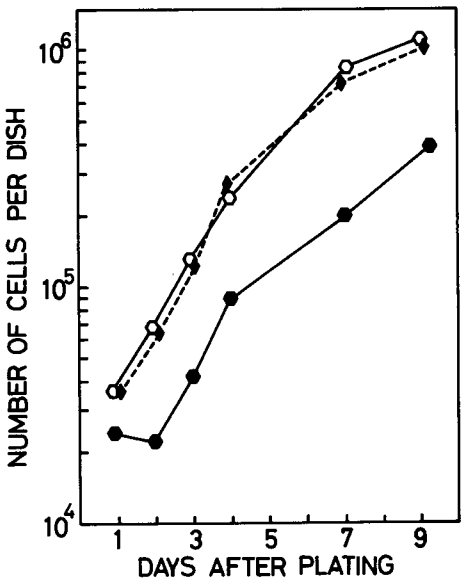

Fig. 5. Fffect of rabbit anti-albumin serum on cell growth of cultured hepatocytes.

Each serum was added to the cultures immediately after plating at a final concentration of 20%.

Normal rabbit serum, ○——○ ;
Rabbit anti-albumin serum, ◆·····◆ ;
Anti-AFP rabbit serum, ●——●.

the periphery of the cells, [3] many dead cells adhering to the dyes. These observations seemed to suggest that the treatment of the cells with the antiserum might lead to cell death.

DISCUSSION

It has been determined in this communication that anti-AFP sera obtained from heterologous animals showed an inhibitory effect on the cell growth of cultured fetal rat hepatocytes. The effect was observed in both antisera from horse and rabbit although some difference of the effect was present. The susceptability of the antisera treated hepatocytes was shown to be dependent on the concentration of the antiserum in the medium. These observations appeared to support our previous study which showed an inhibitory effect of anti-AFP horse serum on cultured ascites hepatoma cells of rat (23). Furthermore similar results were reported by Mizejewski et al. (13, 15) and Allen et al. (2) when mouse hepatoma cells were used as the target cell.

Differences of the effect between the antisera from horse, goat and rabbit could be due to differences in the immunoglobulin class present in the antisera and/or affinity of the AFP antibodies to the antigen. The mechanism of suppression of cell growth by AFP antibodies is not understood although several possibilities were proposed by Sell et al. (19) and Hirai (6). Our present results on growth inhibition and morphological changes may support the notion that AFP antibodies may adhere to the cell membrane, thereby causing injury as a result of cell surface changes. However, the results of cultures with goat anti-AFP and rabbit anti-albumin sera may suggest that serum component(s) other than the antibody are responsible for the phenomenum of growth inhibition.

Allen and Ledford (2) reported that anti-mouse albumin (rabbit serum) showed its cytotoxicity on cultured mouse hepatoma cells. But in our present experiment, the antiserum to rat albumin showed no effect on the cell growth even when the concentration of antiserum added was as high as 20% of the culture. The disagreement of their data and ours may be due to the difference in methodology or participation of unknown substance other than the antibody in the serum. Thus studies utilizing purified polyclonal and/or monoclonal antibody to AFP will better clarify this controversy.

The results from in vivo experiments of rat or mouse (5, 7, 11, 12, 14, 16, 20, 22, 27,) became more complicated because of the differences in animals, target cells, antisera and methodology. But recent observations in mouse and rat by Mizejewski et al. (14) and Wepsic et al. (27) might shed some light on the possibility for immunotherapy of hepatoma patients by anti-AFP antibody and might lead to possible clinical trials (8). There is little doubt that more definitive data

and resolution of these discussed problems are necessary before clinical trial can be implemented.

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