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Basic Investigation

INFLUENCE OF ALPHA-FETOPROTEIN ON THE GROWTH OF TUMOR CELLS *IN VITRO*

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It has been recognized that alpha-fetoprotein (AFP), as an oncofetal antigen, re-expresses in large amounts in adult tumor cells and serves clinically useful purposes as a tumor marker assay. However, its biological activities are still far from clear. In the present study, the ability of AFP to stimulate tumor cell growth was observed by *in vitro* test system. The new finding indicates that AFP contributes to the generation and development of tumor and is an important target action site of tumor therapy.

Key words: Alpha-fetoprotein, Liver neoplasma, Animal model, *In vitro* experiment

An emphasis has been placed on the identification of alpha-fetoprotein (AFP), a tumor-associated embryonic substance, in human serum as diagnostic tools in tumor identification. In addition to the important practical consideration[1] in the management of human tumors, considerable interest has also been shown in the possible biologic roles of AFP as a suppressive agent in immune responsiveness.[1] The effect of AFP administration on the development of experimental tumors in mammals has also been investigated with the use of virus and chemical carcinogens. *In vivo* treatment with AFP in rodents increases their susceptibility to various experimental tumors, which is demonstrated by greater tumor sizes, longer periods for tumor regression, and an increased number of tumor progressions.[2] But, this heightened susceptibility can be explained by immunosuppression of AFP.[3] It is still far from clear whether AFP stimulates tumor cell growth *in vitro*. It has been observed that the passive administration of anti-AFP serum inhibits the growth of AFP-producing hepatoma cells *in vitro* and *in vivo*.[1] These findings have potentially important implications that AFP may also function as a tumor-promoting agent. The availability of large quantities of AFP-enriched umbilical cord sera permits extension of functional analysis of AFP. Hence, in present study, influence of AFP on tumor cell growth was observed by *in vitro* test.

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MATERIALS AND METHODS

Animals and Implantation of Tumors

KM female mice, 6 — 8 weeks old, 20 — 22 g (age and weight matched per experiment), were maintained on a 12: 12 h light-dark cycle at constant temperature (22 ± 1 ℃), and housed five in a cage with free access to food and water. Implantation of tumors was done by intraperitoneal injection of 1×10^7 tumor cells into KM mouse.

Isolation and Identification of Human AFP

The AFP preparations used in this study were isolated from human umbilical cord sera by sequential

precipitation with ammonium sulphate. They were then purified by anti-AFP monoclonal antibody affinity chromatography as described in detail elsewhere.[4] All purified AFP samples revealed only a single band of protein on polyacrylamide electrophoresis. Protein concentrations were determined according to Lowry's method.

Cell Culture

For assay of tumor cell growth, approximately 1 ml of ascites was collected from peritoneal cavity of mouse bearing hepatoma-22 (H-22) or Ehrlich ascites carcinoma (EAC) on 7 — 10 days after implantation of tumors. Cell suspensions were prepared in RPMI 1640 medium supplemented with 100 IU ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin and 10% new-born bovine serum. Cells were dispensed into each well of 96-well flat bottom plates. The culture was incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 in air for 48 h.[5]

MTT-Microculture Tetrazolium Assay

Cell growth in the presence or absence of experimental agents was determined using the previously described MTT-microculture tetrazolium assay.[6] Briefly, after 48 h culture, the cells were incubated with MTT (800 μg ml^{-1}). Four hours later, 100 μl of 10% sodium dodecyl sulphate-5% isobutanol-0. 12% HCl solution was added to each well of 96-well plate to solubilize the MTT-formazan product. The culture plate was incubated for another 12 h. A_{540} value was measured with an enzyme-linked immunosorbent assay (ELISA) plate reader.

Statistical Analysis

Student t-test was used to calculate differences between experimental and control groups. For all statistical analyses, significance was accepted at $P< 0.05$. Data are presented throughout as $\bar{\chi}\pm s$.

RESULTS

Study on the relationship between measured absorbance at 540 nm (A_{540}) and number of viable mouse ascites H-22 cells indicated that cell densities at 2.5 — 80× 10^4 cell ml^{-1} gave rise to a detectable and relatively linear range of absorbance values (r=0.98, Table 1).

Table 1. Absorbance measurement as a function of viable cell density

No. of cultured H-22 cells (× 10^4 cells ml^{-1})	Absorbance at 540 nm
2. 5	0. 20 ± 0. 04
5. 0	0. 27 ± 0. 05
10. 0	0. 37 ± 0. 13
20. 0	0. 48 ± 0. 09
40. 0	0. 88 ± 0. 06
80. 0	1. 16 ± 0. 05

Mouse hepatoma-22 (H-22) cells were cultured at the indicated cell density in RPMI 1640 medium supplemented with 10% new-born bovine serum at 37 ℃ in 5% CO_2 humid atmosphere for 48 h. Each value represents the mean of 6 samples.

To determine the stimulation of tumor cell growth by AFP, ascites H-22 cells (5 × 10^4 cell ml^{-1}) were incubated with 9. 4 — 300.0 μg ml^{-1} of human AFP. The results showed that AFP at 37. 5 — 300 μg ml^{-1} significantly stimulated the growth of H-22 cells (Table 2). It suggested that this effect of AFP resulted from direct action of AFP on H-22 cells rather than indirect suppression of the immune system by AFP. The AFP and albumin genes are believed to arise from a common ancestral gene. Hence, there are striking physicochemical and functional similarities between AFP and albumin.[7] When H-22 cells were incubated with 9. 4 — 300 μg ml^{-1} of human serum albumin (HSA), no obvious influence on the growth of H-22 cells was observed in HSA-treated group (Table 2). It was inferred that growth-stimulatory effect of AFP was not a non-specific nutrition of this protein on cultured cells. In order to further confirm the specificity of action of AFP, H-22 cells were incubated with combined AFP and anti-AFP antibody. It was observed that the growth-stimulatory effect of AFP at 50 μg ml^{-1} and 100 μg ml^{-1}, respectively, was obviously abolished by anti-AFP antibody at 55 μg ml^{-1} and 110 μg ml^{-1}, respectively (Table 3).

5-Fluorouracil (5-FU) is widely used in the chemotherapy of human hepatoma. Thus, we studied effect of 5-FU on growth-stimulatory action of AFP, H-22 cells (3×10^5 cells ml^{-1}) were incubated with 3.

1 — 25 μg ml^{-1} of 5-FU. Direct cytotoxic activity was obtained with higher concentrations of 5-FU. However, in the presence of 100 μg ml^{-1} of AFP, lower concentrations of 5-FU could produce a potent antagonism on AFP -stimulated hepatoma cell growth (Table 4). It indirectly suggested that human AFP exhibited a direct stimulatory activity on the growth of H-22 cells.

Table 2. Effect of alpha-fetoprotein (AFP) and human serum albumin (HSA) on H-22 cell growth in vitro

Concentration	H-22 cell growth (A_{540})	
(μg ml^{-1})	with AFP	with HSA
0	0.27 ± 0.02	0.27 ± 0.02
9. 4	0.35 ± 0.04 (29.6)	0.28 ± 0.04 (6.7)
18. 8	0.44 ± 0.10 (62.9)	0.25 ± 0.01 (-7.4)
37. 5	0.60 ± 0.06 (122.2)*	0.27 ± 0.02 (0)
75. 0	0.61 ± 0.07 (125.9)*	0.29 ± 0.04 (7.4)
150. 0	0.62 ± 0.09 (129.6)*	0.29 ± 0.03 (7.4)
300. 0	0.69 ± 0.07 (155.5)*	0.29 ± 0.04 (7.4)

Mouse H-22 cells (5×10^4 cells ml^{-1}) were incubated with various concentrations of human AFP or HSA for 48 h. Each value represents the mean of 6 samples.. $P<0.05$, compared with 0 μg ml^{-1}. Data in parenthesis indicated stimulation percent.

Table 3. Effect of combined AFP and anti-AFP antibody on H-22 cell growth in vitro

Cells cultured in:	Concentration (μg ml^{-1})	H-22 cell growth (A_{540})
medium	0	0.27± 0.02
AFP	50	0.59± 0.09(118.5)*
AFP + anti -AFP	50 + 55	0.33± 0.06(22.2)#
AFP	100	0.65± 0.03(140.7)*
AFP +anti - AFP	100 + 110	0.32± 0.07(18.5)#

*$P<$ 0.05, compared with 0μg ml^{-1}, #$P<$ 0.05, compared with AFP group. Data in parenthesis indicated stimulation percent. Additional details see Table 2.

When EAC cells (1×10^5 cells ml^{-1}) were incubated with AFP (9. 4 — 300.0μg ml^{-1}), growth-stimulatory activity of AFP was also observed. HSA exhibited no significant influence on EAC cell growth (Table 5). It means that direct stimulation of AFP on tumor cell growth shows no strict tumor specificity.

Table 4. Suppression of 5-FU on H-22 cell growth in vitro

Concentration	H-22 cell growth (A_{540})	
(μg ml^{-1})	without AFP	with AFP
0	0.58± 0.08	1.53± 0.08
3.1	0.54± 0.07(6.9)	1.26± 0.11(17.6)
6.3	0.49± 0.06(15.5)	1.09± 0.05(28.8)*
12.5	0.45± 0.09(22.4)*	0.96± 0.14(37.3)*
25.0	0.38± 0.07(34.5)*	0.81± 0.29(47.1)*

Mouse H-22 cells (3×10^5 cells ml^{-1}) were incubated with various concentrations of 5-fluorouracil (5-FU) for 48 h in the presence or absence of AFP (100 μg ml^{-1}). * $P<0.05$, compared with 0 μg ml^{-1} . Data in parenthesis indicated inhibition percent. Each value represents the mean of 6 samples.

Table 5. Effect of AFP and HSA on EAC Cell growth in vitro

Concentration	EAC cell growth (A_{540})	
(μg ml^{-1})	with AFP	with HSA
0	0.27± 0.09	0.27± 0.09
9.0	0.39± 0.16 (44.4)	0.26± 0.03 (-3.7)
18.8	0.44± 0 08 (62.9)	0.28± 0.03 (3.7)
37.5	0.54± 0.11 (100.0)*	0.26± 0.04 (-3.7)
75.0	0.65± 0.15 (140.7)*	0.25± 0.04 (-7.4)
150.0	0.87± 0.19 (222.2)*	0.32± 0.09 (18.5)
300.0	0.90± 0.11 (233.3)*	0.26± 0.04 (-3.7)

Mouse Ehrlich ascites carcinoma (EAC) cells (1×10^5 cells ml^{-1}) were incubated with various concentrations of human AFP or HSA for 48 h. *$P<$ 0.05, compared with 0 μg ml^{-1} . Data in parenthesis indicated stimulation percent. Each value represents the mean of 6 samples.

DISCUSSION

The results obtained by *in vitro* test system showed that AFP does indeed stimulate tumor cell growth, independent of its immunosuppression. Specificity of its action can be displayed with the use of HSA and anti-AFP antibody. Taking all previous investigations,[2,3] it is strongly inferred that AFP serves as a tumor-promoting agent. The new finding indicates that AFP is an important target action site of tumor therapy.[8] How AFP exhibits growth-stimulatory activity remains to be explained, but the possibility may be considered: AFP may bind with tumor cell membrane for biological purposes. So, tumor cell membrane surface will be a major target of analysis.

In addition to its action on hepatoma cells, AFP can also promote the growth of EAC cells. It is in agreement with the previous studies.[2] *In vivo*, the effect of AFP can be explained by its immunosuppression.[3] But *in vitro*, because of the deficiency of the effective immune system, the influence of AFP on nonhepatoma cell growth is interesting . Further studies will be done to define the meaning and mechanism of non-hepatoma-stimulatory activity of the stimulation of human AFP on the growth of mouse tumor cells may be related with cross immunological reaction in different species. But, whether or not does it also mean that there is homological functional domain in AFP molecules from different species for tumor-promoting purpose? It will be verified by further investigation.

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