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• LIVER CANCER •

The promoting molecular mechanism of alpha-fetoprotein on the growth of human hepatoma Bel7402 cell line

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Abstract

AIM: **The goal of this study was to characterize the AFP receptor, its possible signal transduction pathway and its proliferative functions in human hepatoma cell line Bel 7402.**

METHODS: **Cell proliferation enhanced by AFP was detected by MTT assay, ^{3}H-thymidine incorporation and S-stage percentage of cell cycle analysis. With radioactive labeled ^{125}I-AFP for receptor binding assay; cAMP accumulation, protein kinase A activity were detected by radioactive immunosorbent assay and the change of intracellular free calcium ($[Ca^{2+}]_i$) was monitored by scanning fluorescence intensity under TCS-NT confocal microscope. The expression of oncogenes N-ras, p53, and p21^{ras} in the cultured cells in vitro were detected by Northern blotting and Western blotting respectively.**

RESULTS: **It was demonstrated that AFP enhanced the proliferation of human hepatoma Bel 7402 cell in a dose dependent fashion as shown in MTT assay, ^{3}H-thymidine incorporation and S-phase percentage up to 2-fold. Two subtypes of AFP receptors were identified in the cells with Kds of 1.3×10^{-9}mol.L^{-1} and 9.9×10^{-8}mol.L^{-1} respectively. Pretreatment of cells with AFP resulted in a significant increase (625%) in cAMP accumulation. The activity of protein kinase A activity were increased up to 37.5, 122.6, 73.7 and 61.2% at treatment time point 2, 6, 12 and 24 hours. The level of intracellular calcium were elevated after the treatment of alpha-fetoprotein and achieved to 204% at 4min. The results also showed that AFP(20mg.L^{-1}) could upregulate the expression of N-ras oncogenes and p53 and p21^{ras} in Bel 7402 cells. In the later case, the alteration were 81.1%(12h) and 97.3%(12h) respectively compared with control.**

CONCLUSION: **These results demonstrate that AFP is a potential growth factor to promote the proliferation of human hepatoma Bel 7402 cells. Its growth-regulatory effects are mediated by its specific plasma membrane receptors coupled with its transmembrane signaling transduction through the pathway of cAMP-PKA and intracellular calcium to regulate the expression of oncogenes.**

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INTRODUCTION

Alpha-fetoprotein (AFP) is an oncofetal protein normally produced in the fetal liver and yolk sac, whose higher serum level is a useful marker for hepatocellular carcinoma and yolk sac tumors. Although the physicochemical and structural properties of this 70-kDa glycoprotein have been largely documented, its pathophysiological functions were limited in *in vitro* studies. In the last decade, the growth regulatory properties of AFP have aroused interest as a result of studies involving ontogenetic and oncogenic growth in both cell culture and animal models[1-3]. A myriad of studies has now described that AFP is capable of regulating growth in ovarian, placental, uterine, hepatic phagocyte, bone marrow, and lymphatic cells[4] in addition to various neoplastic cells[5]. This suggests that AFP is not merely a fetal form of albumin-like carrier protein and a marker for cancer and fetal disorders, but should rather now be considered as a potential factor associated with the regulation of growth, differentiation, regeneration, apoptosis and transformation in both ontogenetic and oncogenic growth processes. Although it is currently thought that a 62- to 67-kDa membrane protein on the surface of monocytes and phagocytes is specific for AFP binding[6,7], the properties of the binding sites were still unknown in most tumor cell lines. Furthermore, few studies have focused on its intracellular signaling events and gene expression. The goal of this study is to characterize the AFP receptor, its possible signal transduction pathway and its proliferative functions in human hepatoma Bel 7402 cells.

MATERIALS AND METHODS

Reagents

Purified AFP was from Sigma (USA). Monoclonal antibod against AFP (anti-AFP) was prepared in this laboratory and used to block AFP. The cAMP kit and Na[^{125}I] were purchased from Amersham, UK. Fluo-3 AM was from BIORAD (USA). Monoclonal antibodies for p53 and p21 were purchased from MBI (USA).

Purification of human AFP Human AFP was prepared as previously described[8]. Briefly, human cord blood AFP was precipitated by ammonium sulphate and passed through an anti-AFP affinity chromatography column. AFP-positive fractions were collected and concentrated. The purity of prepared AFP was 92.7% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein was stored at -80℃ until use.

Effect of AFP on the cell proliferation Total 1.5×10^4 cells per well of Bel 7402 cells were plated into 96-well plates and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37℃ in a humidified atmosphere of 5% CO_2 for 48h. The cultures were replaced with medium without FCS for another 24h, and treated with different concentration of AFP (1-80mg.L^{-1}) for 48h. The effects of AFP on the proliferation of cells were measured by MTT assay and ^{3}H-Thymidine incorporation, which were performed following a regular procedure.

Effect of AFP on the cell cycle First, 3×10^4 cells per well of Bel 7402 cells were plated into 6-well plates. Culture and AFP treatment were then performed as described above. After being treated

for 24h, the cells were digested with 0.25% trypsin/0.02% EDTA and washed three times with PBS. A final density of 1×10^6 cells in 1ml was added 20μl (10mg.mL^{-1}) RNase (Promega USA) solution and incubated at 37℃ for 30min. The effects of AFP on the cell cycle were detected with flow cytometry.

AFP receptor binding assay Bel-7402 cells were maintained in a humidified atmosphere of 5% CO_2 at 37℃ in RPMI-1640 medium supplemented with 10% FCS. The cells were initially depleted of serum for 12h and then washed with cold medium. Resuspended cells were passed through a 300-mesh screen and adjusted to 1×10^6 cells per ml. ^{125}I-AFP was radioiodinated by the iodogen method and run through a column of Sephadex-G25 to remove free ^{125}I. The specific activity of ^{125}I-AFP was 2715 Ci per mmol and the purity of radioactivity ratio was 99.4%. Each reaction contained 7×10^5 cells, ^{125}I-AFP of 5×10^4 cpm and different concentrations of non-labeled AFP (0.25-64.5ng). The reaction was triplicated and performed at 4℃ for 2h. All samples were collected onto glassfiber membrane (presaturated with 0. 5% albumin) and washed three times with 15ml of PBS. The radioactivity of ^{125}I was detected by a γ-counter. Human serum albumin (HSA) as a non-labeled ligand was utimized for measuring IC_{50}. The parameters of binding were ed using a program of Radioligand Binding Assay of Receptors (RBA).

Extraction and measurement of cAMP The cells were adjusted to 4×10^4 cells per ml and cultured in 24 well plates. After 24h incubation, the cells were collected and resuspended in the medium supplemented with 0.1% egg albumin and 25mmol.L^{-1} of HEPES (pH 7.4) and 2mmol.L^{-1} IBMX (3-methyl-1-isobutyl-xanthine) at 37℃ for 15min. AFP (20mg.L^{-1}) and/or anti-AFP (40mg.L^{-1}) was added into each well respectively for 4h. Extraction of cAMP was performed according to the method described by Iwashia[9]. In short, the supernatant was removed and replaced with 1ml of cold PBS per well. After wash, the pellet was frozen in -80℃ for 30min and then 0.5ml of HCl (0.05N) was added into each well for another 30min. The samples were thawed and spun at 10000g for 5min. The supernatants were lyophilized, and the content of cAMP was measured by the radio immunoassay following the instruction of cAMP assay kit.

Determination of protein kinase A activity Total 4×10^5 cells per well were cultured in 24-well plates for 48h, changed to fresh medium without FCS for another 24h, and then treated with either AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}) or AFP (20mg.L^{-1}) plus anti-AFP (40mg.L^{-1}) respectively. After 2, 6, 12 and 24h treatment, the cells were washed and resuspended in 1ml PBS. The measurement of PKA activity has been described by Plet[10]. Briefly, 40μl of cell extract was mixed with 160μl of the reaction mixture at the final concentration of 20mmol.L^{-1} Tris-HCl (pH 7.5), 5mmol.L^{-1} $MgCl_2$, 0.25g.L^{-1} BSA, 0.5g.L^{-1} histone, 2×10^{-7}mol.L^{-1} ATP (γ-^{32}P ATP, 3.7×10^4 Bq) and 8.0μmol.L^{-1} cAMP at 37℃ for 10min. Followed by incubation on ice for 5min, the reaction mixture was filtered through Whatmen GF/C filter, washed with 10% TCA-2% phosphoric acid and 5% TCA for 30min. The radioactivities were measured by a liquid scintillation counter, and PKA activity was expressed as pmol value of ^{32}P in histone catalyzed by per mg protein per min.

Determination of intracellular calcium concentration The cell suspension was dispensed into specific culture plates at a density of 2×10^4 cells per ml and incubated at 37℃ in a humidified atmosphere of 5% CO_2 for 48h. The supernatant was removed and replaced with medium without FCS for 6h, followed by washing three times with Hank's solution. The measurement of intracellular calcium concentration has been described by Tsugorka and Petti *et al*[11,12]. Briefly, the cells were loaded with 10ml of Fluo-3AM in Hank's solution at a final concentration of 5μmol.L^{-1} and incubated at 37℃ for 30min. After washing 3 times with Hank's solution, either AFP (20mg.L^{-1}) or anti-AFP (40mg.L^{-1}) was loaded into each well. The change of intracellular free calcium ($[Ca^{2+}]_i$) was monitored by scanning fluorescence intensity under TCS-NT confocal microscopy every 10s.

RNA isolation and Northern blotting Cells were treated with either AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}) or AFP (20mg.L^{-1}) plus anti-AFP(40mg.L^{-1}) for 24h. Total cellular RNA was isolated from cell lines with TRIzol reagent(Promege, Madison,WI, U.S.A) according to the manufacturer's protocol. RNA (10-20μg/lane),quantitated by absorbance at 260nm, and fractionated by eletrophoresis through a 1% formaldehyde agarose gel, and the fractionated RNA was transferred(in 20×SSC) to nitrocellulose membranes(Millipore corporation Bedford, MA; U.S.A), by standard procedure[13] These membranes were hybridized with a ^{32}P labeled probe and washed using standard protocol. The membranes were then exposed to X-ray film at -70℃ for varying periods of time.

Western blot analysis Cells were treated with either AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}) or AFP (20mg.L^{-1}) plus anti-AFP(40mg.L^{-1}) for 24h. After three times wash, the cells in each reaction were lysed in 10μl of lysis buffer containing 0.2% Triton X-100, 500mmol.L^{-1} NaCl, 500mmol.L^{-1} sucrose, 1mmol.L^{-1} EDTA, 0.15mmol.L^{-1} spermine, 0.5mmol.L^{-1} spermidine, 10mmol.L^{-1} HEPES (pH 8.0), 200μmol.L^{-1} phenylmethylsulfonyl fluoride, 2mg leupeptin.L^{-1}, 2mg pepstatin.L^{-1}, 24 IU aprotinin.ml^{-1} and 7mmol.L^{-1} γ-mercaptoethanol. 40μg proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane for immunodetection. SDS- PAGE molecular weight markers (Bio-Rad) verified the correct location of the visualized bands. The membranes were blocked in 5% nonfat milk (w/v) in PBS-Tween, then probed with anti-p53 or anti-p21 and followed by secondary antibodies (goat anti-mouse Ig-alkaline phosphatase). Immunoreactive proteins were detected using color development system (NBT/BCIP).

Statistical analysis Data were analyzed by t test and expressed as mean±SD based on 3 or 4 independent experiments.

RESULTS

Effect of AFP on the cell proliferation

Pretreating Bel 7402 cells with AFP (1-80mg·L^{-1}) resulted in a dose-dependent increase in cell proliferation (Figure 1). The increase was about 2-fold at a dose of 80mg.L^{-1}as compared to the control (0mg.L^{-1}). The effects of AFP (20mg.L^{-1}) on cell proliferation could be blocked by anti-AFP (40mg.L^{-1}) which was observed both in MTT assay and ^{3}H-Thymidine incorporation (Figure 2). The increase was not observed in the HSA-treated group and non-treatment control. Flow cytometric analysis showed that AFP (20mg.L^{-1}) pretreatment increased the S phase cell population by 59.3% of Bel 7402 cells (Table 1).

Table 1 The effects of AFP on the cell cycle progression. Bel 7402 cells maintained in RPMI 1640 medium were respectively treated with either AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}), AFP (20mg.L^{-1}) + anti-AFP (40mg.L^{-1}) or HSA (20mg.L^{-1}) for 24 hours. The effects of AFP on the cell cycle progression were analyzed by flow cytometry. The data represented the mean values of four independent experiments performed each in triplicate

Groups	G_1 (%)	S (%)	G_2 +M (%)
Conrtol	37.0±3.0	42.7±2.8	19.2±1.8
AFP	20.3±1.6[a]	68.0±4.2[a]	12.7±1.3[a]
AFP+anti-AFP	32.0±2.1	48.8±2.51	9.2±1.3
anti-AFP	34.61±1.9	45.8±2.5	19.6±1.9
HSA	36.9±4.3	43.2±2.6	19.9±2.4

[a]$P<0.05$ *vs* control group

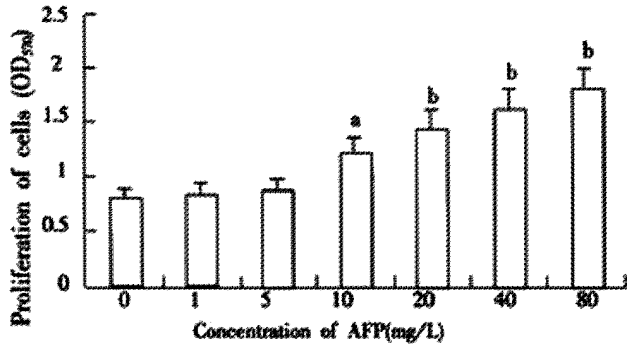


Figure 1 The effects of different concentration of AFP on the proliferation of cells. Bel 7402 cells were incubated with different concentrations of AFP for 48h and the cell proliferation was measured by MTT assay. The data represented the mean values of six independent experiments performed each in triplicate. $^{a}P<0.05$ and $^{b}P<0.01$ *vs* control (0mol.L^{-1}).

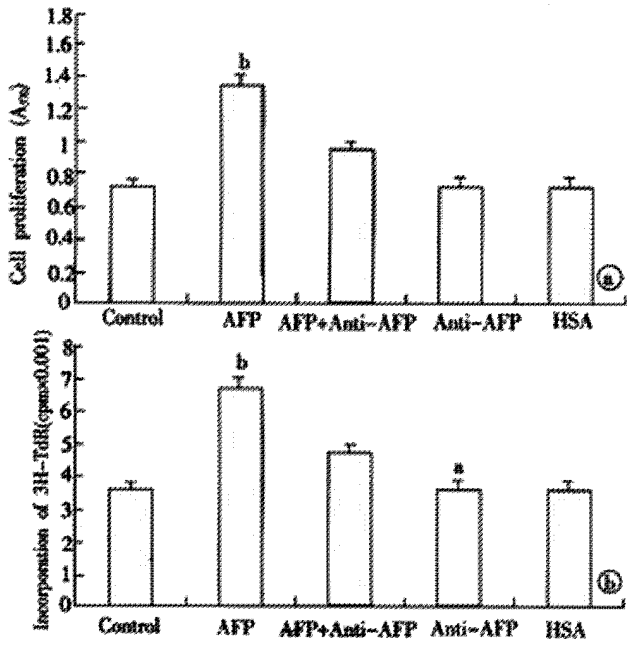


Figure 2 The blockage of anti-AFP to the effect of AFP on the proliferation of cells. A. The data of MTT assay. B. The data of ^{3}H-TdR incorporation. The cells were respectively treated with either AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}), AFP (20mg.L^{-1})+ anti-AFP (40mg.L^{-1}) or HSA (20mg.L^{-1}) for 48h. (MTT assay) or 18h (^{3}H-TdR incorporation). The data represented as the mean value of four independent experiments performed each in triplicate. $^{a}P<0.05$ and $^{b}P<0.01$ *vs* control (0mol.L^{-1}).

Distribution of AFP receptor on the membranes of Bel 7402 cells

The binding sites of AFP on the surface of the cells and Kd values were calculated based on Scatchard plot analysis of ^{125}I-AFP. Scatchard analysis showed that there were two classes of receptors with different affinities on Bel 7402 cells. As for Bel 7402 cells, K_D1 with 89400 sites per cell was 1.3×10^{-9}mol.L^{-1} and K_D2 with 582000 sites per cell was 9.9×10^{-8}mol.L^{-1}(Figure 3). To indicate a higher affinity of the binding sites for AFP, IC_{50} was calculated to achieve 50% inhibition. More than two fold of HSA were needed compared with AFP (data not shown), which indicated a higher affinity for the binding sites on the surface of cells to AFP.

Effect of AFP on intracellular camp

AFP markedly elevated the concentration of cAMP up to 625% in Bel 7402 cells (Figure 4). Anti-AFP could not alter the concentrations of cAMP when added alone, but it reversed the effect of AFP. As a control, HSA did not influence the content of cAMP.

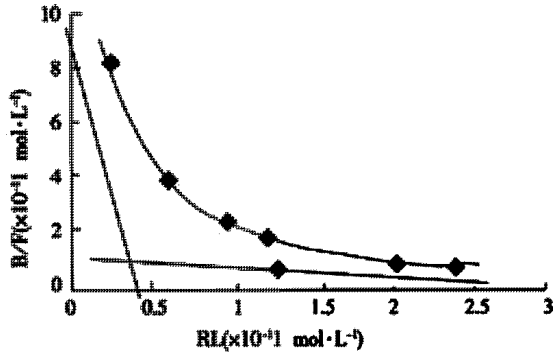


Figure 3 Scatchard analysis of ^{125}I-AFP binding to Bel 7402 cells. The properties of AFP receptor in Bel 7402 cells was detected with receptor binding assay and analyzed by a program of Radioligand Binding Assay of Receptor. The data were selected from three independent experiments.

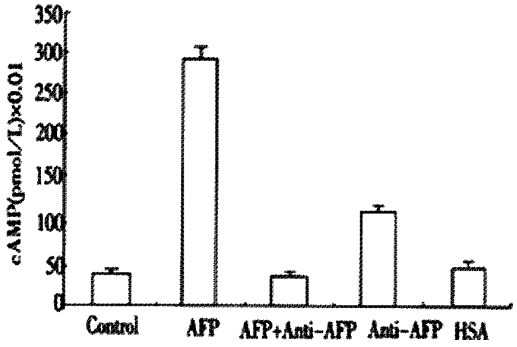


Figure 4 The effects of AFP on the cAMP concentration in cytosol of human hepatoma Bel 7402 cells. 4×10^{4} cells were respectively treated with AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}), AFP (20mg.L^{-1})+ anti-AFP (40mg.L^{-1}) or HSA (20mg.L^{-1}). The data represented the mean values of four independent experiments performed each in triplicate. $^{b}P<0.01$ *vs* control (0mol.L^{-1}).

Effect of AFP on PKA activity

The activities of PKA in the cytosol of Bel 7402 cells were obviously elevated after being treated with AFP (20mg.L^{-1}) for 2, 6, 12 or 24h (Figure 5). The activities of PKA were increased up to 37.5, 122.6, 73.7 and 61.2% in Bel 7402 cells at each time point. The peak value was achieved at 6h and then declined gradually, but still maintained a higher activity for several hours. Anti-AFP or HSA alone did not affect the activity of PKA in Bel 7402 cells, but anti-AFP could block the effects of AFP on the activity of PKA.

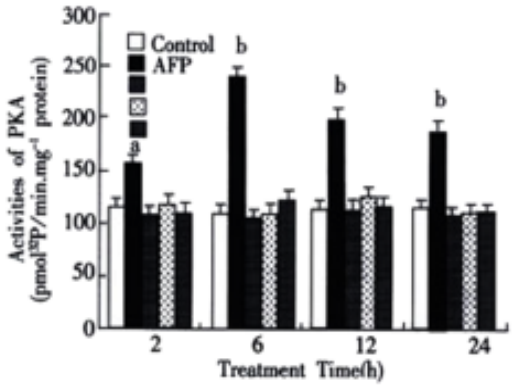


Figure 5 The effects of AFP on the activity of PKA in Bel 7402 cells. 4×10^{5} cells per ml were respectively treated with either AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}), AFP (20mg.L^{-1})+ anti-AFP (40mg.L^{-1}) or HAS (20mg.L^{-1}) for different time and the activities of intracellular PKA were then detected. The data represented the mean values of four independent experiments performed each in triplicate. $^{a}P<0.05$ and $^{b}P<0.01$ *vs* control (0mol.L^{-1}).

Determination of intracellular[Ca^{2+}]i release

Figures 6A1-5 and figure 6B showed that AFP increased the intracellular [Ca^{2+}]i after treatment of 2, 4, 6, 8 and 10 min in Bel 7402 cells. The peak was achieved at treatment time 4 min (increment 204.1% in Bel 7402 cells). Anti-AFP and HSA did not change the content of [Ca^{2+}]i in either cell type. However, anti-AFP could reverse the effect of AFP.

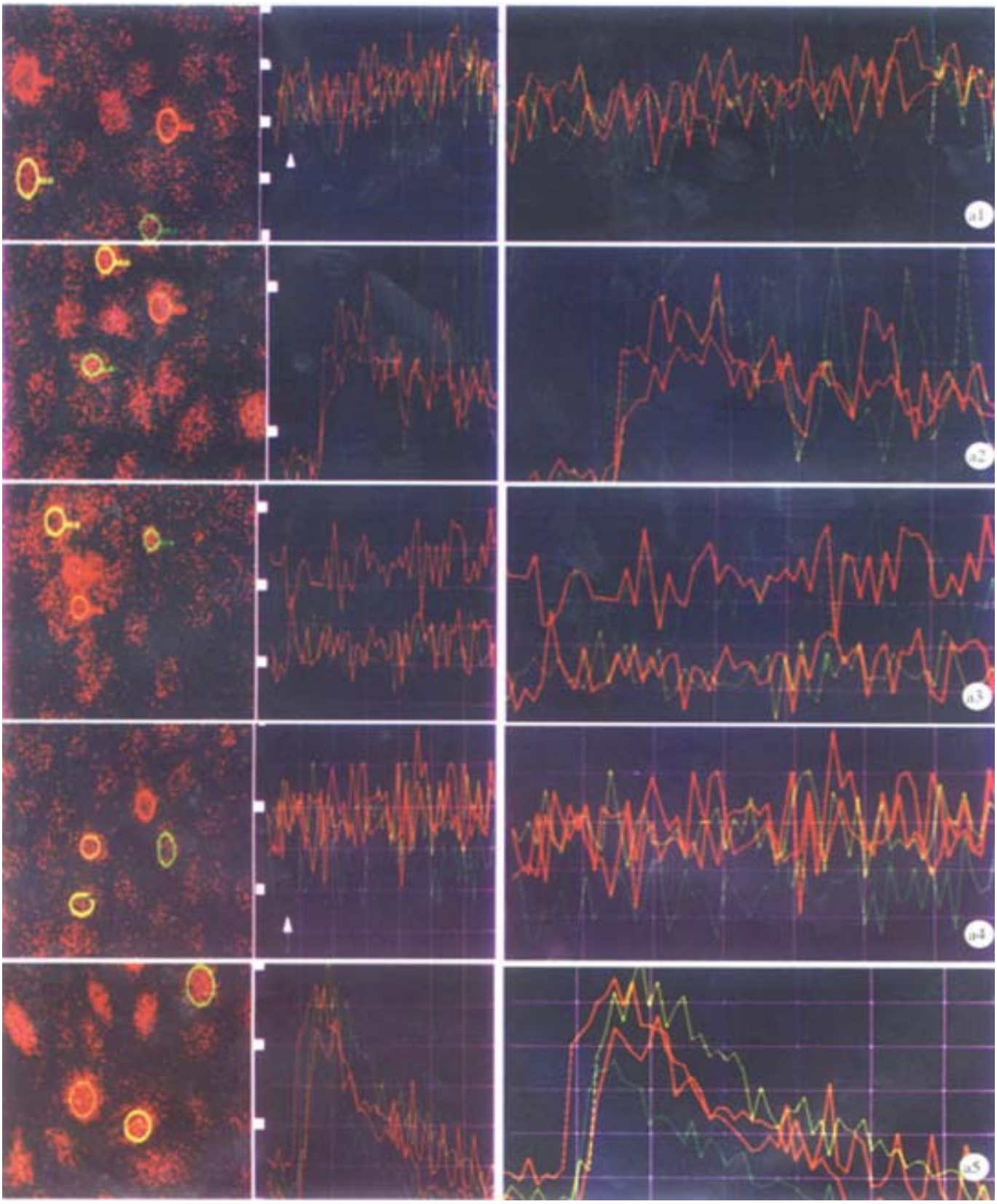
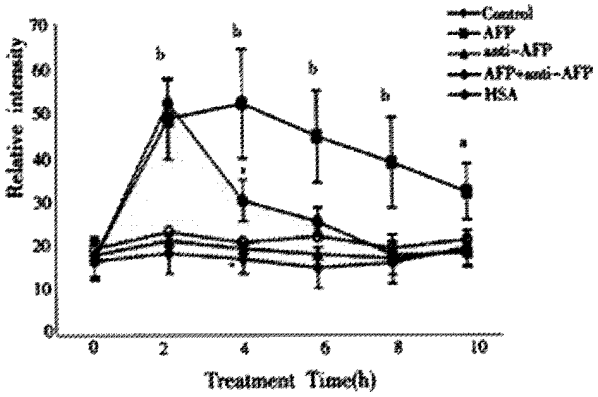


Figure 6A The change of Ca^{2+} concentration in human hepatoma Bel7402 cells was measured by confocal microscopic scanning. Cells were incubated with 5μmol.L^{-1} fluo-3/AM at 37℃ for 30min and then stimulated with Hank's. (1) AFP(20mg.L^{-1}); (2) HSA(20mg.L^{-1}); (3) Anti-AFP; (40mg.L^{-1}); (4) or AFP(20mg.L^{-1}) + Anti-AFP(40mg.L^{-1}); (5) The arrow indicate the stimulated time point.



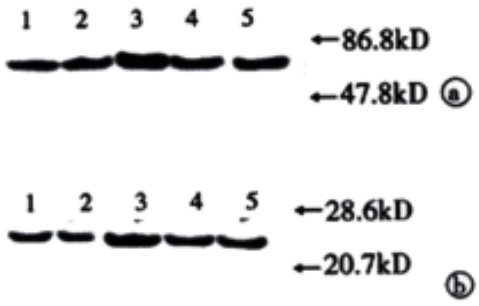


Figure 6B The graph shows the scanning results. The data represented as the mean value of 10 cells ±s. $^aP<0.05$ and $^bP<0.01$ *vs* control (0mol.L^{-1})

Expression of p53 and p21 proteins

The results in Figure 8 demonstrated the overexpression of mutant p53 and p21 protein in AFP-treated group in Bel 7402 cells. Anti-AFP could reverse the upregulated effects of AFP on the expression of p53 and p21 genes. HSA could not influence the amount of these proteins. Each graph was selected from 3 similar results.

Expression of N-ras mRNA

The results in Figure 7 demonstrated the overexpression of N-ras mRNA in AFP-treated group in the Bel 7402 cells. Anti-AFP could reverse the upregulated effects of AFP on the expression of N-ras mRNA. HSA could not influence the mRNA amount of the oncogene. Each graph was selected from 3 similar results.

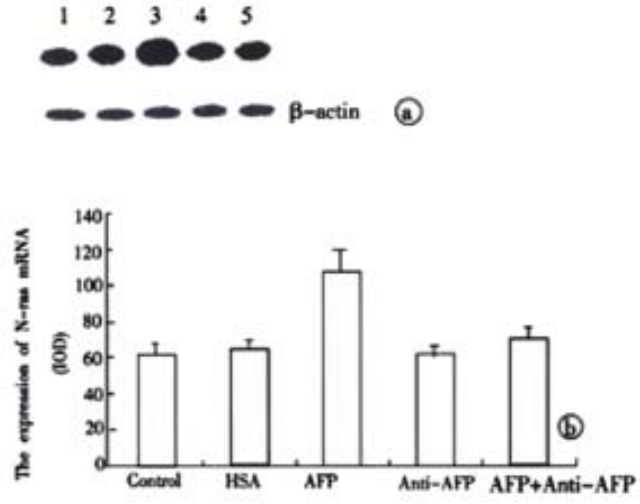


Figure 7 The effects of AFP on the expression of N-ras mRNA in Bel 7402 cells. 1×10^5 cells were respectively treated with AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}), AFP (20mg.L^{-1}) + anti-AFP (40mg.L^{-1}) or HSA (20mg.L^{-1}) for 12 hours and expression of N-ras mRNA was detected by Northern blot assay. Lane 1: control group; Lane 2: HSA treated group; Lane 3: AFP treated group; Lane 4: anti-AFP treated group; Lane 5: AFP plus anti-AFP treated group. The data was selected from 3 independent experiments. A: Autoradiograph of Northern blot. B: Quantitated by densitometric scanning of N-ras mRNA expression blot in Bel7402 cells (relative IOD units). The columns represent the means of triplicate determinations ±s.

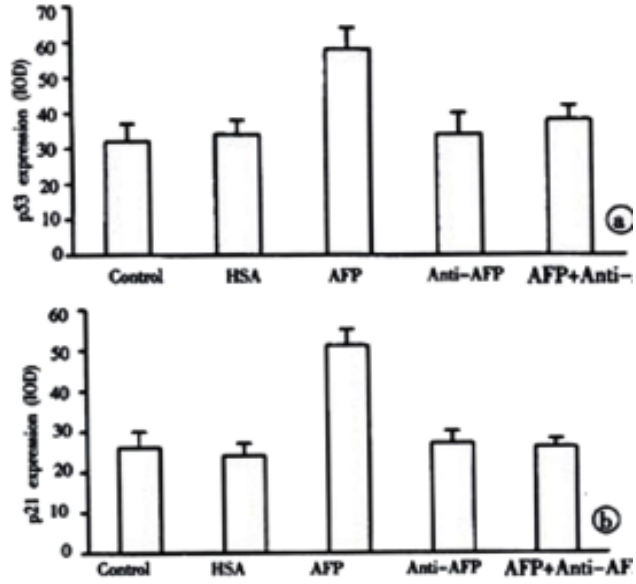


Figure 8 The effects of AFP on the expression of p53 (A) and p21 (B) proteins in Bel 7402 cells. 1×10^5 cells were respectively treated with AFP (20mg.L^{-1}), anti-AFP (40mg.L^{-1}), AFP (20mg.L^{-1}) + anti-AFP (40mg.L^{-1}) or HSA (20mg.L^{-1}) for 24 hours and the expression of p53 and p21protein were detected by Western blot assay. Lane 1: control group; lane 2: HSA treated group; lane 3: AFP treated group; lane 4: anti-AFP treated group; lane 5: AFP plus anti-AFP treated group. The data was selected from 3 independent experiments. A:p53; B:p21. The columns represent the means of triplicate determinations ±s.

DISCUSSION

AFP is an onco-developmental gene product. In the adult, AFP is highly expressed during liver regeneration and hepatocarcinogenesis and used as a maker for the diagnosis of hepatocellular carcinoma[14-16]. The regulation and activation on the expression of the AFP gene have been extensively investigated[17-21]. Although less data indicated AFP could causes apoptosis in tumor cells[22,23], the data from most current research demostrate that AFP is enhancer in tumor growth. The downregulation of expression of alpha-fetoprotein is able to induce the suppression of growth of malignant hepatocyte cell[24-26]. Although the biological role of AFP in cell growth has been reported[27-29], the properties of the AFP receptor as well as the subsequent events after AFP binding were still undefined. Our data indicate that a specific AFP receptor does exist in a human tumor cell line, Bel 7402 cells. There were two kinds of receptors with different affinities in Bel 7402 cells (K_D:10^{-9} and 10^{-8}mol.L^{-1}), which was consistent with similar experiments that characterized the K_D of binding protein in monocytes in the range of 10^{-11}-10^{-7}mol.L^{-1}[6,30]. The AFP-binding protein possibly containing the AFP-receptor has been isolated from human

embryos and human breast cancer tissue[31].

Based on the results of MTT assay, ^{3}H-thymidine incorporation and flow cytometry analysis, as well as the enhanced expression of mutant p53 and p21 and expression of protooncogenes N-ras mRNA, AFP appears to be a potential growth promoting factor.

Since the albumin and AFP genes are similar in structure, they are believed to be derived from a common ancestral gene, even in the same albuminoid gene family. In all our AFP studies, none of the results showed that human serum albumin (HSA) as a control was able to alter the parameters of cell proliferation although it can non-specifically bind to the cell surface.

Little information on the effect of AFP on signal transduction was available. The present experiments demonstrated that intracellular cAMP was significantly elevated 7 fold in Bel 7402 cells. PKA activities were also increased. This indicates that a cAMP-dependent protein kinase pathway is involved in the effects of AFP on the tumor cells, even though some data from other laboratory indicated that the alteration of activity of PKC affected only liver gene expression rather than cell growth in fetal hepatocytes[32]. Other experiments for the relationship between AFP and message has been tested[33].

In addition, the results of intracellular calcium showed that AFP markedly increased intracellular [Ca^{2+}]i. It has been reported that Bcl-2 suppresses apoptosis by inhibiting calcium activation of the permeability transition of mitochondria[34] and the inhibition of calcium influx was related to the suppression of lymphoma cell-line proliferation[35]. Furthermore, a reciprocal regulation between calcium signaling and hypertrophic growth has been identified[36]. According to these findings, a higher intracellular[Ca^{2+}]i elicited by AFP may play a role in tumorigenesis.

Although growing evidence has confirmed the effects of AFP on the growth of tumor cells, little work has focused on the subsequent events in the nucleus. The impacts of overexpression of protooncogenes N-ras, mutant p53, p21 and other genes on tumor growth have been largely documented[37-41]. In the present experiment, mutant p53 and p21 protein were over produced under the treatment of AFP, which was consistent with similar work[42,43]. It suggested that the mechanism by which elevated levels of mutant p53 and p21 proteins might be involved in AFP-induced oncogenesis.

The pattern inducing the hepatocarcinogenesis is multimode[44,45], but the effect of AFP on the growth of tumor has been confirmed. Based on our experiments, the functional mechanism of AFP on the growth of tumors may be attributed, at least in part, to receptor-mediated cAMP pathway and/or calcium signaling resulting in overexpression of certain genes. The clarification of the mechanism will provide a possibility for the gene therapy of liver tumor[46-48]. Further investigations on the function of AFP may shed further light on the mechanism of AFP action.

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