Expression of alpha fetoprotein messenger RNA in BEL-7404 human hepatoma cells and effect of L-4-oxalysine on the expression *

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Subject headings  oxalysine; liver neoplasms; fetoprotein; tumor cell, cultured; RNA, messenger; gene expression; in situ hybridization; immunohistochemistry

Abstract

AIM To investigate alpha-fetoprotein (AFP) mRNA expression in BEL-7404 human hepatoma cells and the effect of L-4-oxalysine (OXL) on the expression.

METHODS Bel-7404 human hepatoma cells were maintained in RPMI 1640 media. Human AFP cDNA probe was labelled with digoxigenin-11-dUTP by the random primer labelling method. The expression of AFP mRNA in Bel-7404 cells was determined by an in situ hybridization technique with digoxigenin-labelled human AFP cDNA probe. The positive intensities of AFP mRNA in cells were analyzed by microspectrophotometer and expressed as absorbance at 470nm. For the experiment with OXL, cells were incubated with various concentrations of the agent for 72h.

RESULTS Essentially all the hepatoma cells contained AFP mRNA in the cytoplasm, although in various amounts. The specificity of the hybridization reaction was confirmed by control experiments in which the use of Rnase-treated BEL-7404 cells, non-AFP producing cells (HL-60 human leukemia cells) or a nonspecific cDNA probe resulted in negative hybridization. When the cells were treated with OXL (25, 50mg/L), the content of AFP mRNA in the cytoplasm was decreased with the inhibition percentages of 34.3% and 70.1%, respectively (P<0.05).

CONCLUSION AFP mRNA was expressed in BEL-7404 human hepatoma cells and OXL suppressed AFP mRNA expression in the cells.

INTRODUCTION

Serum alpha-fetoprotein (AFP) has been widely detected as a marker for primary hepatocellular carcinoma (PHC). However, the relationship between AFP and PHC is still unclear. We found recently that AFP directly stimulated the growth of mouse ascites hepatoma-22 cells and inhibited the immune responses[1-4]. It is suggested that AFP contributes the generation and development of PHC[5-7]. L-4-oxalysine (OXL) is a natural product isolated from a new species of Streptomyces roseo viridofuscus n. sp. in China. Previous studies indicated that OXL exhibited marked antiproliferative activity against several animal tumors. The antitumoral influence of OXL was also detected in mice bearing Lewis lung carcinoma[8-10]. OXL also exhibited immunoregulatory activity[11]. Preliminary clinical studies suggested that oral treatment with OXL induced an improvement in the symptoms of PHC patients, and no serious side effects were observed[12]. Recently, our laboratory also found that OXL antagonized the biological activities of AFP[13]. AFP content in human BEL-7404 hepatoma cells and cultured media was obviously decreased after the OXL treatment[14]. It is inferred that OXL has anti-AFP activities. In this report, an in situ- hybridization (ISH) technique was used to study the level of AFP mRNA expression in BEL-7404 human hepatoma cells. The effect of OXL on AFP mRNA expression was also observed.

MATERIALS AND METHODS

Cell culture
A human hepatoma cell line, BEL-7404, was maintained in RPMI 1640 media (Gibco)
supplemented with 10% calf serum, 100kU/L of penicillin and 100mg/L of streptomycin, at 37°C, 5% CO2 and 100% humidity. The RPMI 1640 media was replaced with fresh media every three to four days. For in vitro experiment with OXL, cells at a density of 5×10⁶ cells/L were grown on circular coverslip in each well of 24-well culture plate. Twenty-four hours later, various concentrations of OXL (Department of Antibiotics of this Institute) were added, and cells were again incubated for 72h. Control group contained cells alone. After incubation, adhesive cells were directly used for ISH assay.

**Labelling of probes**

The probes used in this experiment are shown in Table 1. Recombinant plasmids pHAF-2 containing human AFP cDNA and phalb-7 containing human serum albumin (HSA) cDNA were kind gifts of Drs Yoshitake Hayashi and Kyoskuke Ohta at University of Kobe, Japan. Plasmids were grown in bulk in Escherichia coli - HB101, extracted by the alkaline procedure, purified by phenol and two ethanol precipitations. Plasmids were digested with restrictive enzymes (Promega). The digests were then electrophoresed in 1% preparative agarose gels to separate the purified inserted gene sequences from the residual linearized plasmid band.

| Table 1  Probes used in this experiments |
|-----------------|-------------|-------------|---------------|-----------------|
| Plasmids       | Carrier     | Gene        | Inserted      | Restrictive     | Resistance     |
| pHAF-2         | pBR322      | AFP         | 900bp         | Pst I+Hap II   | Tetracycline   |
| phalb-7        | pBR322      | Albumin     | 727bp         | Pst I+Hind III | Tetracycline   |

A 900bp Pst I-Hap II fragment from the plasmid pHAF-2 and a 727bp Pst I-Hind III fragment from the plasmid phalb-7 were labelled with digoxigenin (Dig)11-dUTP by random primer labelling method with Dig DNA labelling kit (Boehringer Mannheim Company) and used as AFP and albumin probes, respectively. Briefly, the cDNA was denatured by heating for 5min at 100°C and then quickly chilled on ice. The following reagents were added to an Eppendorf tube on ice: 4 µl freshly denatured cDNA (1 µg), 2 µl hexanucleotide mixture, 2 µl dNTP labelling mixture, 11 µl sterile water and 1 µl Klenow enzyme, mixed and incubated at 37°C for at least 1h (usually incubated 2h-3h). Ten µl of 2g/L yeast tRNA (Sigma) was added and the probes were precipitated with the addition of 4 µl of 3mol/L NaAc (pH 5.2) and 3 volumes of 95% prechilled ethanol (-20°C) at -70°C for at least 30min. The supernatant was discarded by centrifugation and the probes were stored in 50 µl of TE (10mmol/L Tris HCl, 1mmol/L EDTA, pH 8.0) at -20°C. The yield of labelled probes in this reaction was 250ng (5ng/µl). To test the sensitivity of each probe, dot-blot hybridization was carried out with Dig-labelled cDNA probe. The size of the probes was 50 to 250bp as estimated by polyacrylamide gel electrophoresis.

**In situ hybridization (ISH)**

ISH was done essentially according to the procedure of Breborowicz et al[19] with some modifications. Briefly, adhesive cells were fixed in 4% paraformaldehyde for 5min-8min at room temperature. The coverslips were serially washed with the following solutions at room temperature: 0.05mol/L Tris-buffered saline (TBS, pH 7.2) three times, 5min each; 100mmol/L glycine once, 15min; TBS three times, 5min each; 0.4% Triton X100 in TBS once, 15min; and TBS three times, 5min each. The coverslips were then treated with 1mg/L of proteinase K (Sigma) in 20mmol/L Tris-HCl (pH 7.4) and 2mmol/L CaCl2 for 15min at 37°C, washed with TBS three times for 5min each, air dried, postfixed in 4% paraformaldehyde for 5min at room temperature, and washed with TBS three times for 5min each. The coverslips were finally washed with 2×SSC (1×SSC: 150mmol/L NaCl, 15mmol/L sodium citrate) or treated with RNase (Sigma,100mg/L in 2×SSC) for 30min at 37°C.

The hybridization mixture contained 50% deionized formamide, 5×SSC, 10% dextran sulfate (Sigma), 5×Denhardt’s solution, 2% sodium dodecyl sulfate, 100mg/L of salmon sperm DNA (Sigma) denatured at 100°C and 25mg/L-50mg/L of Dig-labelled probe denatured at 100°C. 100×Denhardt’s solution contained 2% Ficoll 400 (Sigma), 2% polyvinylpyrrolidone (Sigma) and 2% bovine serum albumin (BSA, Sigma). Two hundred µl of the hybridization mixture was added to each well of 24-well plate. Cells were incubated in a humidified atmosphere for 18h at 37°C. The coverslips were washed at 37°C in 4×SSC three times for 5min each, and then sequentially immered in 2×SSC, 1×SSC, 0.5×SSC and 0.1×SSC at 37°C for 30min each. The coverslips were then washed in TBS containing 1% BSA and 0.4% Triton X-100 for 30min at room temperature. Sheep anti-Dig antibody conjugated to alkaline phosphatase (AP, Boehringer Mannheim Company) was diluted 1: 500 with TBS containing 1% BSA and 0.4% Triton X-100, and applied to specimens. Cells were incubated for 2h at 37°C, then washed in buffer I (100mmol/L Tris-HCl, 100mmol/L NaCl, 10mmol/L MgCl2, pH8.0) and II (100mmol/L Tris-HCl, 100mmol/L NaCl, 50mmol/L MgCl2, pH 9.5), respectively, for 10min each at room temperature. Development
reagent contained 33 µl of nitroblue tetrazolium salt (NBT, 75 µg/L in 70% dimethylformamide) and 25 µl of 5-bromo 4 chloro 3 indocyl phosphate (BCIP, X-phosphate, 50 µg/L in dimethylformamide) in 7.5ml of buffer II. Cells were incubated in the color solution at 37°C for up to 4h in the dark. Color development was periodically checked and reaction was stopped by washing the coverslips for 5min in TE at room temperature. The coverslips were rinsed well in distilled water, air-dried, cleared in xylene and mounted with glycerin jelly.

The following controls were performed. Just before the hybridization, cells were incubated with 2×SSC containing RNase 100mg/L for 30min at 37°C, then were processed for ISH as above; Diglabelled AFP cDNA probe was replaced by Diglabelled albumin cDNA probe; Nonhepatoma cells (human leukemia HL-60 cells) were incubated with hybridization buffer containing the Diglabelled AFP cDNA probe. The positive intensities of AFP mRNA in hepatoma cells were analyzed by microspectrophotometer (Leitz MPV-3) and expressed as absorbancy at 470nm (A470).

**Statistical analysis**

The statistical significance of differences was evaluated using analysis of variance (ANOVA).

**RESULTS**

**Detection of AFP mRNA by ISH**

It was demonstrated with dot-blot hybridization that the sensitivity of the Diglabelled probes was 1.0pg. When Diglabelled AFP cDNA probe was used, purple grains were present in the cytoplasm of BEL-7404 human hepatoma cells, with fewer grains seen in cell nuclei. The number of grains in the cytoplasm varied, but essentially all the hepatoma cells, including those undergoing mitotic division, were considered to contain AFP mRNA -(Figure 1).

In order to establish the specificity of hybridization, BEL-7404 cells were incubated with Diglabelled albumin CDNA probe under the same hybridization conditions. No accumulation of grains over the cells was observed (Figure 2). Pretreatment of BEL-7404 cells with RNase abolished the formation of grains. Other cell lines not producing AFP, such as HL-60 human leukemia cells, also gave negative results.

**Influence of OXL on AFP mRNA expression in BEL-7404 human hepatoma cells**

Hepatoma cells were incubated with OXL 25, 50mg/L. Cell viability was greater than 95% using trypan blue exclusion. Seventy-two hours later, the change in AFP mRNA content in hepatoma cells during OXL treatment was determined by ISH. It was found that AFP mRNA content in hepatoma cells was significantly decreased by OXL; the effect of higher concentration was more obvious. It is suggested that OXL inhibits AFP mRNA expression in hepatoma cells (Table 2).

| Treatment | Concentration (mg/L) | Positive intensities of AFP mRNA (A470±S) | Inhibition (%) |
|-----------|----------------------|------------------------------------------|----------------|
| Control   |                      | 0.67±0.08                                |                |
| OXL 25    | 25                   | 0.44±0.06*                               | 34.3           |
| OXL 50    | 50                   | 0.20±0.05b                               | 70.1           |

BEL-7404 human hepatoma cells (5×10⁶ cells/L) were cultivated for 72h in absence or presence of various concentrations of OXL. The results were expressed as absorbance at 470nm (A470). The percentage of inhibition was calculated in the cultures in absence of OXL. *P<0.05; bP<0.01.

**Figure 1** Detection of alpha-fetoprotein (AFP) mRNA in human BEL-7404 hepatoma cells. In A, BEL-7404 cells were hybridized in situ with digoxigenin (Dig)-labelled AFP cDNA probes. Reaction products were visible in almost all cells. **Figure 2** Same as in Figure 1, except that the Diglabelled albumin CDNA was used as probes. Cells were counterstained with hematoxylin. ×400
DISCUSSION
Various amounts of AFP were present in almost all the BEL-7404 hepatoma cells shown by the avidin-biotin-peroxidase complex (ABC) method\(^\text{14}\). We got the similar finding of the mechanisms of anti-tumor action of OXL. Such investigation on the whole, it is strongly inferred that OXL exhibits significant anti-AFP activities, which may be one of the mechanisms of anti-tumor action of OXL. Such findings could also lead to the development of new anti-PHC drugs based on AFP target.

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REFERENCES
1. Wang XW, Xu B. Effect of alpha-fetoprotein on the growth of mouse ascites hepatoma-22 cells in vitro. Acta Oncol Sin, 1995;4(4):229-231
2. Wang XW, Xu B. Influence of alpha-fetoprotein on the growth of tumor cells in vitro. Chin J Cancer Res, 1997;9(2):79-82
3. Wang XW, Xu B. Effect of alpha-fetoprotein (AFP) on splenocyte proliferation of mice bearing ascites hepatoma-22 in vitro. Shanghai J Immunol, 1995;15(6):327-329
4. Wang XW, Xu B. Effect of human alpha-fetoprotein on immunological functions in mice bearing ascites hepatoma-22 in vitro. Shanghai J Immunol, 1997;17(4):224-226
5. Wang XW, Xu B. Effect of some drugs on alpha-fetoprotein and treatment of primary hepatocellular carcinoma. Chin Oncol, 1995;4(12):24-26
6. Wang XW, Xu B. Several new targets of antitumor agents. Acta Pharmacol Sin, 1997;18(4):289-292
7. Wang XW, Xu B. Alpha-fetoprotein, primary hepatocellular carcinoma and anticancer drugs. In: Cao SR, ed. New theory and technology of oncology. Shanghai: Shanghai Science and Technology Education Press, 1997:485-497
8. Wang XW, Xu B. L-4-oxalysine, a new antitumor agent of natural origin. Med Chem Res, 1996;6(4):225-232
9. Wang XW, Xu B. Mechanisms of antitumor action of L-4-oxalysine, a new natural product. Med Chem Res, 1996;6(4):233-247
10. Wang XW, Xu B. Antitumor and immunological activities of oxalysine. Chin J Oncol, 1997;19(2):115-117
11. Wang XW, Xu B. Immunoregulatory activity of L-4-oxalysine: an in vitro study. Meth Find Exp Clin Pharmacol, 1997;19(7):437-442
12. Wang XW, Xu B. L-4-oxalysine: Its antitumor activities and mechanisms of action. Drugs Fut, 1996;21(7):727-731
13. Wang XW, Xu B. Anti-alpha-fetoprotein activity of L-4-oxalysine. Asia Pacific J Pharmacol, 1996;11(2):25-28
14. Wang XW, Xu B. Effect of L-4-oxalysine on alpha-fetoprotein gene expression in human BEL-7404 hepatoma cells. Acta Pharmacol Sin, 1997;17(4):229-231
15. Urano Y, Sakai M, Watanabe K, Tamaki T. Tandem arrangement of the tandem arrangement of the mRNA expression and the expression regulation. Acta Pharmacol Sin, 1996;17(4):224-226
16. Ohtsuka T, Sakai M, Wegmann TG, Tamaki T. Primary structures of human alpha-fetoprotein and its mRNA. Proc Natl Acad Sci USA, 1983;80(8):4604-4608
17. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. New York: Cold Spring Harbor Laboratory Press, 1989:304-316
18. Furuta Y, Shinohara T, Sano K, Meguro M, Nagashima K. In situ hybridization with digoxigenin labelled DNA probes for detection of viral genomes. J Clin Pathol, 1990;43(3):806-809
19. Breborowicz J, Tamaki T. Detection of messenger RNAs of alpha-fetoprotein and albumin in a human hepatoma cell line by in situ hybridization. Cancer Res, 1985;45(4):1730-1736
20. Wang XW, Xu B. Research advances of research of alpha-fetoprotein gene expression and the expression regulation. Acta Oncol Sin, 1996;6(4):281-284