Synergistic effect of bromocriptine and tumor necrosis factor-α on reversing hepatocellular carcinoma multidrug resistance in nude mouse MDR1 model of liver neoplasm

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Abstract

AIM: To investigate the effect of bromocriptine (BCT) and tumor necrosis factor-α (TNF-α) on hepatocellular carcinoma (HCC) multidrug resistance (MDR) in nude mouse MDR model of liver neoplasm.

METHODS: Human hepatocarcinoma cell line HepG2, drug resistant hepatocarcinoma cell line HepG2/adriamycin (ADM) and hepatocarcinoma cell line transfection with TNF-α gene HepG2/ADM/TNF were injected into the liver of nude mice via orthotopic implantation and MDR model of liver neoplasm in vivo was established (HepG2, ADM, TNF, BCT groups). Among these groups, BCT group and TNF group were treated with BCT through gastric canal. Each group was divided into control group and chemotherapy group. Size and weight of the tumor were measured. Furthermore, tumor histological character and growth of the nude mice were observed and their chemosensitivity was tested. MDR-associated genes and proteins (MRP, LRP) of implanted tumors were detected by immunohistochemistry, reverse transcriptase polymerase chain reaction, and apoptosis rate of hepatocarcinoma cells was detected by TUNEL assay.

RESULTS: The nude mouse model of each cell line was inoculated successfully. The tumor growth rate and weight were significantly different among groups. After chemotherapy, abdominal cavity tumor growth inhibition rate was higher in BCT group (67%) compared to ADM and TNF groups, and similar to HepG2 group (54%). MDR1 and LRP mRNA could be detected in all groups, but TNF-α was detected only in TNF and BCT groups. Furthermore, MDR1 and LRP protein expression of tumors in TNF and BCT groups was low similar to HepG2 group. The apoptosis rate of hepatocarcinoma cells was much higher in BCT group than in other groups with TUNEL assay.

CONCLUSION: BCT and TNF-α can reverse HCC MDR in nude mouse MDR1 model of liver neoplasm.

INTRODUCTION

The development of multidrug resistance (MDR) in human tumors may be a major obstacle to successful cancer chemotherapy[9]. MDR is often associated with increased expression of the MDR1 (also known as P-glycoprotein) gene, encoding P-glycoprotein (Pgp)[2]. Since the MDR1 gene appears to represent a major cause of MDR in tumor cells, MDR1 messenger RNA (mRNA) and Pgp may be important targets in MDR reversal strategies. But almost all MDR reverse studies are only performed in vitro[3-5], and few are performed in vivo. A study[6] indicates that overexpression of Pgp, a product of MDR1, is intimately associated with liver cancer development and therefore inhibitors of Pgp should inhibit the development of liver cancer. Accordingly, we determined the effect of bromocriptine (BCT), a potent inhibitor of Pgp[7,8], and tumor necrosis factor-α (TNF-α)[9-11] which could downregulate expression of the MDR-associated genes LRP and MRP in multidrug resistant model of nude mice via orthotopic implantation of multidrug resistant human hepatocellular carcinoma (HCC) cells HepG2/adriamycin (ADM).

TNF is a cytokine playing an important role in immunoregulation and antitumor mechanisms, and has been extensively tested in in vitro and in vivo investigations as well as in clinical trials for immunotherapy of malignant diseases[12,13]. More recently, it has been demonstrated that TNF can modulate...
MDR1 expression and enhance cytotoxicity of certain MDR-related drugs to various cell lines. The ability of cytokines to influence MDR phenotype is strongly supported by a previous study.

BCT, as a D1 dopaminergic receptor agonist, has been reported to inhibit activity of ATPase and function of Pgp. Moreover, because of its slight side-effects and the MDR-reversing effect, we chose BCT and TNF-α to detect their synergistic antitumor effects.

MATERIALS AND METHODS

Reagents

1640 medium, fetal bovine serum (FBS), lipofectamine 2000, and G418 were purchased from Invitrogen Inc., USA. Upstream and downstream primers were produced by Shanghai Boya Biosynthesis Co., Shanghai, China. dNTPs, RNasin, AMV reverse transcriptase, Taq and DNA enzymes were purchased from Sigma Company, USA. TRIzol reagent was purchased from Gibco Company, USA. BCT mesilate was purchased from Novartis, Italy; 5-fluorouracil (5-FU), ADM, mitomycin (MMC) were from Pharmacia & Upjohn, Italy; and S-P immune test kit was from Beijing Zhongshan Company, Beijing, China. LRP mouse antibody, P170 mouse antibody and Pan actin antibody-5 were purchased from Neomarker Inc., Germany.

Cell lines and cell culture

Human HCC cell line, HepG2 (GDC024), was purchased from the China Center for Type Culture Collection (Wuhan University, Hubei). HepG2 was induced to form a multidrug resistant cell line (HepG2/ADM) by exposure to gradually increased concentration of ADM. Firstly, eukaryotic expression vector pBK-TNF-α was constructed by recombinant DNA technique, and transfected into multidrug resistant cell line (HepG2/ADM) to obtain cell line HepG2/ADM/TNF and cultured in the presence of 800 mg/mL G418. All cells were maintained in 1640 medium containing 10% heat-inactivated FBS at 37 °C in a humid atmosphere of 50 mL/L CO₂.

Multidrug resistant model establishment and grouping

BALB/c, nu/nu, nude mice, male and female from Centers for Disease Control and Prevention of Hubei province, age ranging 4-5 wk, weighing 12.1-15.0 g, bred in specific pathogen-free condition, were used in this study. Firstly, a left subcostal incision was made under anesthesia with 20% urethane, then left lobe of the liver was exposed, and each kind of cells was injected into mice liver and an orthotropic MDR1 hepatoma was obtained successfully (HepG2, ADM, TNF, BCT groups respectively). Each group contained 16 nude mice inoculated with each kind of cell lines, and was divided into control group and experiment group, 8 in each group. Chemotherapy was performed when tumor diameter was 1.5 cm. Among these groups, BCT group and TNF group were treated with BCT (0.25 mg/kg/d) through gastric canal. Association of 5-FU (0.15 g/kg/d) + MMC (1.5 mg/kg/d) + ADM (10 mg/kg/d) were administered through intraperitoneal injection in experiment groups, but 0.9% NS 15 μL/g was given to control groups for 3 d. Nude mice were weighed and the size of tumors was measured every week by B-ultrasound after orthotopic implantation, when the mice were killed 14 d after chemotherapy. Furthermore, some specimens were fixed in 40 g/L formaldehyde and embedded with paraffin for immunohistochemistry (IHC), and other markers were detected by molecular biological techniques.

Evaluation of growth and sensitivity of chemotherapy

The living condition, diet and reaction with stimuli from environment were observed everyday from the first day of chemotherapy. The size of tumors was measured every week by B-ultrasound after chemotherapy using the formula \( V = a \times b^2 / 2 \) (\( V \): volume; \( a \): long diameter; \( b \): short diameter). Meanwhile xenograft tumors were observed and weighed until the mice were killed. Lastly, the inhibition rate of volume and weight was calculated by the formula (inhibition rate of volume or weight = [1-volume or weight of chemotherapy group/volume or weight of control group]×100%).

HE staining and immunohistochemistry (IHC) for detection of MDR1 expression

Firstly, each kind of orthotopic tumor tissues was stained with HE, and fixed in formalin and embedded in paraffin. The expression of Pgp protein was studied using mouse anti-human MDR1 C219 and sections of 5 μm from the tumor tissues were implanted in each kind of cell lines and MDR1 expression was assayed by immunoperoxidase staining with the SP method (SP kit, Zhongshan Biocompany). IHC process was strictly done as the indication of SP kit. In summary, endogenous peroxidase was blocked with H₂O₂ and non-specific antigens were blocked with normal serum. The primary antibodies were diluted to 1:100 for Pgp, then incubated at 4 °C overnight, washed thrice with PBS. The secondary antibody (1:500) was incubated for 15 min at 37 °C, sections were stained with diaminobenzidine tetrahydrochloride (DAB) and restained with hematoxylin for visualization of nuclei. For negative controls, primary antibodies were substituted by PBS instead of Pgp antibodies. The Pgp protein expression intensity was observed through ocular lens and the positive cells per square millimeter were calculated and expressed as mean±SD.

Expressions of TNF mRNA, MDR1 mRNA and LRP mRNA

Total RNA was respectively extracted from tumors in nude mice with TRIzol according to the manufacturer’s instructions. Reverse transcription (RT) was performed with random primers by a complementary DNA (cDNA) synthesis kit (Promega). RT-reaction reagents (25 μL) were added as follows: 0.1 μg OligoDT (100 g/L), 25 U RNasin, 1.25 μL dNTP (10 mmol/L), 5 μL M-MLV 5×buffer, 200 U M-MLVRT. After all the components were mixed, the mixture was incubated at 42 °C for 1 h in a DNA thermal cycler (PTC-100, MJ Research Inc., Watertown, MA, USA), and the reverse transcriptase was inactivated by heating the reaction mixture to 95 °C for 5 min. Then each tube was kept at -20 °C until PCR was performed. The primers of the target genes MDR1, LRP, and the endogenous reference β-actin were designed using the Primer Express Software (Applied Biosystems) (Table 1). The final volume of the reaction (25 μL) of PCR included: 25 μL transcription solution containing 15 pmol upstream and downstream respectively, 2.5 μL 10×buffer, 2 μL cDNA, 0.5 μL dNTP (10 mmol/L),
2 μL MgCl₂ (25 mmol/L), 1 μL Taq polymerase (10×10⁶ U/L), ddH₂O to a final volume of 25 μL. Thirty-five PCR cycles were performed, for each cycle, the sample was denatured at 94 °C for 1 min, annealed at 55 °C for 1 min, extended at 72 °C for 1 min, and finally extended at 72 °C for 10 min.

**TUNEL assay for apoptosis detection**

The paraffin-embedded specimens were cut into 5-μm-thick sections, rehydrated and then incubated with protease K solution for 30 min at room temperature. After being washed twice with PBS, the sections were incubated with TUNEL reaction solution at 37 °C for 60 min. The transforming solution (POD) was added and incubated at 37 °C for 30 min. The sections were stained with DAB for 10 min. Then counterstained with hematoxylin for 10 min, dehydrated in graded alcohol and covered with resin. The criterion of positive staining was that the nuclei were stained pale brown.

**Statistical analysis**

Data were presented as mean±SD, significance of statistical difference was assessed by Student’s t-test and analyzed with SPSS11.0. P<0.05 was considered statistically significant.

**RESULTS**

**TNF-α mRNA expression in orthotopic tumor tissues**

TNF-α mRNA could be amplified only from the tumors of TNF and BCT groups in the orthotopic tumor tissues injected into three kinds of cell lines, and no positive band could be seen from the other two groups. Electrophoresis showed that there was a positive band at 725 bp in the tissues injected into multidrug resistant cells transfected with TNF-α gene (Figure 1) and no band was found, indicating TNF-α mRNA was integrated into the nude mice implanted with multidrug resistant cells transfected with TNF-α gene. Thus, the model of implanted orthotopic tumor was successfully established.

**Evaluation of growth and sensitivity of chemotherapy**

No death occurred. The successful rate of tumor implantation was 100%, and tumor could be seen via B ultrasound after 10 d. The rate of tumor growth in the group transfected with TNF-α was lower compared to other groups (P<0.05). Low appetite and emotion occurred in the mice receiving chemotherapy, especially in BCT group. The tumor volume and weight of BCT group decreased compared to the other group (P<0.01), and tumor growth inhibition rate in HepG₂ group was similar to TNF group (P>0.05), but lower than that in BCT group (P<0.05, Table 2, Figures 2 and 3).

**Table 1 MDR1, LRP primers**

| Gene      | Quantification method | Sequence (5'→3')         | Size (bp) |
|-----------|-----------------------|---------------------------|-----------|
| MDR1      | Forward primer        | CATTGGTGTGGTGAGTCAGG      | 176       |
|           | Reverse primer        | CTCTCTCTCAAACGAGGTG       |           |
| LRP       | Forward primer        | TAAGGCTTCCAGCACCAAC       | 237       |
|           | Reverse primer        | GGAAGTTCGCTCTTCGCC        |           |
| TNF-α     | Forward primer        | GCGGAATGAGCACCAGGAATCC    | 725       |
|           | Reverse primer        | CCCAAGCTTTCACAGGGCAATGACCCAAG |         |
| β-actin   | Forward primer        | GTGGCCGACATTAAGGAG        | 530       |
|           | Reverse primer        | CTAGCTCATAGTCGGCCT         |           |

![Figure 1](image1.png) RT-PCR of TNF mRNA. M: Marker; lanes 1 and 2: TNF group, BCT group; lanes 3 and 4: HepG₂ group, ADM group.

![Figure 2](image2.png) Size of tumor after chemotherapy in groups of HepG₂ (A) and BCT (B).

![Figure 3](image3.png) Growth curve of the tumor of each group time (W).
Histologic characteristics and Pgp protein expression in orthotopic tumor tissues

Histological characteristics of the tumor model in different groups were similar (Figure 4). Pgp protein expression was low. Pgp protein expression was lower in HepG2 group, but high in ADM group, and lower in groups TNF and BCT than in ADM group. There was a significant difference between the groups ($P<0.01$), but no difference among HepG2, BCT and TNF groups ($P>0.05$, Figure 5)

**Table 2** Volume and size of orthotopic tumor tissues (mean±SD)

| Groups   | Mice and incidence of tumor, n (%) | Volume (cm$^3$) | Rate of volume inhibition (%) | Weight (g) | Rate of weight inhibition (%) |
|----------|-----------------------------------|----------------|-----------------------------|------------|-----------------------------|
| HepG2    | Experiment 8 (100)                | 1.25±0.13      | 57                          | 0.72±0.12  | 54                          |
| Control  | 8 (100)                            | 2.92±0.21      |                             | 1.55±0.17  |                             |
| ADM      | Experiment 8 (100)                | 2.03±0.11      | 14                          | 1.22±0.11  | 18                          |
| Control  | 8 (100)                            | 2.37±0.18      |                             | 1.48±0.21  |                             |
| TNF      | Experiment 8 (100)                | 0.67±0.22      | 58$^a$                      | 0.45±0.16  | 57                          |
| Control  | 8 (100)                            | 1.37±0.18      |                             | 0.93±0.18  |                             |
| BCT      | Experiment 8 (100)                | 0.49±0.17$^b$  | 66$^c$                      | 0.29±0.21  | 67                          |
| Control  | 8 (100)                            | 1.45±0.26      |                             | 0.88±0.17  |                             |

$^aP>0.05$ vs HepG2 group, $\chi^2 = 4.32$; $^bP<0.01$ vs ADM group, $t = 2.38$; $^cP<0.05$ vs HepG2 group, $\chi^2 = 2.49$.

**Figure 4** Histological characteristics of tumor in HepG2 group (A) and BCT group (B).

**Figure 5** Pgp protein expression in groups of HepG2 (A), ADM (B), BCT (C), and TNF (D).

Downregulation of MDR1 mRNA expression in orthotopic tumor tissues transfected with TNF-α

Electrophoresis in each group showed 176, 237, and 530 bp bands, which were coincident with the amplification fragments of designed MDR1 mRNA, LRP mRNA and β-actin genes. The relative quantity of MDR1 mRNA in groups of BCT, TNF, HepG2 was 0.21±0.02, 0.15±0.03, 0.13±0.02 respectively. The relative quantity of LRP mRNA in groups BCT, TNF, HepG2 was 0.17±0.04, 0.15±0.01, 0.11±0.01. There was no difference among these groups ($P>0.05$), but the relative quantity of MDR1 mRNA, LRP mRNA was significantly lower in groups of BCT, TNF, and HepG2 than in ADM group ($P<0.05$, Figure 6, Table 3).

Apoptosis of tumor cells

After intra-abdominal chemotherapy with ADM, 5-FU, and mitomycin, the apoptotic rate of tumor cells expressing TNF-α was significantly higher compared to those not expressing TNF-α. The average of apoptotic cells in TNF-α and BCT group was (21.9±2.1) and (30.5±4.2) per field of high-power microscope respectively. There was a difference between the groups ($P<0.05$). TNF-α could reduce the tolerance of hepatoma cells to chemotherapy and increase apoptosis of tumor cells. BCT and TNF-α could induce apoptosis of HCC (Figure 7, Table 4).

DISCUSSION

Since MDR is the leading cause of failure of comprehensive therapy for HCC, investigations aimed at circumvention of intrinsic tumor-mediated drug resistance are warranted. But the efficacy of some drugs used to reverse MDR is limited due to their side-effects[16-18]. BCT has been used to treat hyperprolactinemia for more than two decades because of its slight side-effects[19,20]. It was reported that BCT can...
Table 3 mRNA level of MDR-associated genes in response to TNF-α gene induction (mean±SD)

| Groups | MDR1mRNA | LRPmRNA |
|--------|----------|---------|
| BCT    | 0.21±0.02 | 0.17±0.04 |
| TNF    | 0.15±0.03 | 0.15±0.01 |
| ADM    | 0.89±0.15 | 0.83±0.12 |
| HepG2  | 0.13±0.02 | 0.11±0.01 |

*P<0.05 vs HepG2 group; †P<0.01 vs ADM group.

Table 4 Apoptosis index of each kind of tumors (mean±SD)

| Group | Mice (no) | Experimental (index of apoptosis) |
|-------|-----------|----------------------------------|
| BCT   | 8         | 30.5±4.2                         |
| TNF   | 8         | 21.9±2.1                         |
| ADM   | 8         | 23.1±1.8                         |
| HepG2 | 8         | 23.2±2.9                         |

*P<0.05 vs TNF group; †P<0.01 vs ADM group; ‡P>0.05 vs HepG2 group.

be used as a chemosensitizer against MDR tumor cells\[7,8\] and a current study showed that TNF-α may play a role in MDR\[21\].

In this study, we first demonstrated that TNF-α was expressed in the orthotopic tumor tissues injected into HepG2/ADM cells transfected with TNF-α. Moreover, tumor inhibition rate after treatment with 5-FU, ADM, MMC through intraperitoneal injection was higher in BCT group (67%, P<0.05) than in TNF group (57%) and ADM group (18%), and similar to HepG2 group (54%). As the data shows, the drug-resistant hepatocarcinoma cell line HepG2/ADM transfected with TNF-α could secrete cytokine TNF-α which could induce cancer cell apoptosis and inhibit the growth of tumor\[22,23\]. The result is in coincidence with Stein et al\[24\]. Furthermore, TNF-α gene in combination with BCT could reverse HCC MDR. Moreover, HIC and reverse transcriptase polymerase chain reaction (RT-PCR) showed that transduction and expression of human TNF-α in drug-resistant HCC cell line HepG2/ADM could reverse expression of MDR1mRNA, LRPmRNA, and Pgp, but had no synergistic effect compared to BCT, suggesting that TNF-α can downregulate the expression of MDR-associated genes and proteins\[25]\, and BCT may act on for its potential interaction and transport by Pgp\[26-31\], but has no effect on MDR-associated genes. Additionally, an interesting discovery indicate that the rate of apoptosis after chemotherapy in BCT group is the highest in all experiment groups, and BCT and TNF-α can reverse HCC MDR in nude mice MDR1 model of liver neoplasm.

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