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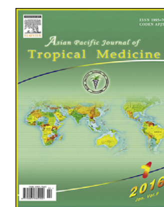

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Influence of *Toxoplasma gondii* on *in vitro* proliferation and apoptosis of hepatoma carcinoma H7402 cell

Gang Wang[1], Ming Gao[2*]

[1]*Department of General Surgery, People's Hospital of Zhengzhou, Zhengzhou 450012, China*

[2]*Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China*

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ABSTRACT

Objective: To discuss the influence of tachyzoite of *Toxoplasma gondii* (*T. gondii*) RH strain on proliferation and apoptosis of hepatoma carcinoma (HCC) H7402 cell.

Methods: The HCC H7402 cell in logarithmic phase and tachyzoite of *T. gondii* RH strain in different concentrations (1×10^7/mL, 2×10^7/mL, 4×10^7/mL, 8×10^7/mL and 16×10^7/mL) were co-cultured. CCK-8 was utilized to determine the inhibition rate of *T. gondii* tachyzoite on H7402 cell growth. Flow cytometry was used to detect the change of cell cycle. RT-PCR method was used to detect the expression of *cyclinB1* and *cdc2*–two genes related to cell cycle. Western blot method was used to detect the expression of apoptosis-related proteins Caspase-3 and Bcl-2.

Results: The tachyzoite of *T. gondii* RH strain can inhibit the proliferation of HCC H7402 cells. The inhibition rate of tumor cell growth increased with the increase of concentration of *T. gondii* tachyzoite. With the increase of concentration of *T. gondii* tachyzoite, the proportion of G_0/G_1 phase of H7402 cell increased, the proportion of S phase decreased, and PI value decreased accordingly. The expression of *cyclinB1* and *cdc2* genes decreased with the increase of the concentration of *T. gondii* tachyzoite. With the increase of the concentration of tachyzoite of *T. gondii* RH strain, the expression quantity of Caspase-3 in H7402 cell increased, but the expression quantity of Bcl-2 protein decreased.

Conclusions: *T. gondii* can inhibit the *in vitro* proliferation of HCC H7402 cell, and induce its apoptosis. This effect shows a trend of concentration-dependent increase. Moreover, it is related to the down-regulation of *cyclinB1* and *cdc2* (cell cycle-related genes), the increase of apoptosis-related protein Caspase-3, and the decrease of Bcl-2 expression.

1. Introduction

Hepatoma carcinoma (HCC) is one of the common malignant tumors. Its mortality in China ranks the 3rd in malignant tumors [1]. Presently, therapeutic methods of HCC include surgery, intervention, radiotherapy, chemotherapy, radiofrequency ablation, immunotherapy, gene target therapy and liver transplantation, *etc*. Since the prevalence of HCC is hidden and there is no specific clinical symptom in early stage, HCC is often in late stage upon definite diagnosis. Operation cannot be conducted, and the sensitivity to chemotherapy declines [2]. Recently, study on *Toxoplasma gondii* (*T. gondii*) brings new hope to oncotherapy [3,4]. After *T. gondii* infection, the body will generate natural killer cell and cytotoxic T cell during the reaction to *T. gondii*. These immune cells can also be used to inhibit the tumor cell. Therefore, *T. gondii* can excite the immune system inhibited by tumor.

T. gondii is an obligate intracellular parasitic protozoon. It is widely parasitized in karyocyte of human and animal [5]. The study indicates that *in vitro* culture of *T. gondii* tachyzoite system plays an important role in genetics, immunology, biochemical metabolism study and selection of

*Corresponding author: Ming Gao, Department of Oncology, The First Affiliated Hospital of Zhengzhou University, NO. 1 Eastern Jianshe Road, Zhengzhou 450052, China.
Tel: +86 371 66295562
E-mail: gaoming981@sina.com
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anti-*T. gondii* drugs [6]. The role of *T. gondii* tachyzoite in inducing tumor cell apoptosis draws increasing attention. Therefore, some studies hold that *T. gondii* has antitumor activity to a certain types of cancers [7–9]. After tachyzoite of *T. gondii* is infected with HCC H7402 cell, this study detects the proliferation of tumor cell, change of cell cycle, and change of expression of corresponding gene and protein from molecular level, which will provide experimental & theoretical basis to explore the action mechanism of *T. gondii* in treating HCC.

2. Materials and methods

2.1. Cell strain and tachyzoite of T. gondii

HHCC cell line was purchased from Shanghai Bioleaf Biotech Co., Ltd. The tachyzoite of *T. gondii* RH strain was provided by Parasitology Institute of School of Basic Medicine of Zhengzhou University. Twenty BALB/c mice in clean grade were provided by Laboratory Animal Center of Henan University of Traditional Chinese Medicine. The mice were male and 4-week-old mice, 18–20 g in body mass, with license No. of SYXK(yu)2010-0001.

2.2. Culture of tumor cell

Thawed H7402 cell was added to RPMI-1640 culture medium containing 10% fetal calf serum, and cultured in a 5% CO_2 incubator at 37 °C.

2.3. Culture of T. gondii tachyzoite

The *T. gondii* tachyzoite frozen by liquid nitrogen was taken out. 40 °C water bath was conducted for 1–2 min to completely thaw the *T. gondii* tachyzoite. It was mixed with isopyknic normal saline. Then it was injected to abdominal cavity of mice. The 0.5 mL was injected to each mouse. After 5–7 d, the ascites of mice after prevalence were extracted under aseptic condition. The ascites were washed and resuspended by PBS. The liquid supernatant of *T. gondii* tachyzoite was obtained through filtration by filter membrane with 0.22 μm aperture, and reserved.

2.4. Inhibiting effect of T. gondii tachyzoite on H7402 cell

H7402 tumor cell in logarithmic phase was taken. RPMI-1640 culture medium was used to adjust the suspension concentration to 5×10^4/mL. Then it is added to 96-well plates (100 μL/well) and placed in a 5% CO_2 incubator at 37 °C overnight. On the second day, 100 μL *T. gondii* tachyzoite suspension was added to each well, to make final concentration be 1×10^7/mL, 2×10^7/mL, 4×10^7/mL, 8×10^7/mL and 16×10^7/mL respectively. In addition, a control group was set up. Only 100 μL RPMI-1640 culture medium was added. Three repeated wells were set in each group. They were placed in a 5% CO_2 incubator for 24 h culture at 37 °C. Four hours before the end of experiment, CCK-8 (Cell Counting Kit-8) reagent (20 μL per well) was added for 4 h further culture. OD value was determined at 450 nm wavelength by ELIASA, reflecting quantity of living cell indirectly. The cell inhibition rate was calculated. Inhibition rate = $(1 - \text{A450 nm}_{\text{Experimental group}}/\text{A450 nm}_{\text{Control group}}) \times 100\%$.

2.5. Change of cell cycle detected with flow cytometry

H7402 cells, which were co-cultured for 24 h with *T. gondii* tachyzoite in different concentrations (1×10^7/mL, 2×10^7/mL, 4×10^7/mL, 8×10^7/mL and 16×10^7/mL) were taken. The count of cells was adjusted to 1×10^6. After propidium iodide dye liquor was added for 30 min dyeing under dark condition, flow cytometry was used to detect cell cycle, and cell proliferation index (PI value) was calculated. Cell proliferation index (PI) $(PI)=(S + G_2/M)/(G_0/G_1+S + G_2/M) \times 100\%$, of which S, G_2/M and G_0/G_1 represent the cell count of corresponding cell cycle, respectively.

2.6. Detection of cyclinB1 and cdc2 (cell cycle-related genes) expression at mRNA level

The cells after 48 h culture of each group were centrifuged and collected. Total RNA of cell was extracted through referring to instruction of Trizol kit. The cDNA was synthesized referring to instruction of reverse transcription kit of Promega Company. The reaction system is: 2 μg RNA, 2 μL 10 × reaction buffer, 4 μL 25 mmol/L $MgCl_2$, 2 μL 10 mmol/L dNTP, 0.5 μL RNA enzyme inhibitor, 0.75 μL 20 U/μL AMV reverse transcriptase, 1 μL 0.5 μg/μL Oligo$(dT)_{15}$. Nuclease-free water was used to achieve 20 μL. It was incubated in a water-bath for 1 h at 42 °C. Then it was heated for 5 min at 95 °C, and immediately incubated in a water-bath for 5 min. PCR amplification was conducted. The total volume of PCR reaction system is 20 μL, including 1.5 μL cDNA, 2 μL 10 × buffer, 1.5 μL 25 mmol/L $MgCl_2$, 1 μL forward primer, 1 μL reverse primer, 0.36 μL dNTPs, 0.1 μL TaqDNA polymerase and 9.75 μL ddH_2O. Reaction shall be conducted as per the following procedure: denaturation at 94 °C for 2 min, followed by 35 cycles of annealing at 94 °C for 30 s, at 58 °C for 30 s and 72 °C for 30 s, with a further 5 min extension at 72 °C, saved for 10 min at 4 °C. All sequences were synthesized by Sangon Biotech (Shanghai) Co., Ltd. After the completion of reaction, 10 μL PCR product was electrophoresed on a 2% agarose gel. The ratio of *cyclinB1* and *cdc2* gene band and reference gene band was used as relative mRNA content of corresponding gene.

2.7. Expression of apoptosis-related proteins Caspase-3 and Bcl-2 detected by Western blot method

The cells after 48 h culture of each group were collected. 20 μL FITC was added respectively. The cell lysis buffer was used to split the cell. The reaction was conducted on ice. After 20 min, centrifugation at 12 000 r/min was conducted for 5 min. The pellet was removed, and the supernatant was saved. 12% SDS-PAGE gel was conducted to isolate the protein. The protein was transferred to nitrocellulose membrane through electransfection. After it was sealed with 5% skim milk–TBST (20 nmol/L Tris-HCl, 150 nmol/L NaCl, 0.05% Tween-20, pH = 7.4) at room temperature for 2 h, it was reacted with mouse-anti-human Caspase-3 (or Bcl-2 or β-actin) monoclonal antibody (1:500). Then it was washed by TBST solution. Then it was reacted with goat-anti-mouse IgG (1:2000) second antibody marked by HRP. Finally, enhanced chemiluminescence was

used to display color. The Caspase-3/β-actin and Bcl-2/β-actin were calculated to represent relative expression of corresponding proteins, respectively.

2.8. Statistical analysis

SPSS17.0 software was used for statistical analysis of data. The *t* test and one-way analyses of variance were used to compare the mean of two groups or multiple groups. SNK-q test was adopted for multiple comparisons. A $P < 0.05$ was taken to indicate a difference of statistical significance.

3. Results

3.1. Inhibiting effect of RH tachyzoite of T. gondii on growth of HCC H7402 cell

The results of CCK-8 test showed that when the concentration of *Toxoplasma gondii* tachyzoite was 1×10^7/mL ~16×10^7/mL, *T. gondii* had obvious inhibiting effect on the growth of H7402 cells cultured *in vitro* ($P < 0.05$ or $P < 0.01$). With the increase of tachyzoite concentration, the absorbance value of H7402 cells reduced, especially when the concentration was $\geq4\times10^7$/mL, the differences compared to control group were significant ($P < 0.01$). In addition, the inhibitory effect of *T. gondii* tachyzoite on H7402 cells enhanced with the increase of concentration of tachyzoite. When the tachyzoite concentration was 16×10^7/mL, the inhibition rate was up to 83.7%.

3.2. Influence of RH tachyzoite of T. gondii on cell cycle changes of HCC H7402 cell

The result of flow cytometry detection (Table 1) indicated: with the increase of concentration of *T. gondii* tachyzoite, the proportion of G_0/G_1 phase of H7402 cell increased, the proportion of S phase decreased, and PI value decreased accordingly. When concentration of *T. gondii* tachyzoite was 2×10^7/mL, PI value was 0.58, which was significantly lower than that of control group (0.61). The difference had statistical significance ($P < 0.05$). When concentration of *T. gondii* tachyzoite was greater than 2×10^7/mL, PI value of each group was significantly lower than that of control group ($P < 0.05$).

3.3. Influence of tachyzoite of T. gondii RH strain on expression of cyclinB1 and cdc2 genes in H7402 cell

According to the gene band gray values determined by Image J software, the relative expression levels of *cyclinB* and *cdc2* genes were calculated. After H7402 cell was infected by tachyzoite of *T. gondii* RH strain, the expression of cell cycle-related genes *cyclinB1* and *cdc2* decreased with the increase of concentration of *T. gondii* tachyzoite. When concentration of *T. gondii* tachyzoite was $\geq4 \times 10^7$/mL, mRNA relative expression of *cyclinB1* in each group was significantly lower than that of control group ($P < 0.05$ or $P < 0.01$). When concentration of *T. gondii* tachyzoite was $\geq2 \times 10^7$/mL, mRNA relative expression of *cdc2* in each group was significantly lower than that of control group ($P < 0.05$ or $P < 0.01$), Table 2.

Table 1

Effect of *T. gondii* on cell cycle of human hepatoma H7402 cells.

Group	G_0/G_1 (%)	S (%)	G_2/M (%)	PI
Control	38.37	58.87	0.12	0.61
1×10^7/mL	40.07	58.91	0.09	0.60
2×10^7/mL	41.52	57.98	0.10	0.58*
4×10^7/mL	42.09	54.68	3.45	0.58*
8×10^7/mL	63.97	24.86	10.27	0.35*
16×10^7/mL	76.55	11.36	12.15	0.23*

Note: Compared with control group, $^*P < 0.05$.

Table 2

Assay of *cyclinB1* and *cdc2* gene expressions by RT-PCR.

Groups	*cyclinB1*	*cdc2*
Control group	1.25 ± 0.04	1.06 ± 0.12
1×10^7/mL	1.21 ± 0.12	1.01 ± 0.15
2×10^7/mL	1.07 ± 0.10	0.69 ± 0.08*
4×10^7/mL	0.51 ± 0.14*	0.32 ± 0.11*
8×10^7/mL	0.26 ± 0.05**	0.12 ± 0.03**
16×10^7/mL	0.08 ± 0.06**	0.11 ± 0.05**

Note: Compared with the control group, $^*P < 0.05$, $^{**}P < 0.01$.

3.4. Influence of tachyzoite of T. gondii RH strain on expression of Caspase-3 and Bcl-2 protein in H7402 cell

With the increase of the concentration of tachyzoite of *T. gondii* RH strain, the expression quantity of Caspase-3 in H7402 cell increased, but the expression quantity of Bcl-2 protein decreased. When concentration of tachyzoite was $\geq4 \times 10^7$/mL, Caspase-3 protein and Bcl-2 protein had concentration-dependent increase or decrease. The difference was significant when compared with control group ($P < 0.05$).

4. Discussion

T. gondii tachyzoite is a conditioned pathogen parasitized in cell. After it infects the host cell, some anti-tumor active substances can be secreted, or it can induce the chance of tumor gene expression. Therefore, it has a certain anti-tumor effect [10]. Nishikawa Y *et al.* [11] find that RH tachyzoite not only has the characteristics of broad-spectrum intracellular parasitism and aggravating cytopathic effect, etc., but also can induce cell apoptosis. Cell apoptosis is of vital significance in anti-tumor study. Effectively utilizing the characteristic that *T. gondii* tachyzoite can induce cell apoptosis will be a bright spot in tumor study.

This study used human HCC H7402 cell as the experiment object, and found that tachyzoite of *T. gondii* had inhibiting effect on its proliferation, and that the inhibition rate of cell growth increased with the increase of concentration of *T. gondii* tachyzoite. Moreover, the abnormity of cell cycle was closely related to the occurrence of tumor. After having been infected by *T. gondii* tachyzoite in a certain concentration, the proportion of G_0/G_1 phase of H7402 cell increased, and the proportion of S and G_2/M phases decreased. This indicates that it can induce the blocking of G_0/G_1 phase, which may be related to its role of inhibiting proliferation of tumor cell. However, the specific mechanism of action is not clear yet. A report shows that after having parasitized in host cell, the *T. gondii* tachyzoite can lead to the apoptosis or necrosis of host cell, and block the cell cycle to G_2/M phase [12]. Three endogenous molecules related to cell

cycle regulation, namely, cyclin, cyclin-dependent kinase and cyclin-dependent kinase inhibitor, coordinate with each other in cell cycle, and are closely related to proliferation, differentiation and apoptosis of cell. CyclinA and B are closely related to G_2/M transition and cell division. The cyclinB1 can form mitosis promoting factor with cdc2. The formed factor has positive regulating effect on detection point of G_2/M phase [13]. Studies show that the overexpression of cdc2/cyclinB1 can disorder the process of cell cycle, and lead to the occurrence of tumor. Presently, this phenomenon has been found in multiple tumors, such as human glioma, esophagus cancer and Non-small-cell lung carcinoma [14–16].

Cell cycle regulation is a complex process. The ordered cell cycle relies on normal function of every cell regulatory factor. A recent report shows that *T. gondii* tachyzoite can influence the process of cell cycle through regulating the expression of cyclinB1 [17]. As a core molecule in M phase check point, cdc2 binds with cyclinB protein, and plays a key role in regulating the cell into mitotic phase [18,19]. This study analyzed the effect of *T. gondii* on HCC H7402 cell through detecting the expression change of *cyclinB1* and *cdc2* genes. The result indicated that under the effect of *T. gondii* tachyzoite at a certain concentration, the expression of *cyclinB1* and *cdc2* decreased. The study on apoptosis-related protein indicated that with the increase of the concentration of *T. gondii* tachyzoite, the expression quantity of Caspase-3 protein increased, but the expression quantity of Bcl-2 decreased. This indicates that *T. gondii* tachyzoite inhibits the proliferation of HCC H7402 cell and promotes its apoptosis, and this effect is concentration-dependent.

Conflict of interest statement

We declare that we have no conflict of interest.

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