Differentiation-inducing action of 10-hydroxycamptothecin on human hepatoma Hep G2 cells

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ABSTRACT

AIM To study the mechanism of differentiation-inducing action of 10-hydroxycamptothecin HCPT on human hepatoma Hep G2 cells. METHODS The proliferating cell nuclear antigen PCNA expression was studied by immunocytochemical staining method. The cell cycle distribution and the wild-type protein p53 expression were measured by flow cytometry. Telomerase activity was assayed with telomeric repeat amplification protocol TRAP. RESULTS After treatment with HCPT at differentiation-inducing concentrations 5–20 μg L⁻¹ for 6 d Hep G2 cells were mainly arrested at G2/M phase and the PCNA expression rate was lower than that of control cells. When Hep G2 cells grew in a medium containing HCPT 5 μg L⁻¹ for 6 d the p53 expression level markedly increased in comparison with the control cells. The telomerase activity did not change in Hep G2 cells treated with HCPT 5–20 μg L⁻¹ for 8 d. CONCLUSION The differentiation-inducing effect of HCPT on Hep G2 cells is related with the cell cycle arrest at G2/M phase down-regulation of PCNA and up-regulation of wild-type protein p53.

INTRODUCTION

10-Hydroxycamptothecin HCPT an alkaloid isolated from the Chinese plant Camptotheca acuminata is an anticancer agent in experimental research and clinical study. HCPT could inhibit the activity of DNA topoisomerase II Topo II. Our previous study showed that HCPT induced differentiation of murine erythroleukemia cells and HL-60 cells. We have observed the differentiation-induction of HCPT 5 μg L⁻¹ on human hepatoma Hep G2 cells. In this work the mechanism of differentiation-induction action of HCPT was investigated.

MATERIALS AND METHODS

Cell culture and drug treatment The human hepatoma Hep G2 cell line was obtained from the American Type Culture Collection. Cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium DMEM and Ham’s F-12 Nutrient Mixture Gibco/BRL Life Technologies Grand Island NY USA containing 15% calf serum. For all experiments cells were treated with various concentrations of HCPT HCPT injection 5 mg HCPT in 2 mL sterilized water Huangshi Feiyun Pharmaceutical Co Hubei China at 24 h after plating. Analysis was performed at different time intervals during culture of the cells.

Immunocytochemical assay of proliferating cell nuclear antigen PCNA expression After a 6-d treatment with HCPT cells 1.0 × 10⁵ per well were collected and smeared on the slides precoated with 0.01% poly-L-lysine Sigma St Louis MO USA. The PCNA immunoreactivity was detected by the ABC avidin–biotin–peroxidase complex method. The primary antibody used was a mouse anti-human PC 10 antibody DAKO Denmark 1:100 the secondary antibody used was a biotinylated-antibody rabbit anti-mouse IgG Shanghai Chinese-American Company China 1:50. Photos were taken and quantitative analysis was conducted with an image analysis system Leica German. The experiment was performed at least three times.

Flow cytometric analysis of cell cycle After a 6-d treatment with HCPT cellular DNA content was detected by flow cytometry via determination of propidium...
iodide][P] on Hep G2 cells. After trypsinization the cells were washed with PBS and cell pellets were fixed in 70% ethanol at 4 °C overnight. After being washed twice with PBS the cells were stained with 1.0 mL of PI solution containing P], Sigma], St Louis], MO], USA 50 mg L⁻¹ RNase A], Sigma], St Louis], MO], USA 10 mg L⁻¹ Triton X-100, 0.5% v/v], and trisodium citrate 0.1% w/v] at room temperature in the dark for 30 min before cytofluorometry. The experiment was performed at least three times.

**Indirect immunofluorescence assay of p53 expression** The protein p53 level in cells was measured by flow cytometry[7]. Each sample contained 1.0 × 10⁶ cells. The primary antibody used was a mouse primary antibody against p53[6], DAKO], Denmark[1]: 1:50[6] and the secondary antibody was a FITC-conjugated-antibody rabbit anti-mouse IgG[6], DAKO], Denmark[1]: 1:50[6]. The antigen density was measured by Becton Dickson FACStar plus flow cytometer and the percentage of p53 positive cells were recorded. The experiment was performed at least three times.

**TRAP assay of telomerase activity** The telomerase activity of Hep G2 cells were measured by TRAP[6,7]. Briefly[7] after exposure to HCPT 5–20 μg L⁻¹ for 8 d[1] cell pellets were resuspended in 3 mL 3-chloroamidopropyl] dimethyl ammonio] -1-propanesulphonate] CHAPS[6] based telomerase lysis buffer 200 mL. The suspension was centrifuged at 15 000 × g at 4 °C for 30 min and the supernatant was used for telomerase assay. The protein concentration was measured by BCA assay[6], Pierce[7], USA. Telomerase reaction products were amplified at 29 PCR cycles at 94 °C for 30 s and 60 °C for 30 s. The PCR products were examined by 10% non-denature polyacrylamide gel electrophoresis. The experiment was performed at least three times.

**Statistical analysis** Data were expressed as x ± s and analyzed by t-test.

**RESULTS**

**Cell cycle distribution of Hep G2 cells** The proportions of Hep G2 cells in the different phases of the cell cycle were determined by flow cytometry. Untreated Hep G2 cells demonstrated a relatively normal distribution pattern[6] with most cells in the G0/G1 phase 62.3%[6] less cells in S phase 19.8%[6] and G2/M phase 17.8%[6]. The change of cell cycle distribution of Hep G2 cells treated with different doses of HCPT was shown in Fig 1. HCPT obviously blocked the progression of Hep G2 cells and arrested in G2/M. After a 6-d treatment of HCPT 5–10[6] and 20 μg L⁻¹[6] the proportion of G2/M phase cells was found to be 29.2%[6], 50.6%[6] and 80.1%[6] respectively Fig 1[6].

![Flow cytometric analysis of the cell cycle distribution](image)


**Tab 1.** Influence of HCPT on PCNA expression in human hepatoma Hep G2 cells. *P* < 0.01 vs control. x ± s.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Positive rate/%</th>
<th>Mean grey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>292</td>
<td>96</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td>HCPT 5 μg L⁻¹</td>
<td>274</td>
<td>59</td>
<td>0.22 ± 0.065</td>
</tr>
<tr>
<td>HCPT 20 μg L⁻¹</td>
<td>287</td>
<td>32</td>
<td>0.23 ± 0.065</td>
</tr>
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**Protein p53 expression** HCPT 5 μg L⁻¹ enhanced the p53 expression in Hep G2 cells. The p53
Fig 2. Immunocytochemical detection of proliferating cell nuclear antigen (PCNA) expression in human hepatoma Hep G2 cells untreated A or treated with HCPT 5 μg L⁻¹ B and HCPT 20 μg L⁻¹ C for 6 d. × 400.

positive rate of control cells was 6.8%. After 2 d, 4 d, and 6 d treatment of HCPT, the p53 positive rate was increased to 30%, 34%, and 26% respectively.

Fig 3. Flow cytometric analysis of p53 expression in human hepatoma Hep G2 cells treated with HCPT 5 μg L⁻¹ for 0–6 d. Shaded profiles cells incubated with anti-p53 antibody. Open profiles cells incubated with secondary antibody alone.

Effect of HCPT on telomerase activity of Hep G2 cells After incubation with HCPT 5–20 μg L⁻¹ for 8 d, the telomerase activity of Hep G2 cells had no significant change. Fig 4.

DISCUSSION

The relationship between the differentiation induction and the cell cycle distribution is not yet clear. It has been reported that different phases of the tumor cells possess different sensitivity to induction of differentiation. Cytarabine was most effective to induce differentiation at the G₁/S phase in K562 cells but Me₂SO at S phase in mouse erythroleukaemia cells and camptothecin at S and M phase in K562 cells. In the present study, we found that HCPT 5 μg L⁻¹ could produce marked differentiation-inducing effect and arrest Hep G2 cells mainly at G₂/M phase.

PCNA is considered as an auxiliary protein of DNA polymerase δ and participates in the DNA replication process of cell proliferation. The expression level of PCNA is related to the proliferation activity of cells and the PCNA positive cells have been reported in G₁ late phase or S early phase cell. In our experiments HCPT
Fig 4. Telomerase activity in human hepatoma Hep G2 cells untreated lane 1 or treated with HCTP 5· 10⁻⁸ and 20 μg L⁻¹ lane 2 - 4 for 8 d. Lane 0 Negative control. Five separate experiments were made in duplicate.

differentiated into mature hepatocytes. Thus× HCTP could not activate the telomerase repressing mechanism which is based on the complete differentiation of Hep G2 cells.

REFERENCES


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