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Inhibition of phosphorylation of histone H_1 and H_3 induced by 10-hydroxycamptothecin, DNA topoisomerase I inhibitor, in murine ascites hepatoma cells

LING Yi-He[1], XU Bin[2] (*Department of pharmacology I, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China*)

ABSTRACT Hydroxycamptothecin (HCPT), isolated from *Camptotheca acuminata*, is a powerful antitumor alkaloid. Previous studies indicated that the molecular target of this agent was DNA topoisomerase I. The present results demonstrated that *in vitro* treatment of murine ascites hepatoma cells with HCPT resulted in a marked reduction in DNA synthesis and the inhibition of phosphorylation in histone was in a time-dependent manner. Gel electrophoresis found that HCPT had a selectively inhibitory effect on the phosphorylation of histone H_1 and H_3, but less effect on the other kinds of histones. *In vivo*, HCPT also exhibited a suppressive effect on histone H_1 and H_3 phosphorylation. These data suggested that HCPT-induced cell killing may be, at least in part, associated with the suppression of histone H_1 and H_3 phosphorylation.

KEY WORDS 10-hydroxycamptothecin; experimental liver neoplasms; cultured tumor cells; DNA untwisting proteins; histone phosphorylation

Histone phosphorylation, a posttranslation modification, plays a key role in the regulation of the cell cycle in eukaryocytes. The level of histone H_1 and H_3 phosphorylation was increased through G_2 to M at mataphase in mammalian cells. The correlation between histone phosphorylation and chromosome condensation was seen during the induction of premature chromosome condensation[1-3]. Another major participant in regulation of cell cycle is topoisomerases which control the topological state of DNA[4,5]. DNA gyrase (DNA topoisomerase II) inhibitor, etoposide and DNA untwisting proteins (DNA topoisomerase I) inhibitor, camptothecin suppressed histone H_1 and H_3 phosphorylation, and led into cells arrested at G_2 in BHK cells[6].

10-Hydroxycamptothecin (HCPT) showed a more potent activity against several kinds of tumor cell lines *in vitro* and *in vivo*, and less side effect in the clinic trials as compared to camptothecin[7]. HCPT induced topo I-associated DNA single strand breaks and inhibited the synthesis of chromatin protein[8,9]. In this paper, we reported the influence of HCPT on the histone phosphorylation in murine hepatoma cells.

MATERIALS AND METHODS

Drug and chemicals HCPT was provided by Department of Phytochemistry in this Institute. [^{3}H]Thymidine (1.48 PBq·mol^{-1}, and triethylammonium ortho[^{32}P]phosphate (0.37 PBq·mol^{-1}) were purchased from Amersham, UK. RPMI-1640 medium and fetal calf serum were purchased from Sigma Co.

Turmor Cells and treatment *In vitro* hepatoma cells (1×10^6 cells) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum at 37℃ in a 5% CO_2/air incubator. Following the incubation in the presence of drugs, the surviving cells were counted by 0.2% trypan blue dye exclusion in a hemocytometer. *In vivo* mice (18—22 g) were injected ip 2×10^5 ascites hepatoma cells. Seven days after inoculation, mice were injected ip HCPT 10 mg·kg^{-1} or equal volume of normal saline as control. At the specific time, mice were killed and hepatoma cells were harvested by centrifugation for 5 min at $1\ 000\times g$, washed twice with cold PBS, and the resulting cell

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[1] Now in *Department of Medical Oncology, M D Anderson Cancer Center, University of Texas, Houston TX 77030, USA*.

[2] To whom requests for reprints should be addressed

pellet was taken for assay of DNA synthesis and histone phosphorylation.

Measurement of DNA Synthesis Exponentially growing hepatoma cells were plated in a multiwell plate and labeled with 3.7 kBq [^{3}H]thymidine at 37℃. After 1-h incubation, reaction was halted by addition of equal volume of 10% TCA in ice bath for 10 min, and then acid-insoluble materials were collected on GF/A filter (Whatman, UK), washed thrice with cold 5% TCA, once with EtOH, and dried at room temperature. The radioactivity on the filter was measured by a liquid scintillation counter.

Preparation of nuclei The procedure for isolation of nuclei from hepatoma cells was carried out as described by Muramatsu *et al*[10] with slight modification. Briefly, hepatoma cells lysed in RSB solution containing Tris - HCl 10, NaCl 10, $MgCl_2$ 1.5 mmol $\cdot L^{-1}$ (pH 7.4), and placed in ice bath for 10 min. The suspension was centrifuged at 1 000×*g* for 5 min and pellet was resuspended in 10 volumes of RSB solution containing 0.1% Triton X-100. The suspension was homogenized with 10—15 up and down stroked in a glass homogenate fitted with a teflon pestle with a clearance of 0.4 mm. The homogenate was centrifuged at 1 200×*g* for 5 min at 4℃, and washed twice with RSB buffer or $MgCl_2$ buffer 2 mmol $\cdot L^{-1}$ containing sucrose 0.25 mol $\cdot L^{-1}$.

Histone pulse-labeled with ^{32}P and extraction Hepatoma cells (1×10^6 cells) were pulse-labeled with 37 kBq [^{32}P]orthophosphate in the phosphate-free RPMI-1640 medium at 37℃ for 1 h, and then the nuclei were isolated as described above. The total histones were extracted from nuclei twice with H_2SO_4 0.25 mol $\cdot L^{-1}$ at 37℃ for 15 min, and the solubilized proteins were precipitated with 20% TCA, washed twice with acetone, and dissolved in acid-urea buffer containing 5% HAc, urea 2.5 mol $\cdot L^{-1}$.

Gel electrophoresis The urea 8 mol $\cdot L^{-1}$ 10% polyacrylamide gel prepared from a 40% (wt/vol) solution of acrylamide (38%) and bisacrylamide (2%) in urea 8 mol $\cdot L^{-1}$, 5% HAc buffer. The sample (40 μg protein) was loaded on the gel and electrophoresed with 5% HAc as a running buffer under 80 V for 4 h at room temperature. The bands of individual histones were visualized after gel stained with Coomassie brilliant blue G-250 (Bio-Rad, Hercules CA, USA).

Assay of DNA and protein Protein and DNA were determined colorimetrically[11,12].

RESULTS

In the time course of DNA synthesis and histone phosphorylation determined with [^{3}H]thymidine and [^{32}P]orthophosphate incorporation into log phase growing ascites hepatoma cells *in vitro*, the rate of DNA synthesis in untreated cells was linearly increased as increasing the incubation time within 20 min, and then achieved a plateau level at 60 min. When cells exposed to HCPT 100 μmol $\cdot L^{-1}$ for 5 min, the rate of DNA synthesis was not different from the control. But it was markedly decreased by 49—56% after 20—50 min incubation as compared to the control (Fig 1). A similar result was seen from assay of histone phosphorylation in hepatoma cells in the absence or presence of HCPT 100 μmol $\cdot L^{-1}$, ie, only 15% of inhibition of histone phosphorylation induced by HCPT occurred at 5—10 min, and 37—58% of inhibition occurred at 20—60 min incubation (Fig 1). These results indicated that the inhibition of DNA synthesis and histone phosphorylation induced by HCPT exhibited a similar pattern of the time course.

To determine whether HCPT could impact the phosphorylation of individual histones, exponentially growing hepatoma cells were pulse-labeled with [^{32}P]orthophosphate for 1 h and then treated with HCPT for another 1 h. The total phosphorylated histones were extracted from nuclei, and the individual histones were further separated by polyacrylamide gel. The results showed that HCPT 50—200 μmol $\cdot L^{-1}$ markedly inhibited the phosphorylation of histone H_1 and H_3 in a concentration-dependent manner, while it slightly affected the phosphorylation in the other kinds of histones. The inhibitions of phosphorylation in histone H_1 and H_3 at HCPT 50 μmol

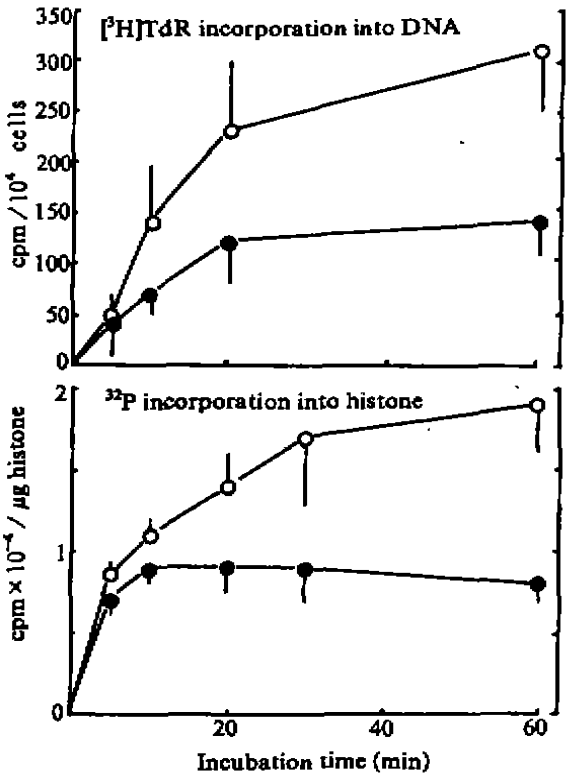

Fig 1. Time course of DNA synthesis and histone phosphorylation in murine ascites hepatoma cells *in vitro* exposed to HCPT 100 μmol·L⁻¹. Hepatoma cells (1×10⁶) were incubated at 37℃ in the absence (○) or the presence of HCPT 100 μmol·L⁻¹ (●). At the time indicated, aliquots of sample were taken and DNA sythesis (panel A), or histone phosphorylation (panel B) was determined as described in Materials and Methods. Each point represents mean of 3 separated experiments, bar; ±s.

·L⁻¹ were about 20% and 12%, respectively (Fig 2). However, only slight inhibitions of phosphorylation were seen in histone H_{2a}, H_{2b}, and H_4. Even at high concentration (200 μmol·L⁻¹), the inhibition of phosphorylation in histone H_1 and H_3 caused by HCPT were about 2-fold higher than those in histones of H_{2a}, H_{2b}, and H_4(Fig 2).

To confirm the evidences obtained from *in vitro*, we examined the effect of HCPT on histone phosphorylation *in vivo*. Eight mice

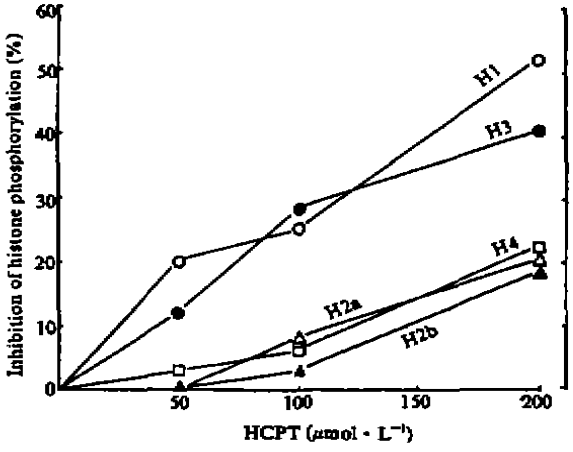

Fig 2. Effect of HCPT on phosphorylation of individual histone *in vitro* in hepatoma cells. ^{32}P-PO_4 was added to the cell culture in phosphate-free medium at 37℃ for 1 h, and then various concentrations of HCPT were added to the culture for another 1 h. The phosphorylated individual histones were separated and measured as described in materials and methods. Each point is mean of two independent experiments.

bearing ascites hepatoma were injected ip HCPT 10 mg·kg⁻¹ or equal volume of saline. After 4 h mice were killed and hepatoma cells were harvested for assay the histone phosphorylation. HCPT 10 mg·kg⁻¹ ip resulted in a decrease in the phosphorylation of histone H_1 and H_3 in hepatoma cells, ie, the inhibitions of phosphorylation in histone H_1 and H_3 were 53% and 57% ($P<0.01$), respectively (Fig 3). No significant inhibitions of phosphorylation were seen in histone H_{2a}, H_{2b}, and H_4 ($P>0.05$).

DISCUSSION

In the present work, we demonstrated that HCPT selectively inhibited the phosphorylation of histone H_1 and H_3 in hepatoma cells, and this effect was correlated to the decrease in DNA synthesis (Fig 1). But we can not distinguish the reduction of histone phosphorylation caused by HCPT was resulted from decrease in the activity of protein kinases

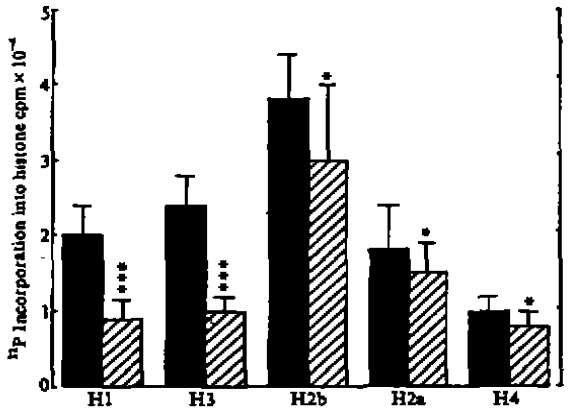

Fig 3. Effect of HCPT on histone phosphorylation *in vivo* in hepatoma cells. Eight mice bearing ascites hepatoma were given ip injection of HCPT 10 mg·kg^{-1} HCPT (▨), or equal volume of normal saline as control (■). Four hour after injection, mice were killed, and hepatoma cells were collected for assay of the histone phosphorylation. Each column represents mean of at least 4 separated experiments, bar, ±s. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$, treatment *vs* control.

or stimulation of phosphatases. Further studies are nacessary to determine whether HCPT could directly interact with these enzymes. It was shown that p34^{cdc2}/cyclin B kinase and histone H_1 kinase were involved the regulation of cell cycle, and the activities of these enzymes were fluctuated as cell cycle in mammalian cells [13]. Topo I and I inhibitors disrupted these enzymes and reduced phosphorylation of histone H_1 and H_3, and finally arrested the cell at G_2[6,14,15]. Flow cytometric analysis found that HCPT blocked HL-60 cells at S/G_2 and G_2[8]. Thus, we presume that HCPT-induced inhibition of histone H_1 and H_3 phosphorylation in hepatoma cells may be due to inactivation of protein kinases, in particular, the cell cycle-associated protein kinases such as p34^{cdc2}/cyclin B and histone H_1 kinase.

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DNA 拓扑异构酶 I 抑制剂羟基喜树碱对小鼠腹水肝癌细胞组蛋白 H_1和 H_3磷酸化的抑制

凌义和[1]，贾 彬[2]
(中国科学院上海药物研究所，上海200031，中国)

R 965.2

摘要 体外用羟基喜树碱(HCPT)处理小鼠腹水肝癌细胞，DNA 合成明显降低，组蛋白磷酸化受抑，此两种效应皆为时间依赖性. 凝胶电泳进一步发现 HCPT 选择性抑制组蛋白 H_1和 H_3的磷酸化，体内实验亦显示 HCPT 显著抑制组蛋白 H_1和 H_3磷酸化. 因此，HCPT 对肿瘤细胞的致死作用至少部分与其抑制组蛋白 H_1和 H_3的磷酸化有关.

关键词 10-羟基喜树碱；实验性肝肿瘤；培养的肿瘤细胞；DNA 解结蛋白；组蛋白磷酸化

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Effect of esculentoside H on release of tumor necrosis factor from mouse peritoneal macrophages[1]

HU Zhen—Lin, ZHANG Jun—Ping, YI Yang—Hua[2], QIAN Ding—Hua
(*Research Laboratory of Natural and Synthetic Drugs, [2] Department of Phytochemistry, College of Pharmacy, Second Military Medical University, Shanghai 200433, China*)

ABSTRACT Effect of esculentoside H (EH) on release of tumor necrosis factor (TNF) from murine peritoneal macrophage (MΦ) *in vitro* was studied. The results showed that EH (12.5—200 $\mu g \cdot ml^{-1}$) induced the thioglycolate-broth elicited peritoneal MΦ to release TNF into supernatants in a dose-dependent manner, and higher levels of TNF activity were detected in the supernatants from EH-stimulated calcimycin-primed MΦ culture. EH-induced TNF release had a different type of kinetics compared with that of lipopolysaccharides (LPS). LPS-induced release of TNF increased rapidly until 6 h after LPS stimulation, then declined gradually, while EH-induced TNF release increased gradually after EH stimulation and reached its peak at approximately 24 h later. These results suggested that the anti-tumor mechanisms of *Phytolaccaceae* may be related to the capacity of EH for TNF release.

KEY WORDS esculentoside H; tumor necrosis factor; macrophages; calcimycin

Tumor necrosis factor (TNF), a macrophage (MΦ)-derived peptide, was originally described as a mediator of lipopolysaccharides (LPS)-induced hemorrhagic necrosis of tumor in animals and as a molecule with cytostatic/cytotoxic activity for tumor cells in culture[1]. The anti-tumor potency of systemically administered TNF has been shown to be disappointing in phase I clinical trails, using a variety of recombinant TNF preparations over last 5 years, as its toxicity severely limited the dosage of TNF administered in humans[2]. Hence the triggering of endogenous TNF production may be a key strategy that lessen the side effects of high TNF dosages administered. In order to search for the inducer of endogenous TNF and investigate the anti-tumor mechanisms of *Phytolaccaceae*, we studied the effects of esculentoside H (EH), a water-soluble saponin isolated from the roots of *Phytolacca esculenta* van Houtte (*Phytolaccaceae*)[3], on release of TNF from thioglyco-

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late (TG)-broth elicited murine peritoneal MΦ primed (or not primed) with calcimycin (Cal) and compared the effects of EH with that of a TNF eliciting-agent, the lipopolysaccharides (LPS).

MATERIALS AND METHODS

Reagents EH was provided by YI Yang—Hua (Faculty of Pharmacy, Second Military Medical University). LPS derived from *E coli* 0 111:B4 and Cal were from Sigma; Dactinomycin (Dac), Xinya Pharmaceutical Co, Shanghai; TG-broth, Shanghai Academy of Biologic Products. The cell culture medium was RPMI-1640 (Sigma) with *l*-glutamine (2 mmol·L^{-1}) supplemented with $NaHCO_3$ (2 g), penicillin (30 mg·L^{-1}), streptomycin (25 mg·L^{-1}), mercaptoethanol (100 μmol·L^{-1}), and 10% heatinactivated new-born bovine serum (NBS) (completed RPMI-1640).

ICR mice ♀, weighing 28±*s* 2 g, from the Animal Center of this university.

Preparation of MΦ ICR mice were injected ip with 1 ml 3% TG-broth and peritoneal exudate cells (PEC) were harvested 4 d later by washing the peritoneal cavity with phosphate-buffered saline (PBS). PECs were washed 3 times with PBS, then adjusted to 5×10^6/ml in completed RPMI-1640. The cell suspension was dispensed into cell culture flask (the area for cell adherence was 5 cm×3 cm) (2 ml/flask). After 6 h of incubation at 37℃, 5% CO_2, the nonadherent cells were removed by washing with RPMI-1640 medium, and the adherent cells were used as TG-elicited MΦ.

Priming MΦ for TNF release In order to enhance the TNF release from MΦ in response to the stimulation of LPS, the TG-elicited PEC were incubated in the presence of priming agent, Cal. Six hours later, the cells were washed with RPMI-1640 medium to remove the agent and the nonadherent cells, and the adherent cells were used as Cal-primed MΦ.

Induction of TNF from MΦ MΦs were incubated for 24 h in the presence of EH (0—200 μg·ml^{-1}) or LPS (100 ng·ml^{-1}) and the supernatants were collected for TNF bioassay. For kinetics analysis of EH or LPS-induced TNF production, MΦs were cultured with EH (100 μg·ml^{-1}) or LPS (100 ng·ml^{-1}) for various periods (2—30 h), and the supernatants were assayed for TNF activity.

Bioassay for TNF activity TNF activity in the supernatants were monitored by crystal violet staining assay *in vitro*[6]. In brief, L929 cells (5×10^4/well) were incubated for 18 h in the presence of Dac (1 μg·ml^{-1}) and serial 1:2 dilutions of test samples in 96-well flat-bottom microtiter plates. Units of TNF were defined as the dilution required to lyse 50% of L929 target cells.

RESULTS

Priming effect of Cal for TNF release from MΦ Incubating the TG-elicited PECs for 6 h in the precence of Cal (0.1—10 μmol·L^{-1}) enhanced the TNF release from MΦ in response to the stimulation of LPS. This indicated that Cal could prime the MΦ for TNF release. Since Cal at 1 μmol·L^{-1} showed the best priming effect, this concentration was selected in the experiments (Tab 1).

Tab 1. Tumor necrosis factor (TNF) activity induced by lipopolysaccharides (LPS) (50 ng·ml^{-1}, 6 h) from the MΦ which had been incubated for 6 h in the presence of calcimycin. $n=3$, $\bar{x}\pm s$. $^*P<0.01$ vs control.

Calcimycin/ μmol·L^{-1}	TNF activity/U·ml^{-1}
0	12±4
0.1	142±19*
1	201±18*
10	54±13*

EH induced release of TNF EH (12.5—200 μg·ml^{-1}) induced the release of TNF from both TG-elicited MΦ and Cal-primed MΦ concentration-dependently. The TNF levels in the supernatants from Cal-primed MΦ cultures were higher than those from TG-elicited MΦ (Tab 2).

Comparison of LPS and EH-induced TNF production TNF levels were detectable in the supernatants 2 h after LPS-stimulation and

Tab 2. Effects of esculentoside H (EH) and lipopolysacchrides (LPS) on the release of tumor necrosis factor (TNF) from thioglycolate (TG)-broth elicited macrophage (MΦ) or calcimycin (Cal)-primed MΦ. n=3, $\bar{x}\pm s$. $^aP>0.05$ $^bP<0.05$, $^cP<0.01$ vs control.

EH/ $\mu g\cdot ml^{-1}$	LPS/ $ng\cdot ml^{-1}$	TNF ($U\cdot ml^{-1}$) induced from TG-elicited MΦ	Cal-primed MΦ
0		1.9±1.0	5.2±1.5
12.5		1.9±0.4[a]	7.2±1.4[a]
25		2.2±0.9[a]	9.8±1.8[b]
50		11±5[b]	43±8[c]
100		13.4±2.5[c]	23±4[c]
200		10.0±1.7[c]	10±4[a]
	100	14±3[c]	59±8[c]

rose rapidly until 6 h, then declined gradually, while TNF levels were not detectable until 6 h after EH-stimulation and increased gradually in 24 h (Tab 3).

Tab 3. EH (100 $\mu g\cdot ml^{-1}$) or LPS (100 $ng\cdot ml^{-1}$)-induced release of TNF from TG-elicited MΦ or Cal-primed MΦ. n=3, $\bar{x}\pm s$.

Time/ h	EH-induced release of TNF ($U\cdot ml^{-1}$) from TG-elicited MΦ	Cal-primed MΦ	LPS-induced release of TNF ($U\cdot ml^{-1}$) from Cal-primed MΦ
2	1.8±1.6	4.1±1.0	60±5
6	3.3±1.1	8.6±0.8	189±30
12	8.8±1.8	16±4	86±3
16	11.0±1.9	18.3±2.1	
24	13.9±2.4	19±4	59±8
36	11.0±0.9	18.4±1.3	

DISCUSSION

The studies reported herein demonstrated that EH could induce TNF release from murine peritoneal MΦ. Since TNF is an important cytokin involved in host immune defence, the capacity of EH for TNF release may be related to the anti-tumor mechanisms of *Phytolaccaceae*.

It would be interesting to note that the kinetics of EH-induced TNF production exhibited some differences compared with that of LPS. This suggested that LPS and EH induced the TNF release through different molecular mechanisms that need to be further investigated.

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商陆皂甙辛对小鼠腹腔巨噬细胞释出肿瘤坏死因子的影响[1]

胡振林，张俊平，易杨华[1]，钱定华

(第二军医大学药学院中西药研究室，

[1]植物化学教研室，上海200433，中国)

摘要 商陆皂甙辛(EH) (12.5-200 $\mu g\cdot ml^{-1}$)可剂量依赖性地诱导硫代乙醇酸钠培养基诱出的小鼠腹腔巨噬细胞(MΦ)以及卡西霉素启动激活的MΦ分泌肿瘤坏死因子(TNF)。时效关系研究发现：脂多糖(LPS)诱导的TNF分泌于6 h左右达峰，而EH诱导的TNF分泌随时间延长逐渐增多，于24 h左右达峰，提示EH和LPS诱导TNF分泌的机制可能不同。

关键词 商陆皂甙辛，肿瘤坏死因子，巨噬细胞，卡西霉素

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Fate of methotrexate albumin microspheres after hepatic intra-arterial injection in dogs[1]

XU Feng, ZHU Yu—Kun
(*Nanfang Hospital, First Military Medical University, Guangzhou 510515, China*)

ABSTRACT Methotrexate (MTX) albumin microspheres (40 μm) were injected into dog hepatic artery. The MTX levels in the hepatic vein maintained at relatively high concentration for over 3 h, whereas in the case of conventional MTX in saline the drug level decreased sharply soon after injection. After ia MTX in microspheres the MTX levels in liver were higher than those after MTX in saline. The microsphere emboli were entrapped in the hepatic precapillary arterioles. Thrombi were found in hepatic arterioles, with microsphere constituting the core. Hence MTX microspheres hepatic intra-arterial injection may be an effective treatment for patients with liver neoplasms.

KEY WORDS methotrexate; albumin microspheres; therapeutic chemoembolization; liver

In the chemotherapy of liver cancer, the hepatic intra-arterial (ia) injection of drug in biodegradable microspheres is superior to conventional drug in saline, for the microspheres prolong the retention time of the drug in the liver[1,2]. Moreover, the microspheres could embolize the arterioles to block the tumor blood supply, resulting in anoxia and ischemic necrosis of the tumor tissue[3]. A new kind of embolizer-albumin microsphere is devised. This paper studied the fate of ia methotrexate (MTX) in human albumin microspheres in dogs.

MATERIALS AND METHODS

MTX-albumin microspheres Microspheres, prepared by emulsion polymerization[4], obtained from Institute of Radiation Medicine, Academy of Military Medical Sciences of the People's Liberation Army, were 40 μm in diameter and the MTX content was about 5% (100 mg microspheres contained 5 mg MTX).

Dog experiment Twelve mongrel dogs of either sex, weighing 13.4±*s* 1.5 kg (Animal Center, First Military Medical University), were anesthetized with iv sodium pentobarbital (30 mg·kg^{-1}). A cannula was inserted into the hepatic artery and another cannula was injected into the inferior vena cava to the inflow junction of the hepatic vein. An amount of 100 mg the microspheres or 5 mg MTX in 5 ml normal saline was injected into the hepatic artery. Inferior vena cava blood and liver tissue were obtained at 0, 5, 15, 30, 60, 120, 150, and 180 min after injection. Dogs were killed on d 1, 3, 5, 10, and 20 after injection, and blood and liver were taken.

Determination of serum MTX concentration Blood samples were kept at 4℃ overnight. Serum MTX was analyzed by fluorescence polarization immunoassay (FPIA)[5]. The TDx System and MTX reagent pack were products of Abbott Laboratories (USA). In this experiment, the range of the MTX calibration curve was 0—1.0 μmol·L^{-1}. Higher concentrations needed dilution. The lowest measurable level was 0.01 μmol·L^{-1}. Reproducibility was measured form 10 runs of five replicates each of human serum with MTX 0.07, 0.40, 0.80, 5.0, 50, and 500 μmol·L^{-1}, yielding CV <10%.

Determination of liver MTX concentration The liver was blotted with filter paper. Liver tissue 0.50 g was hemogenized in KCl (0.15 mol·L^{-1}) 3 ml and stored in ice bath. The supernatant was obtained by centrifugation (3000×*g* for 5 min) and assayed by FPIA.

Data processing The MTX concentrations-time curve was fitted and pharmacokinetic parameters were calculated with a PKBP-N1 program on a SUN 386 computer.

Histology Liver slices were stained with hematoxylin and eosin. The microsphere-embolizing pat-

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terns were examined under light microscope (×400).

RESULTS

Serum MTX levels After the ia MTX in microspheres, the serum MTX concentration remained at a relatively high level for over 3 h, and decreased gradually. By contrast, the serum MTX levels after the ia MTX in saline reached its peak earlier but decreased quickly (Fig 1A). The MTX concentrations-time curves fitted to a 2-compartment model. The pharmacokinetic parameters were shown in Tab 1. Liver MTX levels Within 3 h after ia injection the liver MTX levels after MTX in microspheres were higher than those after MTX in saline (Fig 1B). On d 20 there remained 0.2 nmol·g^{-1} in the liver after MTX in microspheres while almost undetectable after MTX in saline.

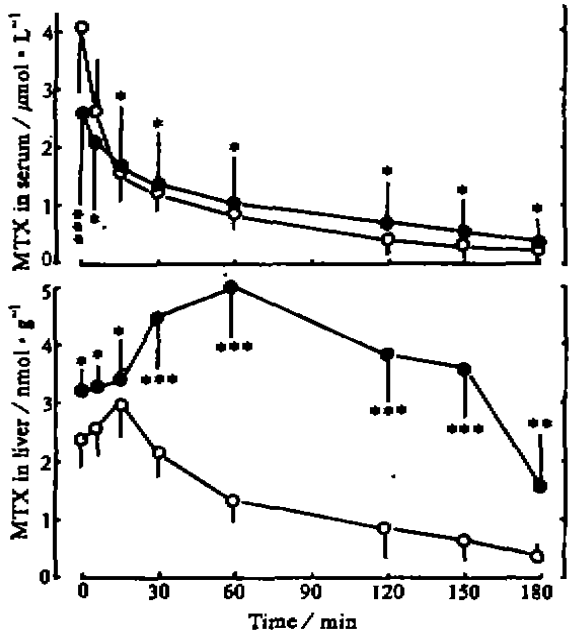

Fig 1. MTX concentrations in serum and liver after ia 100 mg MTX in microspheres (●) or 5 mg MTX in saline (○) in dogs. $n=6$, $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ vs in saline.

Tab 1. Pharmacokinetic parameters of ia MTX in microspheres (100 mg) or MTX in saline (5 mg) in dogs. $n=6$, $\bar{x}\pm s$. $^{a}P>0.05$, $^{b}P<0.05$, $^{c}P<0.01$ vs in saline.

	Microspheres	Saline
$T_{\frac{1}{2}\alpha}$/min	9±5^{b}	3.7±2.1
$T_{\frac{1}{2}\beta}$/min	109±64^{a}	77±55
V_c/L	4.3±1.1^{c}	1.3±0.4
AUC/μmol·min·L^{-1}	272±207^{a}	189±69

Liver/serum MTX ratio After ia MTX in microspheres the ratio increased with time. After ia MTX in saline the ratio remained low (Tab 2).

Tab 2. Liver/serum MTX ratios after ia MTX in microspheres (100 mg) or MTX in saline (5 mg) in dogs. $n=6$, $\bar{x}\pm s$. $^{a}P>0.05$. $^{b}P<0.05$. $^{c}P<0.01$ vs in saline.

Time/min	Microspheres	Saline
0	1.8±0.8^{c}	0.48±0.20
15	3.1±1.5^{a}	2.1±1.2
60	5.4±3.4^{a}	2.1±1.1
120	6.2±3.7^{b}	1.8±1.3
180	10.3±3.2^{b}	5.1±1.5

Embolization pattern The microspheres embolized the precapillary arterioles in a single-beaded arrangement. Thrombi were found in arterioles, each with a microsphere constituting the core. The tissue around the thrombi showed coagulation necrosis. The portal areas and hepatocytes remained unaffected in the non-embolized parts.

DISCUSSION

The albumin is a superior ground-plasma for microspheres to starch or fibrinogen. For it was got from the human plasma, it could not be eliminated by the reticuloendothelial