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Effect of anticancer drug methortrexate on the biliary excretion kinetics of the reduced folate derivatives in rats

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항암제 methotrexate가 랫드 담즙중 환원형엽산유도체의 배설동태에 미치는 영향

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초록 : Dihydrofolate reductase(DHFR) 억제제는 혈중활성엽산유도체의 농도저하를 초래하는데 이에 대한 기전을 밝히고자 DHFR 억제제의 대표적 약물로서 항암치료에 널리 사용되고 있는 methotrexate를 0.3 및 10 mg/kg의 용량으로 랫드에 정맥주사한 후 활성엽산유도체의 담즙배설동태를 검토하였다. 임상적용 용량을 상회하는 용량임에도 불구하고 methotrexate에 의한 환원형엽산유도체의 담즙배설은 유의적 감소를 나타내지 않았다. 이러한 결과는 methotrexate에 의한 엽산유도체의 혈중농도저하가 활성엽산유도체의 담즙배설저하에 직접 관련되지 않는 것을 나타낸다. 따라서 장간순환계가 체내 엽산대사 및 항상성 유지에 중심기구임을 고려해 볼 때 DHFR 억제제에 의한 활성엽산유도체의 혈중농도저하는 담즙배설의 변화에 의한 것 보다는 소화관에 배설된 후 재흡수의 저하에 의해 초래될 가능성이 높은 것으로 사료된다.

Key words : methotrexate, active folate derivatives, biliary excretion kinetics.

Introduction

Methotrexate, a folate analogue, is an inhibitor of dihydrofolate reductase(DHFR)[7], which is responsible for converting folic acid to reduced folate cofactors which participate in important reactions including the DNA synthesis.[7,13,14,20] Human studyies have also shown that methotrexate has an inhibitory effect on the thymidylate synthase(TS)[13,14]. This drug has widely been used for the treatment of various tumors because of its prominent effect during the DNA synthesis in the cycle of cell proliferation[7,13,20]. However, this drug appeared to be severely cytotoxic in normal cells undergoing rapid turnover, such as liver, bone marrow and gastrointestinal tract[6,18].

The enterohepatic circulation of folates plays an important role in the folate metabolism and homeostasis[29-31]. More than 50% of body folates are present in the liver, and the enterohepatic circulation of folates forms the largest folate pool in the body[31]. Pratt et al[19]. suggested that the distribution characteristics of bile folates may reflect those of liver folates, relating to folate metabolism. Although it was well established that folylenzyme inhibitors such as methotrexate[13], pyrimethamine[36], valproic acid[17], trimethoprim[8] and 5-fluorouracil[34] interfere the pathway of folate metabolism in the liver, their effects on the biliary excretion of folate derivatives have never been demonstrated. One of the possible reasons for this may be that the characterization of folate derivatives in rat bile has been unclear. Recently, Shin et al[23,25-27] identified non-menthylated folates including 5, 10-methylenetetrahydrofolate, tetrahydrofolate, 10-formyltetrahydrofolate other than 5-methyltetrahydrofolate in the rat bile as the major active folate derivatives, and also confirmed their critical roles in the hepatic folate metabolism and homeostasis maintenance of the plasma folate *via* the enterohepatic circulation[28].

Kokue and the coworkers[16,32,33] have demonstrated an acute decrease of 5-methyltetrahydrofolate, the principal folate in the plasma, by a toxic effect of pyrimethamine which is a DHFR inhibitor. Similar results for the decrease in plasma folate were also observed by another DHFR inhibitor, methotrexate. As a mechanism for the plasma folate depletion by DHFR inhibitors, we have focused on the enterohepatic circulation of folates, because of its critical role in the regulation of plasma folate homeostasis[29-31]. The enterohepatic circulation system includes the biliary excretion site and the intestinal reabsorption site. This study was, therefore, carried out to clarify the mechanism for the depletion of the plasma folate by DHFR inhibitors by evaluating of the biliary excretion kinetics of active reduced folate derivatives following the administration of methotrexate.

Materials and Methods

Animals : Female Sprague-Dawley rats(Clea Japan), 200~250g of body weight, were used throughout the experiments. The animals were given the pelleted diet(CE-2, Clea Japan) and allowed to drink water *ad libitum*.

Materials : Methotrexate was obtained from Lederle Japan(Tokyo, Japan) and 5-fluorouracil from Kyowa-(Tokyo, Japan). The magnesium salts of 5, 10-methylenetetrahydrofolate(purity, 96.5%) and tetrahydrofolate(purity, >98%) were obtained from Dr. B. Schircks Laboratories(Jona, Switzerland), and the disodium salt of 5-methyltetrahydrofolate(purity 90%) from Sigma(St. Louis, Mo, USA). 10-Formyltetrahydrofolate was prepared from 5-formyltetrahydrofolate(Lederle Japan, Tokyo, Japan) using the method described by Scott[21]. Sodium ascorbate, sodium acetate, acetic acid, the disodium salt of ethylenediaminetetraacetic acid(EDTA) and *p*-formaldehyde were obtained from Wako(Osaka, Japan). All folate-related compounds were dissolved in 0.2% sodium ascorbate solution except 5, 10-methylenetetrahydrofolate which was dissolved in 0.2% sodium ascorbate solution containing 3×10^{-3}% of *p*-formaldehyde. These solutions were stored at −80℃.

Sampling:The rats were anesthetized with an intraperitoneal injection of ethyl carbamate(1g/kg of body weight). After the abdominal wall had been incised, the bile duct was isolated and cannulated with a polyethylene catheter(PE-10) for the bile collection. Methotrexate was intravenously injected into the jugular vein at doses of 0.3 and 10mg/kg and 5-fluorouracil was also injected at a dose of 20mg/kg. The bile samples were collected into ice-cold tubes containing 0.4% sodium ascorbate solution(bile : sodium ascorbate solution, 1 : 1) at intervals of 30min before and after the injection. After measuring the volume, the bile samples were stored at −80℃ until high-performance liquid chromatography(HPLC) analysis.

Analysis : HPLC with electrochemical detection system was employed for the simultaneous determination of active bile folate derivatives including 5, 10-methylenetetrahydro folate, tetrahydrofolate, 10-formyltetrahydrofolate and 5-methyltetrahydrofolate. Setup of the HPLC system was as follows: an electrochemical detector(LC-4C, Bioanalytical systems. IN. USA), analytical column of phenyl-bonded phase(Nova-Pak phenyl, 4mm, 100×8mm I.D., Waters Assoc., Milford, MA, USA). a pump(LC-9A, Shimadzu, Kyoto, Japan), a fixed-loop injector(100ml, M7125, Rheodyne,

Cotani, CA, USA) and a data processor(C-R4A, Shimadzu). The mobile phase of the HPLC with electrochemical detection system was a mixture of 20mM acetate buffer(pH 5.0) containing 0.1mM EDTA and acetonitrile(95 : 5, v/v). The flow-rate was 0.8ml/min. Detection limits were 0.01~0.02ng/injection for the bile reduced folate derivatives. Bile samples were diluted with 0.2% sodium ascorbate solution containing 1.8×10^{-5}% of *p*-formaldehyde(1 : 10, v/v), and were centrifuged at 5,000g for 2min. After filtration(0.45 μm microfilter), the filtrates(100 μl) were injected onto the HPLC phenyl column.

Biliary excretion kinetics of active folate derivatives : The excretion rates of bile folate derivatives were calculated from their concentration in the bile samples. As a sham operation control, the excretion rates of reduced folate derivatives in the bile were observed up to 4 hrs after cannulation at intervals of 30min. After the injection of methotrexate and 5-fluorouracil, the excretion rates were obtained up to 4 hrs. Prior to the injection of the drugs, bile samples were also collected for the initial data of the biliary excretion kinetics.

Statistical anaylsis : All data were presented as the mean±SE. Statistical analysis of the data was carried out by the Student's *t*-test and $p<0.05$ was defined as statistically significant.

Results

Fig 1 showed the biliary excretion profiles of active folate derivatives including 5, 10-methylenetetrahydrofolate, tetrahydrofolate, 10-formyltetrahydrofolate and 5-methyltetrahydrofolate in sham operation control group. The excretion rates of total and individual active folates in the bile were maintained constant during the 4hr period with no significant differences from the inital values.

Following an intravenous injection of methotrexate at doses of 0.3 and 10mg/kg and 5-fluorouracil at a dose of 20mg/kg, the excretion profiles of active folates in the bile were determined. In the group of methotrexate at 0.3mg/kg(Fig 2), the initial levels of excretion rates were also maintained, though one point at 2.5~3 hr showed a significant decrease. Compared to the initial values, no significant decreases in the bile active folates excretion were observed during the period

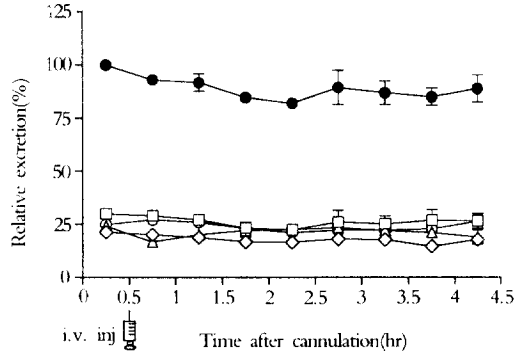

Fig 1. The biliary excretion kinetics of reduced active folate derivatives in sham operation control group. The bile samples were collected during a 4hr period at intervals of 30min. Each point and vertical bar represent the mean±SEM of three different rats. ( ): 5, 10-methylenetetrahydrofolate, ( ): 10-formyltetrahydrofolate, (◇): tetrahydrofolate, (△): 5-methyltetrahydrofolate, and (●): total of bile folate derivatives.

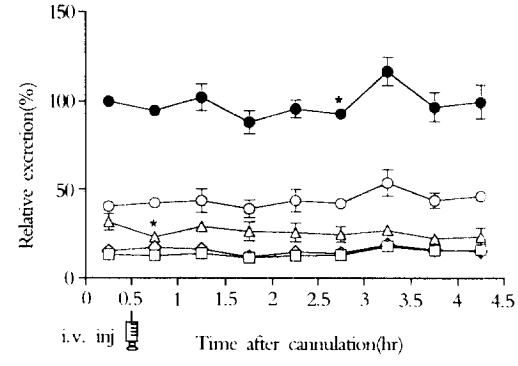

Fig 2. The biliary excretion kinetics of reduced active folate derivative in methotrexate treatment group. The bile samples were collected before and after injection of methotrexate at a dose 0.3mg/kg of body weight up to 4hr at intervals of 30min. Each point represents the meas n±SEM of three different rats. ( ) : 5, 10-methylenetetrahydrofolate, ( ): 10-formyltetrahydrofolate, (◇): tetrahydrofolate, (△): 5-methyltetrahydrofolate, and (●): total of bile folate derivatives.

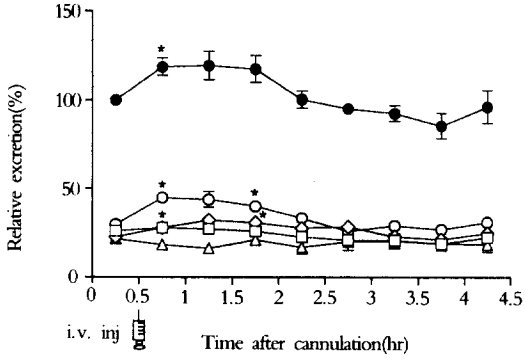

Fig 3. The biliary excretion kinetics of reduced active folate derivatives after an injection of methotrexate 10mg/kg. Each point represents the mean±SEM of three different rats. (○): 5, 10-methylenetetrahydrofolate, (□): 10-formyltetrahydrofolate, (◇): tetrahydrofolate, (△): 5-methyltetrahydrofolate, and (●): total of bile folate derivatives.

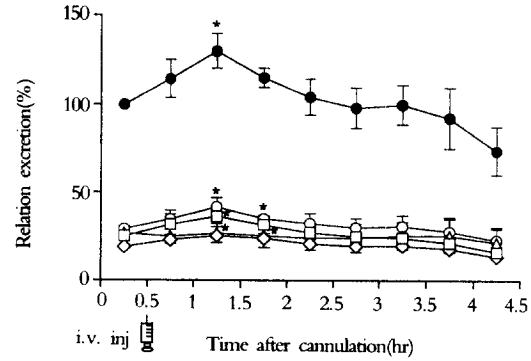

Fig 4. The biliary excretion kinetics of reduced active folate derivatives in 5-fluorouracil 20mg/kg treatment group. The bile samples were collected before and after injection of 5-fluorouracil up to 4hr at intervals of 30min. Each point represents the mean±SEM of three different rats. (○): 5, 10-methylenetetrahydrofolate, (□): 10-formyltetrahydrofolate, (◇): tetrahydrofolate, (△): 5-methyltetrahydrofolate, and (●) total of bile folate derivatives.

of 4 hrs after injection in each group given either one of methotrexate at 10mg/kg(Fig 3) or 5-fluorouracil at 20mg/kg(Fig 4). On the contrary, significant increases in the excretion of bile active folates were observed over 2 hr after injection of the methotrexate at 10mg/kg and 5-fluorouracil at 20mg/kg.

There was no significant difference in absolute values for the initial excretion rates of bile active folates between the groups. Total excretion rates of bile active folate derivatives were approximately 1,500ng/hr/kg in each group. The absolute excretion rates of 5, 10-methylenetetrahydrofolate, tetrahydrofolate, 10-formyltetrahydrofolate and 5-methyltetrahydrofolate were ranged from 250 to 800/ng/hr/kg.

Discussion

The enterohepatic circulation system of folates appeared to be the principal factor for the homeostasis of plasma folate[28,31]. It is well established that the liver is the most important organ in the folate metabolism due to its storage and folylenzyme capacity, and the large flux of folates through the enterohepatic circulation[24,29-31]. Nevertheless, the kinetic properties of folate derivatives in the enterohepatic circulation have poorly been understood. Until recently 5-methyltetrahydrofolate has been considered as the principal folate congener in the enterohepatic circulation system[31]. Recently, Shin et al[23-27] also found non-methylated tetrahydrofolates, including 5, 10-methylenetetrahydrofolate, tetrahydrofolate and 10-formyltetrahydrofolate in the rat bile. Morcover, these non-methylated folates were also reabsorved into the intestinal wall to regulate plasma 5-methyltetrahydrofolate status[28].

Through our serial studies on the toxicity of DHFR inhibitors, we have demonstrated an acute decrease in plasma folate levels by the inhibitors[7,16,32,33]. Since the enterohepatic circulation of folates is the most important system for the regulation of plasma folates[29,30], we have targeted our studies on the system as a possible mechanism for the acute decrease in the plasma folate. It was initially speculated that the decrease in the plasma folate by the inhibitors may be due to either one of the depletion of the bile folate excretion or the intestinal malabsorption of the excreted bile folate derivatives. In the present study, we attempted to investigate the first speculation concerning the biliary excretion

profile employing a DHFR inhibitor, methotrexate, No significant decreases in the excretion kinetics of the bile active folate derivatives were observed after an intravenous bolus injection of methotrexate(Fig 2, 3). Although 10mg/kg of methotrexate was relatively higher dose than that used in clinics for the therapy, there was no acute depletion of bile folates excretion, indicating that the administration of single dose of methotrexate does not affect the hepatobiliary excretion of the folate derivatives. It was considered that the decrease in the plasma folate might not be directly related to the biliary excretion of folates. These results, therefore, suggest that the major factor responsible for the plasma folate depletion by DHFR inhibitors may be the malabsorption of the folate derivatives excreted into the small intestine.

Pharmacokinetic study of methotrexate shows a significant biliary excretion and the intestinal reabsorption profiles[9~11,16], like the bile folate derivatives. Kates et al[15] reported that an inhibition of biliary excretion significantly decreased the elimination of methotrexate. Considering the structure of methotrexate as a folate derivative, it is possible that the drug may cause an impaired absorption of the bile folate derivatives *via* the enterohepatic circulation. The presence of specific transport system, i.e. a folate binding protein, shared by the folate derivatives has been suggested by Selhub et al[12].

Although a main action of methotrexate is the inhibition of DHFR[7], the inhibitory effect on TS was also described elsewhere[1,12~14,20]. Since our present results opened a possibility that methotrexate may reveal a toxic effect *via* the inhibition of TS, the effect of 5-fluorouracil which is a typical TS inhibitor on the biliary excretion of folates was observed in rats. However, the depletion of the bile folate excretion was not observed when 5-fluorourcil was administered at 20mg/kg(Fig 4). On the contrary, the significant increases in the bile tetrahydrofolates excretion were observed at the earlier stage after the injection, similarly to the effect of methotrexate at 10mg/kg. Although we have no evidences for the mechanism of increases in the folates excretion after the injection of methotrexate or 5-fluorouracil, it is possibly due to an elevation of the folate excretion from the hepatic folate pool.

Summary

The biliary excretion kinetics of the active folate derivatives were examined after an intravenous injection of methotrexate at doses of 0.3 and 10mg/kg to clarify the mechanism of the acute decrease in the plasma folate by the dihydrofolate reductase inhibitors. Even at a higher dose than used in the clinical therapy, methotrexate did not cause any acute depletion of folate derivatives in the excreted bile. Therefore, the decrease in the plasma folate appeared not to be related with the biliary excretion process of folates. A factor responsible for the palsma folate depletion by DHFR inhibitors may be due to the malabsorption of folate derivatives excreted into the small intestine.

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