Hepatic methotrexate content and progression of hepatic fibrosis: preliminary findings

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Abstract

Liver tissue from 16 patients with rheumatoid arthritis was studied. The patients had received low dose methotrexate weekly for a minimum of 12 months between two liver biopsies. The progression of pericellular fibrosis was measured by computerised image analysis. Extracts of these liver biopsy specimens were pooled into five samples according to the progression of hepatic fibrosis and analysed by high performance liquid chromatography. The concentrations of methotrexate, 2,4 diamino-*N*¹⁰-methylptericoic acid, and methotrexate polyglutamate were markedly increased in the samples obtained from the three patients who recorded the greatest increase in fibrosis. These preliminary data suggest that progression of hepatic fibrosis is related to the retention of methotrexate and metabolites in the liver.

Hepatic fibrosis is a potential complication of long term methotrexate treatment for psoriasis and rheumatoid arthritis.^{1,2} The relation between the development of fibrosis and the frequency of methotrexate administration in psoriasis^{3,4} and cumulative dose and duration of methotrexate in rheumatoid arthritis⁵ suggests that hepatic accumulation of methotrexate or its metabolites, or both, may play a part in fibrogenesis. Previously used methods of histological grading of methotrexate associated liver injury⁶ are subjective and insensitive to small changes in the degree of fibrosis. In this study we used an image analysis system^{7,8} to measure hepatic collagen in sequential liver biopsy specimens, and describe an assay for hepatic methotrexate and its metabolites, to determine whether there is an association between progressive hepatic fibrosis and the accumulation of hepatic methotrexate and its metabolites.

Patients and methods

Eighteen patients with rheumatoid arthritis were eligible for entry into the study. All patients had had a previous liver biopsy performed and had been taking methotrexate for at least 12 months since the previous biopsy. Five patients had their first biopsy before the start of methotrexate treatment, whereas the remaining 13 patients were taking methotrexate at the time of their first biopsy. Methotrexate was stopped seven days before the second biopsy. At the second biopsy two cores were obtained with a Trucut needle: one core was snap frozen in liquid nitrogen and stored at -90°C for subse-

quent drug analysis and the other core was fixed and processed for histological assessment, including morphometry, as described by Hall *et al* in the preceding paper.⁹

Sixteen patients entered the study—two patients were excluded because insufficient material was obtained for methotrexate analysis. The protocol for this study was reviewed and approved by the medical ethics committees at Flinders Medical Centre and Repatriation General Hospital, Adelaide. All patients gave informed consent.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF METHOTREXATE AND ITS METABOLITES Methotrexate and its metabolites were measured by HPLC by PJH.

Reagents HPLC grade double distilled water was purified by a charcoal filter to remove organic contaminants. Acetonitrile (190 nm grade) and tetrabutylammonium phosphate (Pic A) were obtained from Waters Assoc (Milford, MA, USA). Methotrexate was obtained from David Bull Laboratories (Vic, Australia). Standards for methotrexate polyglutamates G₂, G₃, and G₄—that is, methotrexate with one, two, and three additional glutamyl moieties, were a generous gift of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland, USA; G₅ and G₆ were obtained from American Radiolabelled Chemicals, (St Louis, MO, USA). 2,4-Diamino-*N*¹⁰-methylptericoic acid (DAMPA) was obtained from Sigma (St Louis, MO, USA).

Extraction procedure The extraction and clean up procedure was similar to that of Kamen and Winick¹⁰ with the following adaptations: (1) After extraction and homogenisation samples were laid on diethylaminoethyl-cellulose columns and eluted with 2 ml of 0.15 M ammonium bicarbonate (buffer A) and 2 ml of 0.3 M ammonium bicarbonate (buffer B) to remove impurities. Elution was carried out with 4 ml of 1.0 M ammonium bicarbonate. This elution was subsequently passed through a Sep-Pak column (Waters) as a further clean up procedure. (2) The final eluate was dried under vacuum centrifuge overnight. (3) The dried sample residue was reconstituted in 250 µl of buffer B and 200 µl was injected.

Chromatography Gradient elution was performed on Waters equipment with initially 50% buffer A (5 mM Pic A in water) and 50% buffer

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B (5 mM Pic A in 40% acetonitrile: 60% water) with a slight convex gradient generated over 20 minutes to 100% buffer B where it was held for a further 10 minutes; equilibration time was 15 minutes. Samples were detected at 280 nm wavelength.

Coefficients of variation (for area counts/nmol) for the analysis of DAMPA, methotrexate, and methotrexate diglutamate were 3.4%, 4.7%, and 4.1% respectively. The lower limit of detectability of methotrexate and methotrexate polyglutamates was of the order of 0.2 nmol/sample injection. When this chromatographic assay was used preliminary work suggested that pooling of hepatic tissues would be necessary.

MORPHOMETRY

In a separate study of the same group of patients⁹ we measured increases in hepatic collagen and compared the results obtained using a microcomputer image analysis system with those of a semiquantitative histological grading system. Both methods showed a small but significant increase in pericellular collagen in the group as a whole.

The proportion of pericellular collagen was

Table 1 Details of methotrexate treatment in the study group of 10 women and six men with rheumatoid arthritis

Case No	Age (years)	Sex	Weekly* methotrexate dose (mg)	Total methotrexate dose (mg)	Duration of treatment (months)
1	75	F	10	3245	65
2	41	F	15	5190	80
3	65	F	12.5	4020	58
4	64	M	17.5	5480	72
5	75	F	15	2445	45
6	48	F	15	4505	70
7	64	F	15	1025	13
8	67	M	15	890	13
9	62	F	17.5	4695	49
10	63	M	7.5	405	12
11	52	M	15	5210	73
12	66	F	15	1025	15
13	62	M	15	1075	15
14	61	F	17.5	4060	54
15	67	M	20	3400	40
16	70	F	17.5	4060	54
Mean (SD)	62.6 (9.0)		15 (3)	3171 (1770)	45.5 (24.6)
Range	41–75		7.5–20	405–5480	12–80

*Most commonly used dose.

Time between biopsies: mean (SD) 25.4 (8.3) months (range 12–144).

Dose between biopsies: mean (SD) 1782 (753) mg (range 405–3210).

Table 2 Pooling of samples according to increases in percentage of pericellular collagen

Sample No	Case No	Fibrosis changes (%)	DAMPA* (pmol/mg wet wt)	MTX* (pmol/mg wet wt)	MTX-G ₂ * (pmol/mg wet wt)
1	7	4.11	47.9	232	28.7
	9	4.05			
	2	3.65			
2	1	3.41	9.8	5.64	0
	14	2.87			
	10	2.06			
	6	2.02			
3	5	1.54	14.4	17.3	0
	16	0.77			
	15	0.70			
4	4	0.64	13.7	13.9	0
	12	0.30			
	3	0.05			
5	11	-0.44	8.5	8.3	0
	8	-0.62			
	13	-1.01			

*DAMPA=2,4-diamino-N¹⁰-methylpterico acid; MTX=methotrexate; MTX-G₂=methotrexate diglutamate.

measured in 4 µm sirius stained sections by computerised image analysis.^{7,9} Briefly, software was developed for detecting, editing, and measuring pericellular collagen. A constant threshold was used and the measurements were performed by one person with no knowledge of the patients or their liver biopsies. Collagen measurements were expressed as the percentage area of collagen per tissue area.

To ensure that sufficient quantities of methotrexate and metabolites were present the liver biopsy specimens were pooled. The grouping was determined by ranking the biopsy specimens in order of increase in percentage area of pericellular collagen. The samples were pooled, coded to mask knowledge of the morphometric results, and then assayed.

Results

Table 1 gives details of the 16 patients who entered the study, including their weekly and total methotrexate doses.

Table 2 gives the percentage increase of pericellular collagen in the patients' liver biopsy specimens and shows the method of pooling of hepatic tissue to obtain the samples 1–5.

The pooled hepatic tissue from the three liver biopsy specimens showing the greatest increase in pericellular collagen (sample 1) had markedly raised concentrations of methotrexate, DAMPA, and methotrexate diglutamate (table 2). In the figure (A) shows the profile of methotrexate polyglutamate standards and (B) the hepatic methotrexate polyglutamate profile obtained from sample 1. No methotrexate G₃, G₄, or G₅ were detected in any of the samples. The weekly

(A) The elutions of known synthetically prepared methotrexate polyglutamate standards. G₂, G₃ etc, indicate the number of glutamate residues. The parent molecule of methotrexate (MTX) has one glutamate. DAMPA=2,4-diamino-N¹⁰-methylpterico acid. (B) Pooled hepatic tissue from three liver biopsy specimens (see table 2) showing the greatest increase in pericellular collagen constituting sample 1, and subjected to high performance liquid chromatography. In (A) and (B) the horizontal axis represents elution volumes, the vertical axis represents absorbance units.

methotrexate doses in the three patients whose biopsy specimens constituted sample 1 were 15, 17.5, and 15 mg: their respective cumulative doses were 1025, 4695, and 5190 mg. Because only five samples were assayed statistical analysis of the data is not possible.

Discussion

The aim of this study was to determine if there was an association between progressive hepatic fibrosis and accumulation of hepatic methotrexate and its metabolites. To measure pericellular collagen we used a computerised image analysis system on paired liver specimens.⁹ The hepatotoxic effects of methotrexate cannot be reliably assessed unless a previous liver biopsy specimen, preferably obtained before methotrexate treatment, is available for comparison as methotrexate induced changes are non-specific and pretreatment liver biopsy specimens commonly contain histological abnormalities.^{2 11}

The methotrexate polyglutamates not only have potent enzyme inhibitory activity similar to that of the parent compound but are also retained intracellularly for prolonged periods.¹² For this reason they are thought to play a part in chronic methotrexate toxicity, such as hepatic fibrosis and leucoencephalopathy.¹³ Kremer *et al* were the first to show the presence of methotrexate in a predominantly polyglutamated form in liver tissue of patients given low doses of methotrexate for rheumatoid arthritis.¹⁴ We were able to detect methotrexate in pooled samples 2–5 in similar concentrations to that reported by Kremer,¹⁴ but were unable to find polyglutamates G₃–G₅. There was notably a striking increase in methotrexate and methotrexate diglutamate in our first sample, obtained by pooling tissue from the three patients with the greatest increase in pericellular collagen. Even in this sample, however, the higher polyglutamates did not predominate. Explanations for the differences between Kremer's study and our findings are: firstly, the time between ingestion of methotrexate and the liver biopsy was standardised but might have differed from that in Kremer's study (timing not stated by Kremer). Secondly, different methods of measuring methotrexate and its polyglutamates were used. We used identical means of resolving the individual polyglutamates with HPLC, but we used ultraviolet absorption as a means of detection, which we feel is a more specific method of detecting the substrates than the competitive protein binding assay used by Kremer. Thirdly, as the concentrations of DAMPA, methotrexate, and methotrexate diglutamate are markedly increased in sample 1, one of the patients comprising sample 1 might have inadvertently or deliberately ingested methotrexate immediately before the liver biopsy. This was denied by the patients and their families, however, and serum methotrexate concentrations measured at the time of the biopsy suggested that no ingestion had occurred within the previous 36 hours. Finally, the hydrolysis of the polyglutamates by endogenous hepatic conjugase might have occurred *ex vivo*. The cores were snap frozen in liquid nitrogen

and stored at –90°C; hence this explanation is unlikely, though it is possible that hydrolysis might have occurred immediately before assay. The failure to detect higher polyglutamates (G₃–G₅) supports this explanation. Further experiments are proceeding to investigate this possibility.

Because of the relative insensitivity of the HPLC assay, and because of the small amounts of hepatic tissue that are available on biopsy, pooling of liver tissue was necessary. This pooling increases the risk of error and reduces the amount of material for analysis. These liver biopsies will be repeated in two years, allowing further assessment of the relation between methotrexate, its metabolites, and hepatic fibrosis.

Our data suggest that there is no linear relation between the accumulation of methotrexate or its metabolites, or both, and progression of hepatic fibrosis. Also, our patients showed very minor changes in percentage increase of pericellular collagen, none sufficient to justify stopping the methotrexate treatment. With more marked morphometric changes the relation between hepatic fibrosis and methotrexate metabolites, if it exists, should become more evident.

During enterohepatic circulation of methotrexate the glutamyl residue is removed by intestinal bacterial carboxypeptidases (conjugases) to produce DAMPA, which has 1% of the dihydrofolate reductase inhibitory activity of methotrexate. Its presence in blood and urine may lead to spuriously increased methotrexate concentrations because of substantial cross reactivity in immunoassays.¹⁵ With reverse phase HPLC, methotrexate polyglutamate retention times increase proportionately with the successive addition of glutamyl residues; therefore DAMPA predictably eluted before methotrexate. Kremer *et al*¹⁴ did not report DAMPA in their tissue samples, possibly because they used a competitive protein binding assay, which relies on inhibition of dihydrofolate reductase for detection of methotrexate in column fractions. In our study it is uncertain whether the DAMPA found in the liver originated from the gut or was an intrahepatic degradation product of methotrexate, or, alternatively, occurred as a result of *ex vivo* hydrolysis.

Whether methotrexate polyglutamates mediate chronic toxicity through prolonged inhibition of the enzymes participating in folate metabolism or through folate depletion resulting from methotrexate accumulation is debated.^{11 14 16} In either event cellular injury could occur from deficient nucleotide and methionine biosynthesis. Hendel *et al* found that low erythrocyte folate and high methotrexate concentrations distinguished the six patients with psoriasis who had progressive changes in liver morphology from 25 others without such changes.¹⁷ These six patients merely had increased fatty change and non-specific reactive hepatitis, however.

In a pharmacokinetic study Jones *et al* noted that psoriatic patients with hepatic fibrosis had areas under the concentration versus time curve comparable with those of subjects without

fibrosis despite receiving larger methotrexate doses¹⁸; this suggested that the group with fibrosis had greater hepatic retention of methotrexate.

Although our study does not establish a causal relation between methotrexate, its metabolites, and progression of hepatic fibrosis, it does suggest that there is a relation between hepatic content of methotrexate and the development of fibrosis. Further longitudinal studies required to establish this relation are in progress. Elucidation of the kinetics of polyglutamate synthesis and metabolism in the human liver should lead to a better understanding of the mechanisms of liver injury.

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