Anti-Cancer Drugs 1994, **5**, pp. 194–198

Selective delivery of a greater amount of methotrexate to regional lymph nodes using a new dosage formulation, methotrexate adsorbed on activated carbon particles, in rats

Akeo Hagiwara,[1] Toshio Takahashi,[1] Kiyoshi Sawai,[1] Chouhei Sakakura,[1] Masahide Yamaguchi,[1] Kimihiko Osaki,[1] Hiroyuki Tsujimoto,[1] Takayuki Ohyama,[1] Hiroshi Tomoda,[2] Akira Yamamoto[2] and Shozo Muranish[2]

[1]First Department of Surgery, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602, Japan. Fax: (+81) 75 251 5522. [2]Department of Biopharmaceutics, Kyoto College of Medicine, Misasagi-Nakauchi-cho, Yamashina-ku, Kyoto 607, Japan.

A new dosage formulation (MTX-CH) comprising methotrexate (MTX) adsorbed onto a suspension of activated carbon particles was developed for the treatment of lymph node metastases. *In vitro* studies showed that activated carbon particles adsorbed a great amount of MTX. Subcutaneous injection of MTX-CH into the left hind foot pad of rats distributed a greater amount of MTX selectively to the regional lymph nodes (the left popliteal node and the para-aortic nodes) for a longer period of time than similar administration of MTX aqueous solution with a smaller amount of MTX distributed to the rest of the whole body.

Key words: Methotrexate, activated carbon, drug distribution, lymph node metastasis.

Introduction

Digestive tract cancers in relatively early stages may be treated with endoscopic local resection or local injection of anticancer drugs, especially in patients with poor condition and a high surgical risk due to cardiac disease or old age. These forms of endoscopic local therapy are not always successful because local excision or drug injection may be effective for the primary lesion without influencing regional lymph node metastases.

For the treatment of metastatic lesions as well as the primary lesion, we have developed a new dosage formulation of an anticancer drug. Locally administered drugs in aqueous solution are rapidly absorbed through blood capillary walls into the circulating blood[1] and are not distributed selectively to the regional lymph nodes.[2] In contrast, very small particles such as activated carbon are absorbed through lymphatic capillaries[3] and retained in the regional lymph nodes for a long period of time.[4,5] Utilizing this difference in the absorption of aqueous solutions and particles, a new dosage formulation which comprises methotrexate (MTX) adsorbed onto fine activated carbon particles was designed to distribute a greater amount of MTX to the regional nodes and to enhance the therapeutic effects on the metastases in such nodes.

This paper describes the selective distribution of a greater amount of MTX to the regional lymph nodes using this new dosage formulation in animal experiments.

Materials and methods

Preparation of drugs

Fine activated carbon particles (Activated Carbon M-1500, prepared in our laboratory), which were 20 nm in diameter for primary particles and 1480 m^2/g in specific surface area, and polyvinylpyrrolidone (Polyvinylpyrrolidone K-30®, Nakarai Chemicals Co., Kyoto, Japan) at a ratio of 5:3 were mixed in saline and kneaded with three rollers, with the addition of saline droplets for dilution into a composition of activated carbon at 50 mg/ml and polyvinylpyrrolidone at 30 mg/ml in saline. This procedure resulted in a suspension of particles with an average size of 167 nm.[3] The activated carbon suspension was sealed in a glass tube and sterilized

This work was supported, in part, by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, and from the Ministry of Education, Science, and Culture, Japan.

Correspondence to A Hagiwara

at 120°C for 10 min. Methotrexate (MTX, Methotrexate®, Lederle Laboratories Division, American Cyanamide Co., Pearl River, NY) at 25 mg/ml was added to the activated carbon suspension and the mixture was shaken for 6 h at 37°C to bring the adsorption to equilibrium. Thus, the new dosage formulation (MTX-CH) comprised 25 mg/ml of MTX, 50 mg/ml of activated carbon and 30 mg/ml of polyvinylpyrrolidone in saline.

As control drugs, a MTX aqueous solution (MTX-sol), comprising 25 mg/ml of MTX in saline, and an activated carbon suspension without MTX (carbon suspension), comprising 50 mg/ml of activated carbon and 30 mg/ml of polyvinylpyrrolidone in saline, were prepared.

Adsorption *in vitro*

The adsorption isotherm of MTX onto activated carbon was determined as follows. MTX at 1 mg/ml was dissolved in 5 ml of distilled water. The resulting solution, which had a pH of 7.2, was raised to a pH of 7.4 with phosphate buffer solution. Activated carbon at 1, 2, 2.5 or 5 mg/ml was added to the MTX solution, which was then shaken at 120 c.p.m. for 1 h at 37°C so that the adsorption would be in a state of equilibrium. After the mixture was centrifuged at 3000 c.p.m. for 10 min, activated carbon particles were removed with a filter, and the MTX concentration in the filtrate was measured by high performance liquid chromatography (HPLC, Shimadzu C-R4A Chromatopac®; Shimadzu Co., Kyoto, Japan) and UV detection (Hitachi Model 655-051 UV Detector®, Hitachi Co., Tokyo, Japan) at a wave-length of 305 nm.[6,7] This experiment was repeated three times. The equilibrium points were set on log–log scale abscissa and the adsorption isotherm line was drawn along the points.

The adsorption isotherm was also measured using the same procedures in saline, in which the pH of the MTX solution was 7.9–8.3.

Drug distribution

Thirty rats (Donryu strain, 200 g body weight; Shimizu Animal Center) were divided into two groups (the MTX-CH group and the MTX-sol group) composed of 15 rats each.

MTX at 30 mg/kg was injected subcutaneously into the left hind foot pad, in the form of either MTX-CH in the MTX-CH group or MTX-sol in the MTX-sol group. Three rats in each group were sacrificed at 0.5, 1, 3, 6 and 12 h after injection. Blood was taken from the heart by heart paracentesis, and was separated into plasma and blood cells by centrifugation at 6000 r.p.m. for 5 min, and the blood plasma was stored for MTX concentration assay. The left popliteal lymph node and the para-aortic lymph nodes, which were the first- and second-level regional nodes, respectively, the injection site, kidneys, liver, and spleen were removed for MTX concentration assay. The tissue samples were weighed with a microbalance and were kept at −80°C.

MTX concentration in the samples was measured by HPLC and UV detection as described previously. The effective assay limitation was 5×10^{-7} M of solution or 5×10^{-7} mol/kg of tissue (equal to 0.23 μg/ml or μg/g).

When the MTX concentration was detectable in all three samples from rats sacrificed at one time point, the MTX concentration was compared statistically between the two dosage formulation groups by the analysis of variance. The difference was considered significant when the p value was less than 0.05. When the MTX level was less than the assay limit in any one of the three samples from the same time point, the MTX level was defined as 'not detectable'.

Results

Adsorption and desorption of MTX

The absorption isotherm was defined by $Q = 171\ C^{0.15}$ in distilled water prepared at 7.4 pH by phosphate buffer solution at 37°C (where Q is the amount of MTX adsorbed onto the activated carbon expressed in μg/mg and C is the concentration of MTX in a free state expressed in μg/ml), and by $Q = 270\ C^{0.16}$ in saline at 37°C (Figure 1). Therefore, in the preparation of MTX-CH, MTX at 49 μg/ml (equal to 1.1×10^{-4} M) is in a free state and the other MTX (24951 μg/ml) is absorbed on 50 mg of activated carbon.

Drug distribution in rats

MTX concentration in the left popliteal lymph node (the first level regional node) is shown in Table 1. In the MTX-CH group, the mean MTX concentration was 155×10^{-6} and 240×10^{-6} mol/kg, at 0.5 and 1 h after administration, respectively. These MTX

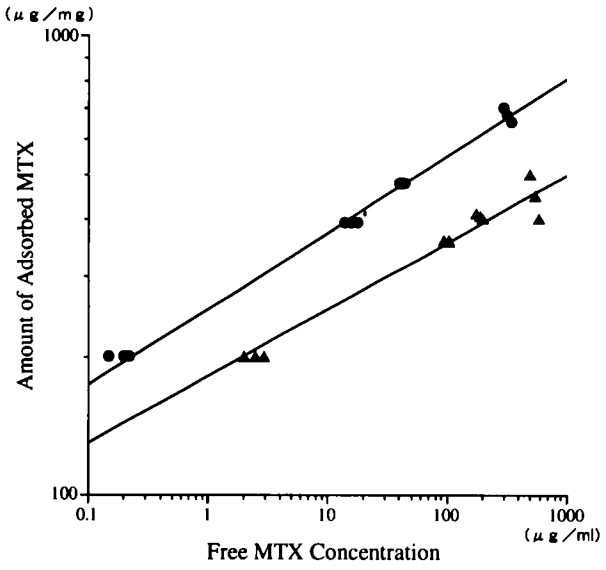

Figure 1. Adsorption isotherm of MTX onto activated carbon. The adsorption isotherms at 37°C in distilled water at pH 7.4 with buffer solution (triangles) and in saline (squares) are given by $Q = 171\ C^{0.15}$ and $Q = 270\ C^{0.16}$, respectively (see text).

concentration values were statistically significantly ($p < 0.025$ and $p < 0.005$) greater, by about 10 and 2.5 times, than those in the MTX-sol group (15.8×10^{-6} and 96.0×10^{-6} mol/kg, at 0.5 h and 1 h after administration, respectively). MTX concentration in the para-aortic nodes (the second-level regional nodes) is shown in Table 2. In the MTX-CH group, the MTX concentration level was also higher (at 0.5 and 3 h after administration, $p<0.05$) than that in the MTX-sol group.

MTX concentration at the injection site is shown in Table 3. In the MTX-CH group, the MTX level was 244 and 254×10^{-6} mol/kg at 0.5 and 1 h after administration, respectively, and slowly decreased with time: at 12 h after administration, the MTX concentration was 15.5×10^{-6} mol/kg. In contrast, in the MTX-sol group, the MTX level was 367×10^{-6} mol/kg at 0.5 h after administration and rapidly decreased to 1.6×10^{-6} mol/kg at 12 h after administration. The MTX level in the MTX-CH group was significantly higher than that in the MTX-sol group ($p < 0.05$ and 0.01 at 3 and 12 h after administration, respectively).

Thus, MTX concentrations in the regional nodes and the injection site were maintained at higher levels for longer periods of time in the MTX-CH group than in the MTX-sol group.

MTX concentration in blood plasma is shown in

Table 1. MTX concentration in the left popliteal lymph node

Time after administration (h)	Methotrexate concentration mean and [95% CI][a] ($\times 10^{-6}$ mol/kg)		Statistical significance[b]
	MTX-CH group	MTX-sol group	
0.5	155 [87.8 to 223]	15.8 [0 to 83.3]	$p < 0.025$
1	240 [205 to 275]	96.0 [62 to131]	$p < 0.005$
3	17.7 [3.2 to 32.2]	1.8 [−12.7 to 16.3]	NS[c]
6	ND[d]	ND	—

[a]Mean and 95% confidence interval for three experiments.
[b]By analysis of variance.
[c]Not significant.
[d]Not detectable.

Table 2. MTX concentration in the para-aortic lymph nodes

Time after administration (h)	Methotrexate concentration mean and [95% CI][a] ($\times 10^{-6}$ mol/kg)		Statistical significance[b]
	MTX-CH group	MTX-sol group	
0.5	11.7 [9.4 to 14.1]	7.7 [5.4 to 10.0]	$p < 0.05$
1	32.3 [2.0 to 62.5]	30.5 [0.3 to 60.7]	NS[c]
3	4.5 [1.7 to 7.2]	0.5 [−2.2 to 3.2]	$p < 0.05$
6	ND[d]	ND	—

[a]Mean and 95% confidence interval for three experiments.
[b]By analysis of variance.
[c]Not significant.
[d]Not detectable.

Table 3. MTX concentration in the injection site

Time after administration (h)	Methotrexate concentration mean and [95% CI][a] ($\times 10^{-6}$ mol/kg)		Statistical significance[b]
	MTX-CH group	MTX-sol group	
0.5	244 [139 to 350]	367 [261 to 473]	NS[c]
1	254 [127 to 382]	105 [−23 to 233]	NS
3	47.4 [21.8 to 72.9]	7.9 [−17.6 to 33.5]	$p < 0.05$
6	39.7 [0.5 to 79.0]	1.0 [−38.3 to 40.3]	NS
12	15.5 [9.8 to 21.1]	1.6 [−4.1 to 7.2]	$p < 0.01$

[a]Mean and 95% confidence interval for three experiments.
[b]By analysis of variance.
[c]Not significant.
[d]Not detectable.

Table 4. MTX concentration in blood plasma

Time after administration (h)	Methotrexate concentration mean and [95% CI][a] ($\times 10^{-6}$ mol/kg)		Statistical significance[b]
	MTX-CH group	MTX-sol group	
0.5	56.0 [0 to 112]	70.7 [0 to 83.3]	NS[c]
1	16.3 [7.3 to 25.3]	28.9 [20.0 to 37.4]	NS
3	3.2 [1.8 to 4.5]	2.1 [0.7 to 3.4]	NS
6	0.7 [0.4 to 1.1]	ND[d] —	—
12	ND	ND	—

[a]Mean and 95% confidence interval for three experiments.
[b]By analysis of variance.
[c]Not significant.
[d]Not detectable.

Table 4. There was no significant difference in MTX concentration between the two dosage formulation groups. At 12 h after administration, MTX was not detectable (less than 5×10^{-7} M) in either group.

MTX concentration in the kidney, from which more than 90% of systemically administered MTX is secreted, is shown in Table 5. MTX concentration in the kidney was maintained at a relatively low level in the MTX-CH group. In the MTX-sol group, the mean MTX level was 32.0×10^{-6}mol/kg at 0.5 h and 17.0×10^{-6} mol/kg at 1 h, which were significantly higher ($p < 0.05$) than the MTX concentrations at the same time in the MTX-CH

Table 5. MTX concentration in the kidney

Time after administration (h)	Methotrexate concentration mean and [95% CI][a] ($\times 10^{-6}$ mol/kg)		Statistical significance[b]
	MTX-CH group	MTX-sol group	
0.5	13.4 [1.6 to 25.3]	32.0 [20.1 to 43.8]	$p < 0.05$
1	11.0 [7.6 to 14.5]	17.0 [13.6 to 20.5]	$p < 0.05$
3	3.5 [2.2 to 4.7]	2.4 [1.2 to 3.6]	NS[c]
6	2.7 [1.4 to 3.8]	2.2 [1.0 to 3.4]	NS
12	1.0 [0.7 to 1.4]	1.3 [0.9 to 1.6]	NS
24	ND[d]	ND	—

[a]Mean and 95% confidence interval for three experiments.
[b]By analysis of variance.
[c]Not significant.
[d]Not detectable.

Table 6. MTX concentration in the liver

Time after administration (h)	Methotrexate concentration mean and [95% CI][a] ($\times 10^{-6}$ mol/kg)		Statistical significance[b]
	MTX-CH group	MTX-sol group	
0.5	12.6 [2.0 to 23.3]	14.4 [3.8 to 25.0]	NS[c]
1	7.0 [3.5 to 10.4]	5.7 [2.3 to 9.2]	NS
3	1.5 [0.2 to 2.7]	1.6 [0.3 to 2.8]	NS
6	1.6 [1.0 to 2.2]	0.9 [0.3 to 1.5]	NS
12	ND[d]	ND	—

[a]Mean and 95% confidence interval for three experiments.
[b]By analysis of variance.
[c]Not significant.
[d]Not detectable.

group. In both groups, the MTX concentration became non-detectable at 24 h after administration.

MTX concentrations in the liver and the spleen are shown in Tables 6 and 7. In these organs, there were no remarkable differences in MTX concentration between the two dosage formulation groups.

Discussion

Adsorption experiments *in vitro* showed that activated carbon adsorbed a great amount of MTX onto the surface of particles.

Drugs composed of water soluble small molecules, such as most anticancer drugs, are not distributed selectively to the regional lymphatic system but are absorbed smoothly into the circulating

Table 7. MTX concentration in the spleen

Time after administration (h)	Methotrexate concentration mean and [95% CI][a] ($\times 10^{-6}$ mol/kg)		Statistical significance[b]
	MTX-CH group	MTX-sol group	
0.5	5.3 [−16.2 to 26.7]	14.6 [20.1 to 43.8]	NS[c]
1	8.4 [6.3 to 10.7]	8.3 [6.1 to 10.5]	NS
3	2.5 [2.0 to 3.0]	3.1 [2.6 to 3.6]	NS
6	ND[d]	ND	—

[a]Mean and 95% confidence interval for three experiments.
[b]By analysis of variance.
[c]Not significant.
[d]Not detectable.

blood, following injection into the tissues in the form of aqueous solutions.[1] This is because water soluble small molecules in aqueous solution easily pass through blood capillary walls into the circulating blood.[2] On the other hand, corpuscular particles, such as fine activated carbon particles, are absorbed gradually through lymphatic capillaries,[3] and are retained at the injection site and in the regional lymph nodes.[4,5]

The present drug distribution experiment showed that MTX concentrations at the injection site and in the regional nodes were maintained at relatively higher levels for longer periods of time in the MTX-CH group than in the MTX-sol group. On the other hand, MTX levels in the blood plasma did not differ between the two groups. Moreover, in the MTX-sol group, the MTX concentration in the kidney, from which most of the systemically administered MTX is secreted into the urine,[8] was higher at 1 and 3 h after administration than in the MTX-CH group. Thus, locally injected MTX-CH maintains the MTX concentration selectively at higher levels for longer periods of time in the regional nodes and the injection site than can be achieved by MTX-sol. The anti-cancer effects of MTX depend on the duration of acting time rather than the exposure concentration.[9] Therefore, the results of the drug distribution experiment suggest that the therapeutic effects on metastatic lesions in the regional lymph nodes as well as on the primary lesion will be greater when MTX-CH is injected into and around the primary cancer tissues, as compared with MTX aqueous solution.

Conclusion

We conclude that locally injected MTX-CH distributes greater amounts of MTX for longer periods of time selectively to the regional lymph nodes and the injection site than MTX aqueous solution does.

References

1. Ballard BE. Biopharmaceutical consideration in subcutaneous and intramuscular drug administration. *J Pharmac Sci* 1968; **57**: 357–78.
2. Rusznyak I, Foldi M, Szabo G. Structure of the lymph-capillary wall—passage of corpuscular particles into the lumen of capillaries. In: Youlten L, ed. *Lymphatics and lymph circulation—physiology and pathology.* Oxford: Pergamon Press 1967: 419–22.
3. Hagiwara A, Takahashi T, Sawai K, *et al.* Lymph nodal vital staining with newer carbon particle suspensions compared with india ink—experimental and clinical observations. *Lymphology* 1992; **25**: 84–9.
4. Hagiwara A, Iwamoto A, Ahn T, *et al.* Anticancer agents adsorbed by activated carbon particles—a new form of dosage enhancing efficacy on lymph nodal metastasis. *Anticancer Res* 1986; **6**: 1005–8.
5. Hagiwara A, Takahashi T, Iwamoto A, *et al.* Selective distribution of aclarubicin to regional lymph nodes with a new dosage form: aclarubicin adsorbed on activated carbon particles. *Anti-Cancer Drugs* 1991; **2**: 261–6.
6. Canfell C, Sad'ee W. Methotrexate and 7-Hydroxymethotrexate—serum level monitoring by high-performance liquid chromatography. *Cancer Treat Rep* 1980: **64**: 165–9.
7. Howell SK, Wang Y-M, Hosoya P, *et al.* Plasma methotrexate as determined by liquid chromatography, enzyme inhibition assay, and radioimmunoassay after high-dose infusion. *Clin Chem* 1980; **26**: 734–7.
8. Henderson ES, Addamson RH, Denham C, *et al.* The metabolic fate of tritiated methotrexate—1. Absorption, excretion, and distribution in mice, rats, dogs and monkeys. *Cancer Res.* 1965; **25**: 1008–17.
9. Keefe AD, Capizzi RL, Rudnick SA. Methotrexate cytotoxicity for L5178/Asn$^-$ lymphoblast—relationship of dose and duration of exposure to tumor cell viability. *Cancer Res* 1982; **42**: 1641–5.

(Received 8 December 1993; accepted 16 December 1993)