

Methotrexate Bound to Carbon Particles Used for Treating Cancers with Lymph Node Metastases in Animal Experiments and a Clinical Pilot Study

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BACKGROUND. A new dosage formulation, methotrexate adsorbed onto activated carbon particles (MTX-CH), was developed for treating cancer patients with lymph node metastases.

METHODS. MTX-CH injected subcutaneously into the left hind foot pad of rats delivered greater amounts of methotrexate selectively to the regional lymph nodes and to the injection site for longer periods than did the same dose of methotrexate in aqueous solution. Seven days after mice received a subcutaneous inoculation with 5×10^5 P388 leukemia cells in the left hind foot pad, when metastasis had occurred in the left popliteal lymph node, methotrexate was injected subcutaneously into the left hind foot pad in the form of either MTX-CH or methotrexate aqueous solution. In clinical trials, patients with cancers in relatively early stages (5 patients with gastric cancer and 1 with esophageal cancer) each received local injections of MTX-CH at MTX doses of 250 to 500 mg under fiberscope.

RESULTS. MTX-CH extended the survival time better than did methotrexate aqueous solution. In mice who received an inoculation of P388 leukemia cells and drug treatment using the same procedures, the treatment effects on metastases to the regional lymph nodes were significantly greater in mice treated with the MTX-CH than in those given methotrexate aqueous solution. MTX-CH controlled the disease well for 25 to 35 months.

CONCLUSIONS. Local injection of MTX-CH is apparently useful for treating digestive cancers with lymph node metastases in patients who have difficulty tolerating surgery. *Cancer* 1996; 78:2199–209. © 1996 American Cancer Society.

KEYWORDS: chemotherapy, methotrexate, activated carbon, P388 leukemia, lymph node metastasis, drug-delivery system, digestive cancer.

Examinations of surgically resected specimens revealed that cancers in the upper digestive tract metastasize to the regional lymph nodes in 20–30% of patients, even when cancer invasion is limited to the mucosa or submucosa.^{1,2} Therefore, even in patients with such superficial cancers, treatment for potential lymphatic metastases is essential. Patients with upper digestive tract cancer can not always receive surgical treatment due to the risks it poses them. For those patients, endoscopic mucosal resection or local injection of anticancer drugs is commonly selected.^{3,4} Although these therapies are effective on the primary lesion, no therapeutic effects on metastatic lesions in the regional lymph nodes are confirmed. This is because locally injected drugs in aqueous solution form are rapidly absorbed through blood capillaries into circulatory blood and therefore do not work on the regional lymph nodes.⁵

To enhance the delivery of drugs to the metastatic lesions in the

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regional lymph nodes, we have developed a new formulation of methotrexate. We previously reported that very small activated carbon particles are absorbed through the lymphatic capillaries and are retained in the regional lymph nodes for long periods.⁶ Based on this difference in retainability between aqueous solutions and very small activated carbon particles, we developed a new formulation (MTX-CH), composed of methotrexate (MTX) adsorbed onto activated carbon particles, to distribute a large amount of drug selectively to the regional lymph nodes as well as to the injection site.

A method has been established to indicate the therapeutic effects on lymph node metastases by quantifying the number of viable malignant cells in the lymph nodes.⁷ Applying this method, we describe in the current article a novel way to compare and assess the effects of different anticancer agents on regional lymph node metastases in mice. This article also reports drug distribution of MTX-CH and MTX aqueous solution in rats and describes our clinical trials using MTX-CH in patients with relatively early stage digestive cancers.

MATERIALS AND METHODS

Drug Preparation

The new dosage formulation (MTX-CH) is composed of methotrexate adsorbed onto fine activated carbon particles in suspension. Fifty mg/mL of activated carbon M-1500 (with a specific surface area of 1500 m²/g and a diameter of 20 nm, prepared in our laboratory) and 30 mg/mL of polyvinylpyrrolidone (PVP K-30, Nakarai Chemicals Co., Ltd., Kyoto, Japan) in saline were kneaded with rollers to make a suspension of 167 nm average particle size.⁶ Twenty-five mg/mL of MTX for clinical use (Methotrexate Sodium Parenteral, Lederle Laboratories Division, American Cyanamid Co., Pearl River, NY) were dissolved in the activated carbon suspension, and the resulting solution was shaken for 6 hours to allow the dissolved MTX to be adsorbed onto the carbon particles. As a control, the same concentration of MTX for clinical use was dissolved in saline (MTX-sol). Drugs were used within 6 hours of preparation.

Adsorption of MTX onto Activated Carbon In Vitro

The adsorption isotherm of MTX onto activated carbon was determined. MTX at 1 mg/mL was dissolved in 5 mL of physiologic saline. Activated carbon at 1, 2, 2.5, or 5 mg/mL was added to the MTX solution, which was then shaken at 120 cycles per minute (cpm) for 1 hour at 37 °C so that the adsorption would be in a state of equilibrium. After the mixture was centrifuged at 3,000 cpm for 10 minutes, activated carbon

particles were removed with a filter, and the MTX concentration in the filtrate was measured by high-performance liquid chromatography (C-R4A Chromatopac, Shimadzu Co., Ltd., Kyoto) and UV detection (655-051 UV detector, Hitachi Co., Ltd., Tokyo) at a wavelength of 305 nm.^{8,9} 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.

Drug Distribution in Rats

Thirty rats (Donryu strain, 200 g body weight, Shimizu Laboratory Animal Center, Kyoto) were divided into 2 groups of 15 rats each (MTX-CH group and MTX-sol group). MTX, 30 mg/kg, was injected subcutaneously into the left hind foot pad as either MTX-CH or MTX aqueous solution. Three rats in each group were sacrificed 0.5, 1, 3, 6, and 12 hours after injection. Blood was taken by heart puncture. Plasma for assay of MTX concentration was separated from blood cells by centrifugation at 6,000 cpm for 5 minutes. The left popliteal lymph node (the first-level regional lymph node), the lumbar lymph nodes (the second-level regional lymph nodes), and the injection site were sampled to determine the concentration of MTX. Samples were stored at -80 °C until assayed. The tissue samples were homogenized in three times their volume distilled water. The tissue fractions and carbon particles were removed by centrifugation at 6,000 cpm for 5 minutes. The supernatant was subjected to measurement of MTX concentration by high-performance liquid chromatography, as in our earlier description of the measurement of the adsorption of MTX on activated carbon *in vitro*. The assay was triplicated for each tissue sample. The result of MTX assay for each tissue sample was expressed by the mean triplicate values in terms of the MTX content in 1 g of the tissue sample or in 1 mL of blood plasma.

The lower limit of the assay was 5×10^{-7} to 5×10^{-10} mol/L or mol/kg of sample (equal to 0.23–0.0023 µg/mL or µg/g), since the sample was sometimes smaller than 5×10^{-3} cm³. When the MTX concentration was below the assay limit in any one of the three samples taken at the same time, the MTX concentration was reported as not detectable. When the MTX concentration was detectable in all of the samples taken at the same time, the MTX concentration was compared statistically between the two groups by analysis of variance.

Experimental Model in Mice

Animals and cancer cell line

Mouse leukemia P388 cell line, maintained through intraperitoneal implantation in DBA2Cr mice, was

used as an experimental tumor because subcutaneously implanted P388 leukemia is known as an experimental model of lymph node metastasis of cancer in mice.^{7,10} Ascites fluid containing P388 leukemia cells taken from a carrier mouse was diluted with Hanks' solution to make a suspension containing 10^7 P388 leukemia cells/mL. The viability of tumor cells exceeded 95%, as determined by the trypan blue exclusion test.

CDF1 male mice (5 weeks old), obtained from the Shimizu Laboratory Animal Center, Kyoto, were used. They were kept under standard conditions (pathogen-free, room temperature of 22 °C, relative humidity of 60%, day-night cycle of 12 hours) during the experiment.

Lymph node metastases in experimental model

To determine whether P388 leukemia cells had metastasized into the regional lymph nodes 7 days after inoculation, 20 mice were injected subcutaneously with a suspension of P388 leukemia cells at 0.05 mL/mouse (equal to 5×10^5 cells/mouse) in the left hind foot pad on Day 0. The mice were sacrificed on Day 7. The lymph node in the left popliteal fossa (the left popliteal lymph node; usually there is one such lymph node per mouse) was removed from each mouse and minced with scissors in one mL of Hanks' solution to make a tissue-fraction suspension under aseptic conditions. Twenty more normal mice (assay mice) were prepared. The tissue-fraction suspension made from the popliteal lymph node from each mouse was transferred intraperitoneally to an assay mouse. Thus, each of 20 assay mice received an intraperitoneal transfer of tissue-fraction suspension of a popliteal lymph node taken from an individual mouse. The survival of the assay mice was evaluated 60 days after the transfer. Mice that died were autopsied to determine the cause of death.

Using the same procedures, a tissue-fraction suspension was made from the lymph nodes located both along the left iliac artery and the lower abdominal aorta (the lumbar lymph nodes, several per mouse), taken from each of the 20 mice who had received an inoculation with P388 leukemia cells. The tissue-fraction suspension was transferred intraperitoneally into each of 20 other assay mice. Thus, a total of 40 assay mice were injected with tissue-fraction suspensions.

Correlation between survival time of mice and P388 leukemia cell number

One hundred twenty mice were divided into 6 groups of 20 each so that the correlation between the survival time and the number of viable P388 leukemia cells could be evaluated. Six concentrations of P388 leuke-

mia-cell suspensions containing tumor cells of 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 cells/mL were prepared. The 20 mice in each group received an intraperitoneal injection, 1 mL/mouse, of one of the 6 concentrations of P388 leukemia-cell suspensions. For 60 days after inoculation, the mice were checked daily for survival. The date of death for each mouse was marked against the number of inoculated P388 leukemia cells on the semi-log abscissa. A calibration line was drawn along the plotted points.

Therapeutic Experiments in Mice

Therapeutic effects were examined by two kinds of experimental methods. One was a survival experiment, in which the therapeutic effects on survival time were evaluated in mice bearing cancer with lymph node metastases. The other was a lymph node transfer experiment, in which the effects on lymph node metastases were examined after the transfer of the regional lymph node to an assay mouse. The effects of treatment on lymph node metastases were indicated by the median survival time of the assay mice who received intraperitoneal transfer of the lymph node.

Survival experiment

A total of 120 mice were inoculated subcutaneously with a P388 leukemia-cell suspension at 0.05 mL/mouse (5×10^5 cells/mouse) in the left hind foot pad on Day 0. Drug administration was carried out on Day 7, when tumor cells had metastasized to the left popliteal lymph node of all mice. The 120 mice were divided into 6 groups of 20 each. The mice in the first group (the MTX-CH group) received a subcutaneous injection of MTX at 25 mg/kg of body weight in the form of MTX-CH into the left hind foot pad. The mice in the second group (the MTX-sol SC group) received a subcutaneous injection of the same dose of MTX in the form of an aqueous solution at the same injection site. The mice in the third group (the MTX-sol IV group) received a systemic administration of aqueous MTX solution at the same dose by way of an intravenous injection into the tail vein. The mice in the fourth group (the carbon group) received a subcutaneous injection into the left hind foot pad of the same volume of activated carbon suspension without MTX. The mice in the fifth group (the saline group) received a subcutaneous injection of the same volume of saline at the same injection site. The sixth group (the non-treatment group) received no drugs. For 60 days after the drug therapy, daily checks were made for mice who had died. Survival curves were compared between the treatment groups with the generalized Wilcoxon test. The therapeutic effect was indicated by T/C%, calculated as (the median survival days in the treat-

ment group)/(the median survival days in the non-treatment group) × 100 (%).

Lymph node transfer experiment

Using the procedures described earlier in the "Survival experiment" section, 120 mice were inoculated with P388 leukemia cells on Day 0 and divided into 6 groups of 20 each, then received 1 of the 6 drug therapies on Day 7. They were sacrificed on Day 10, and the left popliteal lymph node (the first-level regional node) was excised. Using the procedures described earlier in the "lymph node metastases in experimental model" section, a tissue-fraction suspension of the left popliteal lymph node taken from each mouse was transferred intraperitoneally to a corresponding assay mouse. Thus, six assay groups were formed, namely, the MTX-CH assay group, the MTX-sol SC assay group, the MTX-sol IV assay group, the activated carbon assay group, the saline assay group, and the nontreatment assay group. Each assay group was composed of 20 mice who each received an intraperitoneal transfer of a tissue-fraction suspension of popliteal lymph node taken from the groups of mice that received MTX-CH, subcutaneous MTX-sol, intravenous MTX-sol, activated carbon, saline, and no treatment, respectively.

Survival was checked daily among the assay mice 60 days after transfer. Comparison of the survival curves between two assay groups, using the generalized Wilcoxon test, was made to evaluate the differences in the effects of treatment on lymph node metastases. The effects of treatment were indicated in terms of T/C (%) = (the median survival days of the assay mice in the treatment group)/(the median survival days of the assay mice in the nontreatment group) × 100 (%).

Using the same procedures as with the popliteal lymph node, the lumbar lymph nodes taken from each mouse who received each different drug therapy were made into a tissue-fraction suspension. Each suspension was transferred to a corresponding assay mouse (120 assay mice in total). Comparison of the survival curves of two assay groups was made to evaluate the differences in the effects of treatment on metastases in the lumbar lymph nodes. The effects of treatment were indicated in terms of T/C (%) in the same manner.

Clinical Trials

In a pilot study, MTX-CH was administered to six patients at Kyoto Prefectural University of Medicine Hospital and its associated hospitals from 1992 to 1995. These patients all had medical conditions of some kind that contraindicated the surgery. Five had adenocarcinoma of the stomach (T2NxM0H0 in two and

T1NxM0H0 in three, according to TNM classification of gastric cancer¹²) and one had squamous cell carcinoma of the esophagus (T1NxM0, according to TNM classification of esophageal cancer¹³), as proven histologically in biopsy specimens. Out of five carcinomas of the stomach, two were relatively small Borrmann type II advanced carcinomas, and the other three were type IIc early-stage carcinomas,¹² and one esophageal cancer was superficial,¹³ as diagnosed by esophago- or gastro-fiberscopy and barium swallow X-ray studies.

In 1 cycle, MTX-CH was administered at an MTX dose of 50 mg (once a week or once every 2 weeks) 5 times to each patient. Thus, 1 cycle contained a total MTX dose of 250 mg/person). A patient older than 80 years received 1 cycle of the chemotherapy (a total MTX dose of 250 mg/kg), and patients younger than 80 years principally received 2 cycles of the therapy (a total MTX dose of 500 mg/person) with an interval of 3 to 6 months in between. One patient refused to continue the therapy when the total MTX dose had become 300 mg. The drug was injected into the primary lesion and the adjacent normal submucosal tissues with an endoscopic injector (NM-III K injector, Olympus Optical Co., Ltd., Tokyo, Japan) guided by esophago- or gastro-fiberscopy. No other anticancer therapies were administered before, during, or after MTX-CH therapy.

The therapeutic effects were evaluated by gastro- or esophago-fiberscopy combined with biopsy, X-ray, and computed tomography (CT) scan, repeated monthly for the initial 3 months and at 3- to 6-month intervals over the next two years and at 6-month to 1-year intervals subsequently. To check lymphatic metastases, the size of the regional lymph nodes was observed using endoscopic ultrasonography every 3 months during the last 2 years. Complete response of the primary lesion was defined as total disappearance of the tumor on all examinations, with negative histologic findings of cancer cells in endoscopic biopsy specimens for more than 3 months.

The surgical department of the hospital approved the ethics of the study, and all patients gave informed consent.

Statistical Significance

When a *P*-value was 0.05 or less, the difference was considered statistically significant.

RESULTS

Adsorption of MTX on Activated Carbon In Vitro

The adsorption isotherm was shown as $Q = 270 C^{0.16}$ in physiologic saline at 37 °C (by regression analysis with $P < 0.01$, where Q was the amount of MTX adsorbed on the activated carbon expressed in $\mu\text{g}/\text{mg}$,

TABLE 1
Concentration of Methotrexate in the Left Popliteal Lymph Node of Rats

Time after administration (hrs)	Mean [95% CI] methotrexate concentration ^a ($\times 10^{-6}$ mol/kg)		Statistical significance ^b
	MTX-CH group	MTX-sol group	
0.5	155 [87.8–223]	15.8 [0 to 83.3]	$P < 0.025$
1	240 [205–275]	96.0 [62–131]	$P < 0.005$
3	17.7 [3.2–32.2]	1.8 [0–16.3]	NS
6	ND	ND	
12	ND	ND	

CI: confidence interval; NS: not significant; ND: not detectable.

^a Mean and 95% CI for three experiments are represented. The lower limit of the 95% CI was calculated as a minus value. It was considered to be zero because there is no minus value in drug concentration.

^b Statistical significance was determined by analysis of variance.

TABLE 2
Concentration of Methotrexate in the Lumbar Lymph Nodes of Rats

Time after administration (hrs)	Mean [95% CI] methotrexate concentration ^a ($\times 10^{-6}$ mol/kg)		Statistical significance ^b
	MTX-CH group	MTX-sol group	
0.5	11.7 [9.4–14.1]	7.7 [5.4–10]	$P < 0.05$
1	32.3 [2–62.5]	30.5 [0.3–60.7]	NS
3	4.5 [1.7–7.2]	0.5 [0–3.2]	$P < 0.05$
6	ND	ND	—

CI: confidence interval; NS: not significant; ND: not detectable.

^a Mean and 95% CI for three experiments. The lower limit of the 95% CI was calculated as a minus value. It was considered to be zero because there is no minus value in drug concentration.

^b Statistical significance was determined by analysis of variance.

and C was the concentration of free MTX expressed in $\mu\text{g}/\text{mL}$. Based on this adsorption isotherm, it was calculated that in MTX-CH $49 \mu\text{g}/\text{mL}$ of MTX-CH (equal to $1.08 \times 10^{-1} \text{ mM}$) was in a free state, while the remainder ($24,951 \mu\text{g}/\text{mL}$) was adsorbed onto 50 mg of activated carbon.

Drug Distribution in Rats

In the left popliteal lymph node (Table 1), the mean MTX concentration in the MTX-CH group was 155×10^{-6} and $240 \times 10^{-6} \text{ mol/kg}$, at 0.5 and 1 hour after administration, respectively. These MTX concentrations significantly ($P < 0.025$ and $P < 0.005$) exceeded (by about 10 times and 2.5 times) those in the MTX-sol group. In the lumbar lymph nodes (Table 2), the mean

MTX concentration in the MTX-CH group was also significantly higher at 0.5 and 3 hours after administration ($P < 0.05$) than in the MTX-sol group. At the injection site (Table 3), the mean MTX concentration in the MTX-CH group was 244 and $254 \times 10^{-6} \text{ mol/kg}$ at 0.5 and 1 hour after administration, respectively, and slowly decreased to $15.5 \times 10^{-6} \text{ mol/kg}$ at 12 hours after administration. In contrast, in the MTX-sol group, the MTX concentration was $367 \times 10^{-6} \text{ mol/kg}$ at 0.5 hours after administration, and rapidly decreased to $1.6 \times 10^{-6} \text{ mol/kg}$ at 12 hours after administration. The MTX concentration in the MTX-CH group was significantly higher than in the MTX-sol group at 3 hours ($P < 0.05$) and 12 hours ($P < 0.01$) after administration.

The MTX concentration in blood plasma is shown

TABLE 3
Concentration of Methotrexate at the Injection Site

Time after administration (hrs)	Mean [95% CI] ^a methotrexate concentration ($\times 10^{-6}$ mol/kg)		Statistical significance ^b
	MTX-CH group	MTX-sol group	
0.5	244 [139-350]	367 [261-474]	NS
1	254 [127-382]	105 [0-233]	NS
3	47.4 [21.8-72.9]	7.9 [0-33.5]	$P < 0.05$
6	39.7 [0.5-79]	1.0 [0-40.3]	NS
12	15.5 [9.8-21.1]	1.6 [0-7.2]	$P < 0.01$

CI: confidence interval; NS: not significant.

^a Mean and 95% CI for three experiments. The lower limit of the 95% CI was calculated as a minus value. It was considered to be zero because there is no minus value in drug concentration.

^b Statistical significance was determined by analysis of variance.

TABLE 4
Concentration of Methotrexate in Plasma

Time after administration (hrs)	Mean [95% CI] methotrexate concentration ^a (μ M)		Statistical significance ^b
	MTX-CH group	MTX-sol group	
0.5	56 [0-112]	70.7 [0-83.3]	NS
1	16.3 [7.3-25.3]	28.9 [20.0-37.9]	NS
3	3.2 [1.8-4.5]	2.1 [0.7-3.4]	NS
6	0.7 [0.4-1.1]	ND	
12	ND	ND	

CI: confidence interval; NS: not significant; ND: not detectable.

^a Mean and 95% CI for three experiments.

^b Statistical significance was determined by analysis of variance.

in Table 4. In the blood plasma, the mean MTX concentration in the MTX-sol group was 1.26-fold that in the MTX-CH group at 0.5 hours after administration and 1.77-fold at 1 hour, then decreased rapidly to 0.66-fold at 3 hours and less than the assay limit at 6 hours, whereas in the MTX-CH group, MTX concentration was detectable up to 6 hours after administration. There were no significant differences between the groups receiving the two different formulations.

Experimental Model in Mice

Lymph node metastases in experimental model

The 20 assay mice who received an intraperitoneal transfer of tissue-fraction suspension of the left popliteal

lymph node all died during the 60-day period of observation. Autopsy revealed that all of them died of peritoneal carcinomatosis induced by P388 leukemia cells.

For the mice who received an intraperitoneal transfer of tissue-fraction suspension of the lumbar lymph nodes, 19 of the 20 mice died during the observation period of peritoneal carcinomatosis induced by P388 leukemia cells. The only survivor showed no cancer cells on autopsy.

Correlation of mouse survival time and P388 leukemia cell number

The correlation was expressed as $T = -2.5 \log. N + 24$ (by regression analysis with $P < 0.01$, where T was

TABLE 5
Therapeutic Results of the Survival Experiment

Treatment	Median survival time (days)	T/C% ^a	Difference ^b
MTX-CH	17	142	*
MTX-sol SC	14	117	**
MTX-sol IV	13	108	**
Activated carbon	11	92	†
Saline	12	100	††
Nontreatment	12	100	††

MTX-sol SC: subcutaneous injection of methotrexate aqueous solution; MTX-sol IV: intravenous injection of methotrexate aqueous solution.

^a T/C% = (the median survival days in the treatment group)/(the median survival days in the nontreatment group) × 100%.

^b Represents significant difference in the survival curves by the generalized Wilcoxon test. * versus **.

** versus †: significant ($P < 0.05$); * versus † and ††: significant ($P < 0.01$).

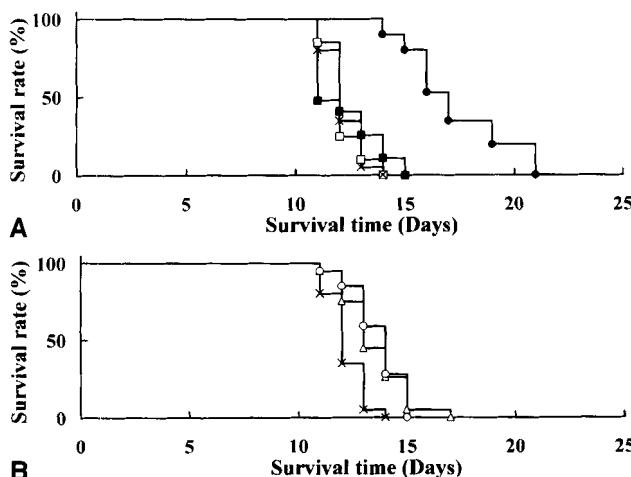


FIGURE 1. (A, B) Survival curves of mice bearing cancer with lymphatic metastases are represented. The survival curve was significantly better in the methotrexate bound to fine activated carbon particles (MTX-CH) group than in the other treatment groups ($P < 0.05-0.01$). ●: MTX-CH group; ■: activated carbon group; □: saline group; ○: subcutaneous methotrexate aqueous solution group; △: intravenous methotrexate aqueous solution group; X: nontreatment group.

the survival time in days and N was the number of viable P388 cells inoculated intraperitoneally).

Results of Therapy in Mice

Survival experiment

Results are shown in Table 5 and Figure 1. All the mice died of P388 leukemia. The median survival time was 12 days in the nontreatment group. The median survival time and T/C% were 17 days (142%), 14 days (117%), 13 days (108%), 11 days (92%), and 12 days (100%) in the MTX-CH group, the MTX-sol SC group,



FIGURE 2. The left popliteal lymph node (regional lymph node) has turned black after MTX-CH injection (left), whereas the right popliteal lymph node, the control, has not (right). Therefore, methotrexate bound to fine activated carbon particles has distributed selectively to the regional lymph node.

the MTX-sol IV group, the activated carbon group, and the saline group, respectively. The survival curve in the MTX-CH group improved significantly as compared with the five other groups ($P < 0.05-0.01$). The survival curves of the MTX-sol SC group and the MTX-sol IV group were significantly better than that of the activated carbon group ($P < 0.05$). There were no significant differences between the latter three control groups.

Lymph Node Transfer Experiment

The left popliteal lymph node and the lumbar lymph nodes of the mice that received a subcutaneous injection of MTX-CH were obviously blackened with MTX-CH. The right popliteal lymph node (the control) was not blackened (Fig. 2).

The results of the lymph node transfer experiment for the left popliteal lymph node were as follows (Fig. 3): The survival curve for the MTX-CH assay group was significantly improved as compared with those in 5 other groups ($P < 0.05-0.01$). The survival curve of the MTX-sol SC assay group was significantly better than those of the saline assay group ($P < 0.05$) and the activated carbon assay group ($P < 0.05$). There was no significant difference between two groups of any combination among the other groups. The median survival time and T/C (%) of the assay mice (Table 6) were 10 days (143%) in the MTX-CH assay group, 8 days (114%) in the MTX-sol SC assay group, 7.5 days (107%) in the MTX-sol IV assay group, and 7 days (100%) in the other three groups.

Results of the lymph node transfer experiment for

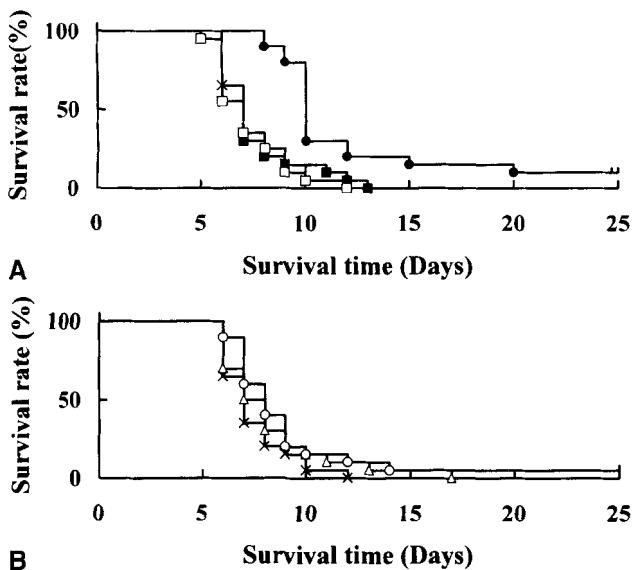


FIGURE 3. (A, B) Survival curves of assay mice receiving the left popliteal lymph node transfer are represented. The survival curve was significantly better in the assay mice that received a transfer of the popliteal lymph node of mice treated with methotrexate bound to fine activated carbon particles (MTX-CH) than in the assay groups that received a transfer of the lymph nodes of mice treated with the other drug therapies. ●: MTX-CH assay group; ■: activated carbon assay group; □: saline assay group; ○: subcutaneous methotrexate aqueous solution assay group; △: intravenous methotrexate aqueous solution assay group; X: nontreatment assay group.

TABLE 6
Popliteal Lymph Node Transfer Experiment

Treatment group	Median survival time (days)	T/C (%)	Difference ^a
MTX-CH	10	143%	*
MTX-sol SC	8	114%	**
MTX-sol IV	7.5	107%	
Activated carbon	7	100%	†
Saline	7	100%	†
Nontreatment	7	100%	

MTX-sol SC: subcutaneous injection of aqueous methotrexate solution; MTX-sol IV: intravenous injection of aqueous methotrexate solution.

^a Represents statistical difference in the survival curves by the generalized Wilcoxon test. * versus **: significant ($P < 0.05$); * versus four others: significant ($P < 0.01$); ** versus †: significant ($P < 0.05$).

the lumbar nodes were as follows (Fig. 4): The survival curve for the mice in the MTX-CH assay group was significantly better than for the five other groups ($P < 0.01$). There was no significant difference between two groups of any combination among the other groups. The median survival time and T/C (%) (Table 7) were

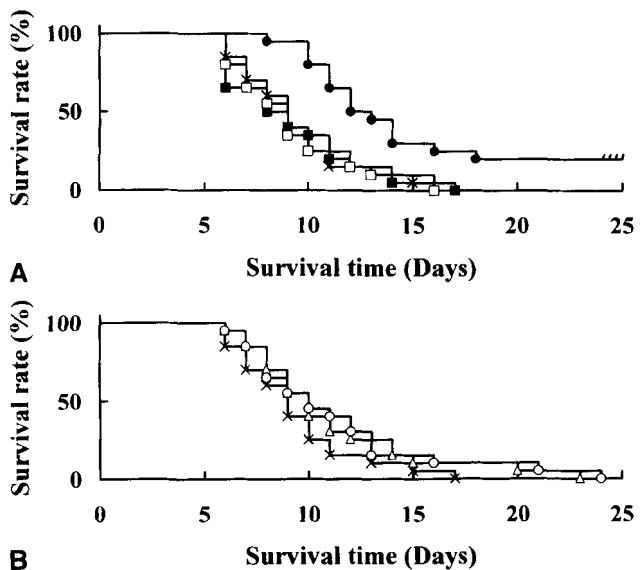


FIGURE 4. (A, B) Survival curves of assay mice receiving the lumbar lymph nodes transfer are represented. The survival curve was significantly better in the assay mice that received a transfer of the lumbar lymph nodes of mice treated with methotrexate bound to fine activated carbon particles (MTX-CH) than in the assay groups that received a transfer of the lymph nodes of mice treated with the other drug therapies. ●: MTX-CH assay group; ■: activated carbon assay group; □: saline assay group; ○: subcutaneous methotrexate aqueous solution assay group; △: intravenous methotrexate aqueous solution assay group; X: nontreatment assay group.

TABLE 7
Lumbar Lymph Nodes Transfer Experiment

Treatment group	Median survival time (days)	T/C (%)	Difference ^a
MTX-CH	12.5	125%	
MTX-sol SC	10	100%	
MTX-sol IV	9	90%	
Activated carbon	8.5	85%	
Saline	9	90%	
Nontreatment	10	100%	

MTX-sol SC: subcutaneous injection of aqueous methotrexate solution; MTX-sol IV: intravenous injection of aqueous methotrexate solution.

^a Represents statistical difference in the survival curves by the generalized Wilcoxon test; * versus five others ($P < 0.01$).

12.5 days (125%) in the MTX-CH assay group, 10 days (100%) in the MTX-sol SC assay group, 9 days (90%) in the MTX-sol IV assay group, 8.5 days (85%) in the activated carbon assay group, 9 days (90%) in the saline assay group, and 10 days (100%) in the nontreatment assay group.

TABLE 8
MTX-CH for Cancer in Patients Who Could Not Undergo Gastrectomy or Esophagectomy

Age, sex	Gross type of primary lesion	TNM classification	Total dose of MTX	Response of primary cancer (duration of cancer free condition)
75, male	Gastric cancer small Borr. II	T2NxM0	500 mg	Cancer free (31 months)
79, female	Gastric cancer small Borr. II	T2NxM0	500 mg	Cancer free (35 months)
84, female	Gastric cancer IIc	T1NxM0	250 mg	Cancer free (25 months)
77, male	Gastric cancer IIc	T1NxM0	300 mg	Cancer free (12 months)
76, male	Gastric cancer IIc	T1NxM0	500 mg	Cancer free (12 months)
71, male	Esophageal cancer I	T1NxM0	500 mg	Cancer free (16 months)

Small Borr. II: a relatively early stage advanced cancer lesion classified as Borrmann type II; IIc: an early stage gastric cancer lesion classified as type IIc;¹¹ I: a superficial esophageal cancer lesion classified as type I;¹² TNM classification: classification of pretreatment state as diagnosed by computed tomography, ultrasonography, plain X-ray, and esophago- or gastro-fiberscopy;^{11,12} T1: tumor invasion of mucosa or submucosa; T2: tumor invasion of muscularis propria or subserosa (Nx: diagnosis of the regional lymph node metastases is unclear because lymph node metastases cannot be clearly diagnosed when surgical treatment is not performed; M0: no evidence of distant metastasis).

Clinical Trials

All patients had a complete response. For 12 to 35 months from the completion of MTX-CH therapy up to the time of last review, repeated gastro- or esophago-fiberscopy showed that the primary cancerous lesions disappeared completely, and every biopsy guided by fiberscopy showed that the cancerous tissues were not found histologically (Table 8). Examinations using X-ray, CT scan, and endoscopic ultrasonography did not demonstrate any hematogenous metastases or increased volume of the regional lymph nodes. As for side effects, epigastralgia was reported in 3 patients, and fever lower than 38 °C was seen in 4 patients. Other side effects, including bone-marrow suppression, were not observed.

DISCUSSION

MTX was used as the anticancer drug in this study because it shows anticancer effects on many kinds of digestive cancers^{13,14} and can be safely injected into subcutaneous or submucosal tissues.¹⁵

Adsorption isotherm of MTX into activated carbon shows that adsorbed MTX remains in dynamic equilibrium with the concentration of MTX in a free state around the activated carbon particles. When the free MTX is consumed by washout or binding to tissues and the concentration of MTX in a free state decreases around the carbon particles, the carbon releases the adsorbed MTX, replacing the decreased concentration. Thus, the free MTX concentration is maintained at the same level in the region. Since the adsorbency is much greater than the free MTX concentration, the free MTX concentration is maintained for a long period of time, even when the free MTX is consumed rapidly.

We have previously shown that very small acti-

vated carbon particles are smoothly absorbed through lymphatic capillaries and retained in the regional lymph nodes for a long period of time.⁶ In the current MTX assay, carbon particles and tissue fractions were removed by centrifugation, and the supernatant was subjected to the assay of MTX concentration. Therefore, the MTX concentration shown in this study is considered to indicate the action of MTX on the tissue in the region, and is not thought to include the MTX bound to carbon particles. The distribution of MTX in rats showed that MTX-CH selectively distributed greater amounts of MTX to the regional lymph nodes and the injection site for longer periods than did the MTX aqueous solution. This result means that the particles of MTX-CH are delivered to the lymph nodes, where the particles of MTX-CH slowly release free MTX and maintain it at a constant level for a long time. Since the anticancer effects of MTX depend on the length of its active exposure period rather than on its concentration,¹⁶ the drug-delivery characteristics that we have cited suggest that MTX-CH can enhance the therapeutic effects of MTX on the metastatic lesions in the regional lymph nodes and in the primary lesion when MTX-CH is injected into the primary lesion.

In the blood plasma, the mean MTX concentration in the MTX-CH group was lower at 0.5 and at 1 hour after administration, then higher at 3 and 6 hours. Thus, the MTX level in the MTX-CH group decreased slowly as compared with the MTX-sol group. This suggests that MTX was absorbed slowly into the circulating blood due to sustained release of the drug from the carbon particles in the MTX-CH preparation. The MTX level in the blood plasma did not differ significantly between the two formulations. This is probably due to the broad ranges of the 95% confidence intervals.

For the experimental model (see "Lymph node

metastases in experimental model"), we examined whether metastasis had occurred in the regional lymph nodes 7 days after inoculation and found that metastases occurred in the left popliteal lymph node in all mice and in the lumbar lymph nodes in 19 of 20 mice. We therefore carried out the drug therapy 7 days after inoculation in the therapeutic experiments. In those experiments, aqueous MTX solution was administered intravenously as a control therapy of systemic administration of the solution. Intravenous MTX solution plus local injection of activated carbon was not given as another control group because preliminary examinations revealed that there were no differences in the survival effects and therapeutic effects on lymph node metastases between the intravenous MTX solution and the intravenous MTX solution plus local injection of carbon.

For mice bearing cancer with lymph node metastases, the therapeutic effects on survival time and the effects on lymph node metastases were also studied (see "Survival experiment" and "Lymph node transfer experiment"). In the survival experiment, MTX-CH extended the survival time of the mice with lymph node metastases more than did the MTX aqueous solution.

The logarithm (the number of intraperitoneally inoculated P388 leukemia cells) correlates linearly and inversely with the assay mouse's survival time. On this basis, Tsuruo et al.⁷ reported that the number of P388 leukemia cells in the lymph node can be estimated from the survival time of an assay mouse after tissue fractions have been made from a lymph node containing P388 leukemia cells and injected intraperitoneally into the mouse. Using this method, the therapeutic effects of drugs on lymph node metastases were indicated by the number of the residual P388 leukemia cells in the lymph nodes estimated from calibration line. In the current study (see "Lymph node transfer experiment"), effects of treatment on the metastases were compared statistically between two treatment groups. When the lymph nodes taken from each mouse in the treatment group were transferred to a corresponding mouse in an assay group, the shortening of the survival time of the assay mice expressed the lethal effects induced by the P388 leukemia cells in the lymph nodes. The strength of the lethal effects on the assay mice is considered to be an indication of the magnitude of a cancerous lesion's activity. Therefore, the effects of treatment on metastases can be expressed as the improvement of the survival curves of the assay mice. We evaluated the differences in the effects of treatment on lymph node metastases between the treatment groups by comparing the survival curves of the two groups of assay mice. The survival

curve of the MTX-CH assay group was significantly improved as compared with those of the other groups. This result means that the treatment made P388 leukemia cells in the metastatic lesions significantly less active in the MTX-CH group as compared with the other treatment groups.

In the clinical pilot study, locally injected MTX-CH controlled the lesion for long periods without recurrence in the primary site or in regional lymph nodes. MTX-CH therapy will likely become one of the successful and safe therapies for the control of early stage or relatively early stage cancers of the upper digestive tract in patients for whom surgery presents high risks.

If future clinical trials reveal that MTX-CH therapy controls the primary lesions and their lymph node metastases in early stage or relatively early stage digestive cancers, surgical treatment will not be chosen principally. Instead, MTX-CH therapy alone or combined with the fiberoptic local resection will be warranted. We are initiating this therapy without surgery for relatively early stage cancers at the ampulla of the rectum.

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