Electrical Stimulation Prolongs the Survival Days of Leukemic Mice Treated With Methotrexate

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ABSTRACT—To investigate effects of electrical stimulation on survival days of leukemic mice treated with methotrexate (MTX), L1210-bearing mice were treated by MTX and calcium folinate (leucovorin®) rescue therapy (MTX: 400 mg/kg, followed by leucovorin at the dose of 7.5 mg/kg at 8, 15 and 24 hr after MTX) under electrical stimulation (foot shock: shock amplitude, 0.4 mA; voltage, 60–100 V/cm; shock duration, 5 sec; frequency, 0.5 Hz) of various lengths. The survival days were significantly prolonged by 6-hr electrical stimulation in combination with MTX, while plasma MTX concentrations and pharmacokinetic parameters such as the area under the curve (AUC$_{0-12}$) and clearance (CL) were not significantly altered. Psychological stress did not alter the efficacy of MTX in the communication box paradigm. Amplified efficacy of MTX was shown in a length-dependent manner when electrical stimulation of various lengths were applied to L1210-bearing mice.

Keywords: L1210, Methotrexate, Electrochemotherapy, Stress, Communication box

When a cell is exposed to short and strong electric pulses, its membrane undergoes a remodeling process characterized by the occurrence of transient permeation structures ("electropores") (1, 2). Electrical stimulation has been reported to allow insertion of nonpermeant substances into cell cytosols in specific cell lines and animals (3–5) and it may be possible to transport an anticancer drug across the cell membrane by electroporation (electrochemotherapy) (6–17). In electrochemotherapy, cell membranes, through which some anticancer drugs will not readily pass, can be made easily permeable and the cytotoxicity of the drugs markedly increased. The dose of anticancer drugs with such high toxicity could be reduced by enhancing in situ drug delivery to tumor cell cytosols by electrochemotherapy, and eventually, systemic adverse reactions may be reduced followed by the decreased plasma concentrations of drugs. Electrochemotherapy may be applicable to cancers that are resistant to conventional chemotherapy. However, so far, it has been applied only to limited types of tumors in animals and humans (6–17). Those tumors were localized and solid in most studies. The effect of electrochemotherapy on a non-solid highly malignant cell line such as L1210 murine leukemia is not clear.

L1210 experimental murine leukemia is resistant to a conventional dose of methotrexate (MTX), which is one of the folic acid analogues widely used as an anticancer drug against leukemia and other neoplastic diseases. Therapy with a high dose of MTX has become possible by addition of the leucovorin rescue regimen (18–21). However, the dose of the drug is often limited because of its high toxicity and low efficacy to the tumor cells.

Electrical stimulation actually contains both physical and psychological stresses that may influence the pharmacokinetics of drugs with renal elimination (22). Effects of electric stimulation on pharmacokinetics could result in changes in efficacy of MTX, when electrochemotherapy is applied to tumors in vivo. Therefore, the present study was designed to investigate the effects of electrical stimulation on survival days of L1210-bearing mice and MTX pharmacokinetics in plasma and to determine if psychological stress is involved in the mechanism of, if any, survival prolongation in electrochemotherapy. Furthermore, the relationship between the length of electrical stimulation and the efficacy of MTX was investigated in the treatment of L1210-bearing mice.
MATERIALS AND METHODS

Animals
All animals were obtained from Charles River Japan, Inc. (Yokohama) and housed 6 per cage on standardized light-dark cycle of lights on during 7:00 am–7:00 pm, at a room temperature of 24±1°C and a humidity of 60±10%, with food and water ad libitum. The treatment of the animals was based on the Guidelines for Animal Experiments at Oita Medical University and all experiments were approved by the Oita Medical University Animal Experiment Center Committee (Approval No. 3105403).

Materials
L1210 cells and its preparation: Ascitic forms of the L1210 cells were maintained by weekly intraperitoneal transplantation in male DBA/2 mice. The initial cell line was a bank of frozen tumor cells in our laboratory. Tumor cells were harvested from the peritoneal cavity as suspensions in cold RPM-1640 (Kojin Bio, Co., Ltd., Sakado) at 7 to 10 days after previous transplantation. The number of tumor cells was microscopically determined immediately after staining by Turk’s solution.

Drugs: MTX and leucovorin were dissolved by saline and were administered to mice in a volume of 10 ml/kg. Drugs were purchased from Leadeley (Tokyo) through its local distributor.

The MTX and leucovorin rescue regimen
L1210-bearing mice were treated by the MTX and leucovorin rescue regimen modified from the previous report (21). In our preliminary study, untreated L1210-bearing mice died in 8.9±0.5 days (mean±S.D.), and the MTX and leucovorin rescue regimen was confirmed to double the survival time of L1210-bearing mice. Mice were treated with a high dose MTX (400 mg/kg, i.p.) followed by leucovorin (7.5 mg/kg at 8, 15 and 24 hr after MTX).

Measurement of plasma MTX concentrations and pharmacokinetic parameters
Plasma MTX concentrations were measured by homogeneous enzyme immunoassay (Syva Corporation’s EMIT® system; Syva Corp., San Jose, CA, USA) (23). Pharmacokinetic parameters (area under the curve: AUCₙ₋₁₂ hr, clearance: CL) were calculated by the trapezoidal method.

Electrical stimulation
Mice were isolated in each compartment during the stress session. Parallel electrodes, 3 mm in diameter, located every 10 mm, were set on the floor of the compartment (10×10 cm). Each compartment was separated by transparent plastic walls. The electric current was automatically regulated at 0.4 mA by the shock generator (special order; Muromachi Kikai Co., Ltd., Tokyo). Five-second stimulation every 120 sec was given repeatedly (shock amplitude, 0.4 mA; voltage, 60–100 V/cm; shock duration, 5 sec; frequency, 0.5 Hz). Lengths of time applied to mice were 0–12 hr according to the experiment. Applied voltage was automatically regulated at approximately 60–100 V/cm to maintain the shock amplitude fixed at 0.4 mA, the minimum amplitude that the shock generator could produce. In the preliminary study, no animal was injured or died of this electrical stimulation.

The communication box paradigm
Psychological stress was given to mice in the compartment of the communication box paradigm. Parallel electrodes, 3 mm in diameter, located every 10 mm, were set on the floor of the compartment (10×10 cm). Experimental mice were isolated in each compartment as the “responder” during the stress session. The electrodes for “responder” mice were covered by plastic panels to shield electricity. The same series of electrical stimulation described in the Electrical stimulation section was given to sex and age-matched “sender” mice, which were located in the next door compartment, so that “responder” mice would communicate with them. “Responder” mice received only psychological stimuli.

Effect of electrical stimulation (foot-shock) on the survival days of L1210-bearing mice
Forty-eight male specific-pathogen-free DBA/2 mice at 6 weeks of age were injected intraperitoneally with 0 (saline control) or 400 mg/kg of MTX at 24 hr after intraperitoneal transplantation of 1×10⁶ of L1210 leukemic cells suspended in RPM1640 (0.1 ml). MTX was injected intraperitoneally at 1:00 pm. Zero (saline control) or 7.5 mg/kg of leucovorin was administered subcutaneously 3 times at 8, 15 and 24 hr after MTX injection (17–20). Immediately after MTX administration, electrical stimulation (foot-shocks) was given to each mouse for 0 (control) or 6 hr. Survival days of each group (n=12/group) were observed every 24 hr at 1:00 pm.

Pharmacokinetic effect of electrical stimulation (foot-shock) in plasma of L1210-bearing mice
Sixteen male DBA/2 mice at 6 weeks of age were injected intraperitoneally with 400 mg/kg of MTX. Leucovorin (7.5 mg/kg) was administered subcutaneously 3 times at 8, 15 and 24 hr after MTX injection. Immediately after MTX administration at 1:00 pm, the same series of electrical stimulations were given to each mouse over 0 (control) or 6 hr. Plasma MTX concentrations of each
Effect of psychological stress on survival days of L1210-bearing mice treated with MTX in the communication box paradigm

Forty-eight male specific-pathogen-free DBA/2 mice at 6 weeks of age were injected intraperitoneally with 0 (saline control) or 400 mg/kg of MTX at 24 hr after intraperitoneal transplantation of $1 \times 10^5$ of L1210 leukemic cells suspended in RPM1640 (0.1 ml). MTX was injected intraperitoneally at 1:00 pm. Zero (saline control) or 7.5 mg/kg of leucovorin was administered subcutaneously 3 times at 8, 15 and 24 hr after MTX injection. Immediately after MTX administration at 1:00 pm, psychological stress was given to each mouse in the compartment of the communication box paradigm for 0 (control) or 6 hr. Survival days of each group ($n=12$ /group) were observed every 24 hr at 1:00 pm.

Effect of durations of electrical stimulations on survival days of L1210-bearing mice treated with MTX

Eighty male specific-pathogen-free DBA/2 mice at 6 weeks of age were injected intraperitoneally with 0 (saline control) or 400 mg/kg of MTX at 24 hr after intraperitoneal transplantation of $1 \times 10^5$ of L1210 leukemic cells suspended in RPM1640 (0.1 ml). MTX was injected intraperitoneally at 1:00 pm. Zero (saline control) or 7.5 mg/kg of leucovorin was administered subcutaneously 3 times at 8, 15 and 24 hr after MTX injection. Immediately after MTX administration at 1:00 pm, electrical stimulation was given to each mouse over 0 (control), 1, 3, 6 and 12 hr. Survival days of each group ($n=8$ /group) were observed every 24 hr at 1:00 pm.

Statistical analyses

Survival days were analyzed by factorial (2 × 2) analysis of variance (ANOVA) to identify the interactive effect of MTX and electrical stimulation or psychological stress and by one-way ANOVA to clarify between-subgroup difference and a length-dependent response of electrical stimulation in electrochemotherapy, followed by Fisher's protected least significant difference (PLSD). Plasma MTX concentrations were analyzed by ANOVA with repeated measurements and pharmacokinetic parameters were analyzed by the unpaired t-test. A P value less than 5% was considered to be statistically significant. Statistical calculations were performed using the personal computer software StatView 4.01.

RESULTS

Effect of electrical stimulations (foot-shock) on the survival days of L1210-bearing mice

The time courses of survival rates in subgroups are shown in Fig. 1. MTX and leucovorin rescue regimen significantly prolonged the survival days of L1210-bearing mice ($P<0.001$). Six-hour electrical stimulation (foot-shock: 6 hr) significantly prolonged the survival days of L1210-bearing mice treated by MTX and leucovorin rescue therapy ($P<0.001$). There was a significant interaction between electrical stimulation and treatment (electrical stimulation × treatment, $P<0.001$). Table 1 shows the

![Fig. 1. Effects of electrical stimulation (ES) and MTX treatment on survival rate of L1210-bearing mice. MTX: 400 mg/kg, leucovorin: 7.5 mg/kg at 8, 15 and 24 hr after MTX. Solid and dotted lines indicate MTX-treated groups and saline controls, respectively. Triangles and circles indicate the groups with and without ES, respectively. Treatment, $P<0.001$; ES, $P<0.001$; Treatment × ES, $P<0.001$ (2×2 ANOVA). n=12/group.](image-url)
Table 1. Survival days of L1210-bearing mice treated with ES and MTX

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ES</th>
<th>n</th>
<th>Survival days</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>+</td>
<td>12</td>
<td>15.6±0.6 **</td>
</tr>
<tr>
<td>MTX</td>
<td>−</td>
<td>12</td>
<td>13.3±1.4 **</td>
</tr>
<tr>
<td>SAL</td>
<td>+</td>
<td>12</td>
<td>8.9±0.7 **</td>
</tr>
<tr>
<td>SAL</td>
<td>−</td>
<td>12</td>
<td>8.2±0.4 **</td>
</tr>
</tbody>
</table>

MTX: MTX and leucovorin rescue regimen, SAL: saline control, +: electrical stimulation (ES) for 6 hr, −: without ES. Data are shown as the mean±S.D. There was a significant difference in survival days between subgroups, P<0.001 (ANOVA). *P<0.05, **P<0.01 (Fisher’s PLSD).

Table 2. Effect of electrical stimulation (ES) on pharmacokinetic parameters of MTX in L1210-bearing mice treated by MTX and leucovorin rescue regimen

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ES (+) (n=8)</th>
<th>ES (−) (n=8)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0-12 hr} (mg/l·hr)</td>
<td>86.1±54.3</td>
<td>63.4±32.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>CL (l/hr/kg)</td>
<td>6.0±2.5</td>
<td>7.9±4.4</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Data shown as the mean±S.D.

Mean survival days in each subgroup. There was a significant difference in survival days between subgroups (P<0.001). The mice treated with MTX and electrical stimulation survived longest among all subgroups.

Pharmacokinetic effect of electrical stimulation (foot-shock) in plasma of L1210-bearing mice

The time-concentration profiles of L1210-bearing mice treated by MTX and leucovorin rescue regimen with and without electrical stimulation are shown in Fig. 2. AUC_{0-12 hr} and clearance (CL) of MTX are shown in Table 2. Six-hour electrical stimulation did not significantly change the plasma MTX concentrations or pharmacokinetic parameters in the L1210-bearing mice. AUC_{0-12 hr} of the stimulated and non-stimulated group were 86.1±54.3 and 63.4±32.4 mg/l·hr, respectively.

Effect of psychological stress on survival days of L1210-bearing mice treated with MTX in the communication box paradigm

Survival days of subgroups are shown in Fig. 3. The treatment with MTX and leucovorin significantly prolonged the survival days of L1210-bearing mice (P<0.001). The mean survival days of the MTX-treated group and untreated group were 16.3±2.4 and 7.8±0.9 days, respectively. Six-hour psychological stress did not significantly prolong the survival days of L1210-bearing mice treated with MTX and leucovorin rescue therapy.

Effect of duration of electrical stimulation on survival days of L1210-bearing mice treated with MTX

Figure 4 shows the mean survival days of each subgroup. There was a significant difference among the groups according to electrical stimulation of different lengths in the L1210-bearing mice treated by MTX and leucovorin rescue therapy (P<0.001). The mean survival...
days of the MTX-treated group and untreated group were 14.7±2.2 and 8.3±0.5 days, respectively. The longer duration (6 and 12 hr) of the electrical stimulation significantly prolonged the survival days of L1210-bearing mice treated with MTX compared with the control (0 hr) group. The mean survival days of MTX-treated groups with 0, 6 and 12-hr electrical stimulation are 13.3±2.0, 14.8±1.3 and 17.4±2.3 days, respectively (0 hr vs 6 hr, P<0.05; 0 hr vs 12 hr, P<0.01).

DISCUSSION

In Fig. 1, the therapy with a high dose of MTX followed by leucovorin was obviously effective. MTX significantly prolonged the survival days of L1210-bearing mice. Interestingly, the effect of MTX was enhanced by electrical stimulation (foot shock: shock amplitude, 0.4 mA; shock duration, 5 sec; frequency, 0.5 Hz; length, 6 hr). The electrical stimulation was chosen to be 6-hr-long because our preliminary data indicated that such stimulation of short duration (1 hr) did not alter the survival days of L1210-bearing mice. Six-hour electrical stimulation prolonged the survival days of L1210-bearing mice.

Yatsuka et al. previously showed a decreased clearance of amikacin when rats were under 6-hr electric foot shock (22). Amikacin, one of the aminoglycoside antibiotic agents, is mainly eliminated from the kidney, and so is MTX. There was a question whether the change of the MTX efficacy was due to pharmacokinetic alteration induced by electrical stimulation. In Table 2, electrical stimulation decreased CL of MTX by 24% and increased AUC by 35%, although these changes were not statistically significant. These changes might have been significant if more sampling points had been examined.

There still remains a question whether efficacy of anticancer agents is totally explained by the plasma concentration. The plasma concentration is certainly useful parameter to judge therapeutic efficacy in clinical practice. However, the tissue MTX concentration, which was not measured in the present study, may be playing a more important role. When radiolabeled cobalt (57)-bleomycin was injected to LPB tumor-bearing mice, the tumors treated by the electric pulses retained 3.6- to 4-fold more 57Co-bleomycin than those that did not receive the electric pulses (5). In the present study, electrical stimulation may have promoted the efficacy of MTX in the treatment of L1210-bearing mice by elevating the tissue MTX concentration. Treatments of cancer by combination of an electric field with chemotherapeutic agents have been reported by investigators (6–17). However, those tumors were localized and solid in most studies, and the effect of electrochemotherapy on a non-solid cell line was not clear. Also, the applied electrical stimulation was stronger than that in the present study. For example, Okino and Mohri first introduced in vivo electrochemotherapy using AH-109A hapatocellular carcinoma in rats and showed greater reduction in the tumor size treated by combination of bleomycin and an electrofield of 5000 V/cm (6). In recent clinical trials, a clear local anticancer efficacy was found following bleomycin and intense pulses of 1300 V/cm (14, 15). L1210 is not a localized tumor, so we gave mice a systemic electrical stimulation of smaller strength; the applied voltage in the present study was 60–100 V/cm. Neither local nor general side effects were observed. Recent investigation indicates that the immunological response may be involved in killing tumor cells in electrochemotherapy (9). The efficacy of electrochemotherapy was enhanced by small amounts of interleukin (IL)-2 or IL-2 secreting cells (9). Thus, the inflammatory reaction may have some influence on efficacy of anticancer drugs in electrochemotherapy. Further studies are needed to investigate whether or not electric stimulation increases the permeability of MTX into L1210 cell cytosol with the immunological response in local sites.

Electrical stimulation actually contains both physical and psychological stress. The communication box is a special device designed to give psychological stress to the "responder" mice without giving physical stress. The responder mice put into small compartments communicate psychologically with the "sender" mice that are located in the next door compartments. The floor for the sender mice are metal rods to give electrical pulses, and that for the responder mice is electrically shielded by plastic plates. The communication box was used to investigate the change in efficacy of MTX by adding psychological stress. The results in Fig. 3 indicated less possibilit-
ity that psychological stress was involved in the mechanism of electrochemotherapy, and therefore that stress as a physical reaction was more involved in the elevated efficacy of MTX in Figs. 1 and 4.

To clarify the relationship between the strength of physical stress and the efficacy of MTX, the last experiment was designed to observe the alteration of survival days of L1210-bearing mice treated with MTX by adding electrical stimulation of different lengths. The length-dependency was demonstrated by the result that the longer (6 and 12 hr) physical stress significantly prolonged the survival days of L1210-bearing mice treated with MTX compared with the control (0 hr) group. In this experiment, reproducibility of the effect of electric stimulation on MTX chemotherapy was also confirmed.

Thus, amplified efficacy of MTX was experimentally revealed when L1210-bearing mice were electrically stimulated. The pharmacokinetic alterations of MTX in plasma were not clearly involved in the mechanisms of this phenomenon. As for the results obtained from our experiments, further studies should be accomplished to reveal cellular pharmacokinetic changes by developing an accurate method for measuring the tissue or the tumor cell concentration of MTX. At the same time, it may be needed to clarify unknown mechanisms such as local involvement of specific inflammatory or immunologic reactions that might cause alteration of the efficacy of anticancer drugs.

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