

High-dose methotrexate with citrovorum factor for malignant fibrous histiocytoma of soft tissue: a cell culture study

Research Article

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Abbreviations: high-dose methotrexate (HD-MTX), citrovorum factor (CF), malignant fibrous histiocytoma (MFH), (CellTiter One Aqueous Solution) MTS, (Analysis Of Variance) ANOVA

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Summary

Spindle-pleomorphic sarcomas of soft tissues are resistant for various chemotherapy regimens that are currently used. Although high-dose methotrexate (HD-MTX) with citrovorum factor (CF) rescue regimen is widely used in chemotherapy for skeletal osteosarcoma, the effect of HD-MTX-CF on spindle-pleomorphic soft tissue sarcomas remains unknown. The purpose of the present study is to investigate the pertinence of clinical application of the HD-MTX-CF regimen for chemotherapy of malignant fibrous histiocytoma (MFH) in soft tissue. A human MFH cell line TNMY1 was used. The cytotoxic effect by MTX and the cell revival effect by CF were determined by MTS tetrazolium assay. The chemosensitivity was compared to that of an osteosarcoma cell line KHOS/NP. The growth inhibition rates by MTX in the MFH cells significantly increased time-dependently by the subsequent incubation. The growth inhibition rate of the MFH cells, however, was lower than that of the osteosarcoma cells. The decreased cell proliferation of MFH by MTX was not significantly reversed by CF. Our data indicate that a HD-MTX-CF protocol is potentially applicable to the treatment of MFH

I. Introduction

Malignant fibrous histiocytoma (MFH) is one of the most common high-grade sarcomas in late adult life that develops in bone and soft tissue. MFH has a predilection for extremities, and the prognosis of the disease is reported to be poor due to its chemo-resistance (Papai et al, 2000; Reichardt, 2002; Spira and Ettinger, 2002). Recent studies introduced combination adjuvant chemotherapy protocols consisting of doxorubicin, cisplatin, and high-dose methotrexate (HD-MTX) for skeletal osteosarcomas, and the successful clinical results have been reported (Bacci et al, 2001, 2002; Thompson et al, 2002). Of these, HD-MTX with citrovorum factor (CF) rescue regimen is one of the frequently used chemotherapy protocol for osteosarcoma (Breithaupt and Kuenzlen, 1983). In contrast, the effectiveness of adjuvant chemotherapy for spindle-pleomorphic sarcomas including MFH of soft tissue remains controversial. Although many chemotherapy protocols currently used for spindle-

pleomorphic soft tissue sarcomas consist of doxorubicin, epirubicin, cisplatin, and ifosfamide (Edmonson et al, 2002; Reichardt, 2002), there is little consensus concerning the doses and the combinations of these chemotherapeutic agents. To our knowledge, little has been reported concerning the clinical use of HD-MTX for soft-tissue MFH. The purpose of the present study is to experimentally examine the effects of the HD-MTX with CF rescue regimen on proliferative activity of a MFH cell line, comparing that of an osteosarcoma cell line.

II. Materials and methods

A. Cell lines and cell culture

The MFH cell line TNMY1 that was previously established in our laboratory was used (Nakatani et al, 2001). The osteosarcoma cell line KHOS/NP was purchased from American Type Culture Collection (CRL-1427, Rockville, MD, USA). Each cell line was maintained as a monolayer culture in a humidified 5% CO₂ incubator at 37°C. The culture medium consisted of Eagle's minimum essential medium (MEM)

containing 0.292 g/L L-glutamine (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma Chemical Co.), 100 U/ml penicillin G (Sigma Chemical Co.), and 100 U/ml streptomycin (Sigma Chemical Co.).

B. Chemicals

Methotrexate and CF were kindly provided by Lederle Laboratories (Pearl River, NY, USA).

C. The effect of MTX on the cell proliferation

The cells were suspended in the culture medium at a concentration of 5.0×10^3 /ml. A hundred microliter aliquot of the cell suspension was dispersed to each well in 96-well cell culture plates (Costar, Cambridge, Massachusetts, U.S.A), and the cells were kept quiescent for 48 hours. The culture medium of each well was replaced by 100 μ l of fresh medium, and MTX was pulsed to each well at a final concentration of 1.0×10^{-2} to 1.0×10^{-8} mol/L. An equivalent volume of fresh medium, instead of MTX, was added to certain wells to serve as controls. At this time, the serum concentration in the culture medium was reduced to 1%. After the cells were incubated for 24 hours, the culture medium was replaced by a new medium following cell rinse with phosphate buffer saline (PBS). Finally, the cells were incubated another 0, 48, or 96 hours.

The cell viability was determined using a modified MTS tetrazolium assay (Malich et al, 1997). The medium in each well was replaced by 100 μ l of Eagle's MEM with 0.292 g/L L-glutamine without FBS, and 20 μ l of MTS reagent (CellTiter 96-AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega Co., Madison, WI, USA) was pulsed to each well. After 2 hours of further incubation at 37°C in a humidified 5% CO₂ incubator, the optical density (OD) value of each well was measured at 490nm using an automatic microplate reader (Microplate Reader, Bio Rad, USA). The growth inhibition (Gi) rate of the cells in each well was determined by the following equation (Reinecke et al, 2000).

$$Gi = \{1 - (OD_s - OD_B) / (OD_C - OD_B)\} \times 100$$

Gi; growth inhibition rate (%)

OD_s; OD value of each sample

OD_C; OD value of control

OD_B; OD value of blank well

The data were shown in mean \pm SD from six replicate wells for each MTX concentration and three replicate experiments for each cell lines.

D. The reversal effect of CF on the cytotoxicity of MTX

The monolayered cell culture of TNMY1 cell line was prepared in a plastic 96-well plate in the same way as was described above. After the cells were exposed to subsequent doses of MTX for 24 hours, the culture supernatant was replaced by refresh culture medium containing 1% FBS, 100U/ml penicillin G, and 100U/ml streptomycin. Then, the cells were cultured for 96 hours in absence or presence of CF at the concentration of 10^{-7} mol/L. The cell growth inhibition rate of each well was determined by MTS assay.

E. Statistical analysis

The data was analyzed using One-way repeated measures ANOVA for comparing the Gi rates in each group, and Two-way repeated measures ANOVA for comparison between each cell line or each incubation time. The p-values less than 0.05 were considered statistically significant.

III. Results

A. The effect of MTX on the cell proliferation

The Gi rates of the KHOS/NP cells after 24 hours exposure to MTX and additional 0 hour incubation are shown in **Table 1A** and **Figure 1**, and those in the TNMY1 cell line are shown in **Table 1B** and **Figure 2**, respectively. One-way repeated measures ANOVA showed that MTX significantly inhibited the cell growth of the KHOS/NP cell line at the concentrations of 10^{-8} to 10^{-2} M dose-dependently, but no significant effect was observed on the TNMY1 cell line.

The Gi rates of the cells after 24 hours exposure to MTX and additional 48-hour incubation in the KHOS/NP cell line are shown in **Table 2A** and **Figure 1**, and those in the TNMY1 cell line are shown in **Table 2B** and **Figure 2**, respectively. One-way repeated measures ANOVA showed that MTX significantly inhibited the cell growth at the concentrations of 10^{-8} to 10^{-2} M in the KHOS/NP cell line, while MTX inhibited the cell growth at the concentrations of 10^{-6} to 10^{-2} M in the TNMY1 cell line.

The Gi rates of the cells after 24 hours exposure to MTX and additional 48-hour incubation in the KHOS/NP cell line are shown in **Table 3A** and **Figure 1**, and those in the TNMY1 cell line are shown in **Table 3B** and **Figure 2**, respectively. One-way repeated measures ANOVA showed that MTX significantly inhibited the cell growth at the concentrations of 10^{-8} to 10^{-2} M in the KHOS/NP cell line, while MTX inhibited the cell growth at the concentrations of 10^{-7} to 10^{-2} M in the TNMY1 cell line.

Two-way repeated measures ANOVA revealed that the growth inhibitory effect of MTX on both KHOS/NP and TNMY1 cell lines significantly increased by incubating for 48 and 96 hours after the cells were exposed to MTX.

Table 1A. Gi rates of the KHOS/NP cells after 24 hours exposure to MTX and additional 0 hour incubation

Gi rate (%) KHOS/NP cell line	MTX concentration (M)
35.0 \pm 15.4	10^{-8}
49.8 \pm 6.2	10^{-7}
50.1 \pm 6.7	10^{-6}
51.0 \pm 7.9	10^{-5}
50.3 \pm 6.7	10^{-4}
51.4 \pm 6.7	10^{-3}
60.5 \pm 4.6	10^{-2}

Table 1B. Gi rates of the TNMY1 cell line after 24 hours exposure to MTX and additional 0 hour incubation

Gi rate (%) TNMY1 cell line	MTX concentration (M)
1.4 \pm 2.2	10^{-8}
2.5 \pm 2.6	10^{-7}
4.5 \pm 1.7	10^{-6}
4.7 \pm 2.9	10^{-5}
6.9 \pm 5.9	10^{-4}
7.6 \pm 5.5	10^{-3}
4.0 \pm 7.9	10^{-2}

Table 2A. Gi rates of the KHOS/NP cells after 24 hours exposure to MTX and additional 48-hour incubation

Gi rate (%) KHOS/NP cell line	MTX concentration (M)
67.4±3.1	10 ⁻⁸
69.2±2.6	10 ⁻⁷
69.2±2.8	10 ⁻⁶
69.9±2.8	10 ⁻⁵
70.5±2.6	10 ⁻⁴
72.1±3.3	10 ⁻³
0.6±1.8	10 ⁻²

Table 2B. Gi rates of the TNMY1 cell line after 24 hours exposure to MTX and additional 48-hour incubation.

Gi rate (%) TNMY1 cell line	MTX concentration (M)
2.6±3.9	10 ⁻⁸
7.0±4.3	10 ⁻⁷
20.2±5.9	10 ⁻⁶
35.8±4.5	10 ⁻⁵
34.7±4.8	10 ⁻⁴
34.2±3.9	10 ⁻³
35.1±5.5	10 ⁻²

Table 3A. Gi rates of the KHOS/NP cells after 24 hours exposure to MTX and additional 96-hour incubation

Gi rate (%) KHOS/NP cell line	MTX concentration (M)
34.7±13.8	10 ⁻⁸
71.9±12.2	10 ⁻⁷
74.8±9.9	10 ⁻⁶
75.6±9.4	10 ⁻⁵
75.1±9.3	10 ⁻⁴
74.3±8.7	10 ⁻³
77.3±6.2	10 ⁻²

Table 3B. Gi rates of the TNMY1 cell line after 24 hours exposure to MTX and additional 96- hour incubation

Gi rate (%) TNMY1 cell line	MTX concentration (M)
0.9±2.1	10 ⁻⁸
15.1±4.5	10 ⁻⁷
43.5±16.6	10 ⁻⁶
63.4±5.0	10 ⁻⁵
62.9±4.2	10 ⁻⁴
63.1±6.0	10 ⁻³
60.3±9.4	10 ⁻²

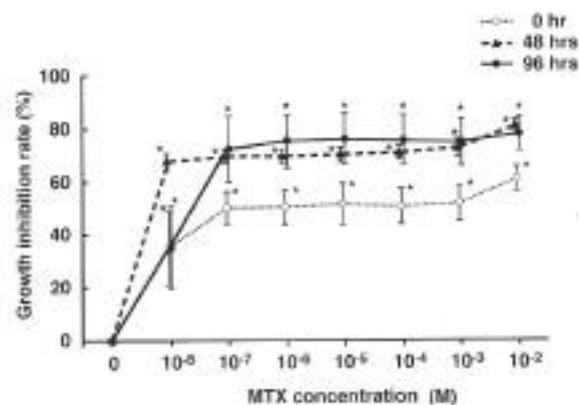


Figure 1: The effect of MTX on the cell proliferation of the osteosarcoma cell line KHOS/NP. Each value represents the mean ± standard deviation from MTS assays of six replicate wells per microtiter plate and three replicate experiments per cell line (n=18). An asterisk beside each error bar indicates statistical significance (p<0.05) compared to the control specimen. MTX: methotrexate

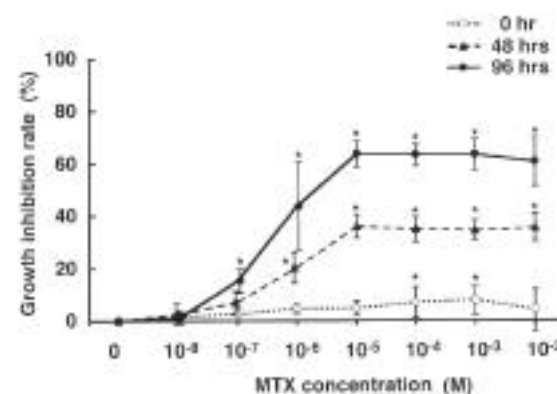


Figure 2: The effect of MTX on the cell proliferation of the MFH cell line TNMY1. Each value represents the mean ± standard deviation from MTS assays of six replicate wells per microtiter plate and three replicate experiments per cell line (n=18). An asterisk beside each error bar indicates statistical significance (p<0.05) compared to the control specimen. MTX: methotrexate

B. The reversal effect of CF on the cytotoxicity of MTX

The Gi rates of the TNMY1 cells after 24 hours exposure to MTX and subsequent 96-hour incubation in the absence of CF at the MTX concentrations of 10⁻⁷, 10⁻⁶, 10⁻⁵, 10⁻⁴, and 10⁻³ M, are shown in **Table 4A** and **Figure 3**, and those in the presence of 10⁻⁷ M CF are shown in **Table 4B** and **Figure 3**, respectively. Two-way repeated measures ANOVA showed no significant decrease in the Gi rates in the presence of 1.0 x 10⁻⁷ mol/L CF. One-way repeated measures ANOVA revealed no significant decrease in the Gi rates at any concentrations of MTX in the presence of CF except for at 1x10⁻⁶ M.

Table 4A. Gi rates of the TNMY1 cell line after 24 hours exposure to MTX and additional 96- hour incubation in the absence of CF.

Gi rate (%) TNMY1 cell line	MTX concentration (M)
6.1±11.1	10 ⁻⁷
36.1±15.0	10 ⁻⁶
63.1±5.7	10 ⁻⁵
61.7±4.2	10 ⁻⁴
59.7±2.7	10 ⁻³

Table 4B. Gi rates of the TNMY1 cell line after 24 hours exposure to MTX and additional 96- hour incubation in the presence of 10⁻⁷M CF.

Gi rate (%) TNMY1 cell line	MTX concentration (M)
6.0±11.6	10 ⁻⁷
20.9±9.4	10 ⁻⁶
57.4±7.4	10 ⁻⁵
60.7±3.6	10 ⁻⁴
60.8±3.0	10 ⁻³

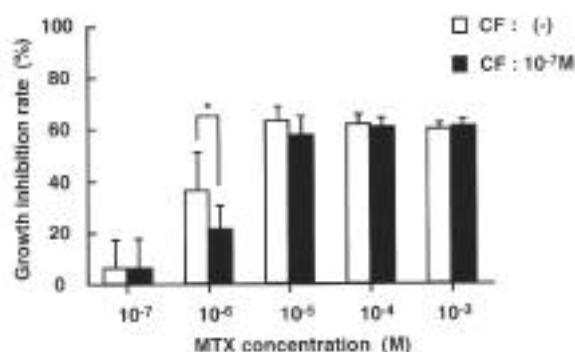


Figure 3: The reversal effect of CF on the cytotoxicity of MTX. Each value represents the mean±standard deviation from MTS assays of six replicate wells per microtiter plate and three replicate experiments per cell line (n=18). An asterisk beside each error bar indicates statistical significance (p<0.05). MTX: methotrexate, CF: Citrovorum factor.

IV. Discussion

Chemotherapy for locally advanced or metastatic spindle-pleomorphic soft tissue sarcomas remains highly investigational. Doxorubicin, epirubicin, and ifosfamide are chemotherapeutic agents commonly used, with a clinical response rate of approximately 20% (Reichardt, 2002); little has been known concerning the effect of HD-MTX with CF rescue on spindle-pleomorphic soft tissue sarcomas either in vivo or in vitro. The present experimental study was designed to provide a conceptual basis for further optimization of the HD-MTX with CF rescue protocol that may be useful in the practical use. The concentration ranges of MTX used in this study were determined to simulate the actual serum levels attained clinically 24 hours after cessation of intravenous administration of HD-MTX in osteosarcoma

chemotherapy (Breithaupt and Kuenzlen, 1983). The standard dose of CF in the practical regimen in osteosarcoma chemotherapy is 15 mg/m² for every six hours, bearing a serum concentration of 1x10⁻⁶ to 1x10⁻⁷ M (Diddens et al, 1987).

In the present study, the data obtained showed that the response to MTX in the MFH cells was significantly lower than that in the osteosarcoma cells shortly after the cells were exposed to MTX for 24 hours. However, a delayed effect of MTX on the MFH cell proliferation was observed; the Gi rates in the MFH cells significantly increased 48 and 96 hours after 24-hour exposure to MTX time-dependently, with the maximal Gi rate of approximately 65%.

The cause of such a delayed effect of MTX on the MFH cell growth is of an important consideration. MTX is a potent inhibitor of dihydrofolate reductase (DHFR), a key enzyme for intracellular folate metabolism (Bertino et al, 1996). After transportation into the cell, MTX needs to be polyglutamylated by folylpolyglutamate synthetase (FPGS) before functioning as DHFR inhibitor. DHFR inhibition by polyglutamylated MTX results in decreased regeneration of tetrahydrofolate from dihydrofolate, which is a product of thymidylate synthase, leading to decrease in DNA biosynthesis (Bertino et al, 1996). The decreased transport of MTX into the cells, impaired polyglutamylation of MTX, and increased DHFR enzyme activity of the cells have been suggested to be the main factors of cell resistance to MTX (Bertino et al, 1996). Assuming that the MFH cells had increased activities of the latter two pathways, the growth inhibition rates should have remained low despite the subsequent incubation after MTX exposure. There is a possibility that a decreased transport of MTX into the MFH cells could result in a delayed increase of intracellular concentration of polyglutamylated MTX, leading to the late response to MTX.

Our data showed that the growth inhibition effects of MTX on both MFH and osteosarcoma cells increased dose-dependently. The maximal Gi rate was attained at the concentrations of over 1x10⁻⁵ M in the MFH cells, and at the concentrations of over 10⁻⁷ M in the osteosarcoma cells when the cells were subjected to 96 hours incubation following 24-hour exposure to MTX. In the clinical HD-MTX chemotherapy, the serum concentrations of MTX have been reported to be 1x10⁻⁵ to 1x10⁻³ M during 24 hours after administration (Breithaupt and Kuenzlen, 1983). These data indicate that the MTX dosages used in the clinical chemotherapy for osteosarcomas could sufficiently cover the effective serum concentration levels for MFH of soft tissue. Although the maximum Gi rate in MFH cells was lower than that in osteosarcoma cells, the MFH cells are considered to be sensitive to MTX.

Citrovorum factor is readily converted to reduced folate independently of DHFR within the cells and bypasses the block of DHFR by MTX, thereby replenishing cellular pools of tetrahydrofolate depleted by MTX action (Diddens et al, 1987; Haskell, 1980). Since a certain neoplastic cell has an insufficient active transporting ability of CF in its cell membrane, such a protective effect of CF from MTX cytotoxicity is often

more effective in normal tissues than in neoplasms (Haskell, 1980). The data in the present study showed that 10^{-7} M CF did not reverse the cell proliferation of TNMY1 cell line when the cells were pretreated with over 10^{-5} M of MTX, although a significant reversal effect of CF was observed after pretreatment with 10^{-6} M of MTX. The reversal effect of CF after pretreatment with the lower dose of MTX could be explained by the fact that MTX and CF competitively share an active transporting mechanism, hence high levels of MTX may require large doses of CF to prevent cell death (Haskell, 1980). These data indicated that the standard concentration of CF administration after HD-MTX could act as the rescue for normal cells without preventing the cytotoxicity of MTX for TNMY1 cells.

V. Conclusions

Our data in the present study have indicated that MFH of soft tissue is potentially sensitive to MTX. The HD-MTX with CF rescue chemotherapy protocol that is frequently used for osteosarcoma is possibly applicable to treatment of MFH of soft tissue, although clinical prospective studies are required to verify this.

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