Pharmacology, Biochemistry and Behavior 95 (2010) 428–433

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Intrathecal methotrexate induces focal cognitive deficits and increases cerebrospinal fluid homocysteine

Yan Li [a], Veena Vijayanathan [a], Maria Gulinello [b], Peter D. Cole [a,*]

[a] Department of Pediatrics, Albert Einstein College of Medicine, 1300 Morris Park Ave, Forchheimer 702, Bronx, NY 10461, United States
[b] Behavioral Core Facility, Dept of Neuroscience, Kennedy 925, 1410 Pelham PKWY S, Albert Einstein College of Medicine, Bronx, NY 10461, United States

ARTICLE INFO

Article history:
Received 29 January 2010
Received in revised form 20 February 2010
Accepted 13 March 2010
Available online 21 March 2010

Keywords:
Acute lymphoblastic leukemia
Methotrexate
Intrathecal injection
Folate
Homocysteine
Cytokines
Interleukin-6

ABSTRACT

Although most children with acute lymphoblastic leukemia (ALL) can be cured, a significant subset of survivors manifests focal deficits in cognitive function, even when the treatment regimen does not include cranial radiation. Intrathecal administration of the folate antagonist methotrexate (MTX) is necessary to prevent leukemic relapse within the central nervous system, but is suspected to contribute to treatment-induced cognitive dysfunction. To better elucidate the underlying pathophysiology, we sought to establish a rodent model of the cognitive and neurotoxic effects resulting from direct administration of MTX into the cerebrospinal fluid (CSF). MTX or artificial CSF was injected via transcutaneous puncture at the level of the cisterna magna. Subsequent behavioral tests were designed to assess cognitive domains frequently impaired among children treated for ALL. MTX administration produced both recognition and spatial memory deficits, without altering general activity or motor coordination. In addition, MTX significantly reduced folate levels in both CSF and serum and increased CSF homocysteine. Thus, we have established an animal model that mimics the clinical effects of prophylactic intrathecal MTX on cognitive function. Using this model we can further study the pathophysiology of MTX-induced cognitive dysfunction and test protective interventions.

1. Introduction

Prevention of central nervous system relapse is a necessary component of all therapeutic regimens for patients with acute lymphoblastic leukemia (ALL). To avoid the neurotoxic side effects and the risk of secondary malignancies associated with cranial radiation, most treatment protocols for childhood ALL instead employ repeated administration of prophylactic intrathecal chemotherapy (Pui et al., 2009). Nevertheless, a substantial minority of pediatric ALL patients still manifests cognitive deficits during and after treatment (Buizer et al., 2009). Reduction of these persistent deficits is an emerging focus of research in ALL, as they may impair educational or occupational competences.

Methotrexate (MTX) is an important chemotherapeutic agent for the treatment of patients with ALL, but also is implicated in treatment-induced neurotoxicity. Both systemic and intrathecal administration of MTX have been associated with cognitive dysfunction among pediatric ALL survivors (Hill et al., 1997; Montour-Proulx et al., 2005), as well as biochemical alterations (Cole and Kamen, 2006) and brain morphological changes (Hill et al., 2004). The pathophysiology of MTX-induced neurotoxicity is not fully understood. An analog of folic acid, MTX exerts anti-neoplastic effects by inhibiting folate-dependent thymidine and purine synthesis. This disruption of folate homeostasis within the central nervous system (CNS) may also explain its neurotoxic effects via inhibition of critical physiological processes, toxic substance accumulation, and direct neuronal damage. Furthermore, an inflammatory response, initiated by microglial cells in response to irritating stimuli such as intrathecal chemotherapy, may also affect cognitive function and trigger secondary neuronal injury.

Rodent models are valuable for studying the detrimental effects of MTX in isolation, without the complication of a co-existing disease state or multi-agent chemotherapy. We recently reported that systemic MTX-induced focal cognitive deficits and disturbances of folate homeostasis in rats after intraperitoneal (i.p.) administration (Li et al., 2010). We now present a rat model of intrathecal MTX delivery, established to mimic prophylactic intrathecal therapy. Using assays targeting domains of cognitive function frequently impaired in pediatric ALL patients, we detected focal cognitive deficits in both recognition and spatial memory, without altered motor coordination or basal activity levels. These cognitive deficits were associated with decreased CSF and serum folate and increased CSF homocysteine, but not with a specific increase in CSF pro-inflammatory cytokines.

* Corresponding author. Tel.: +1 718 741 2342; fax: +1 718 920 6506.
E-mail address: Peter.Cole@Einstein.YU.edu (P.D. Cole).

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doi:10.1016/j.pbb.2010.03.003

2. Methods

2.1. Subjects and chemicals

Wild-type male adult Long Evans rats were obtained from Charles River Laboratories (Wilmington MA). Rats were housed in groups of 2 with *ad lib* food (LabDiet 5001) and water with a 12–12 light/dark cycle. All studies were conducted following the 'Guide for the Care and Use of Laboratory Animals' and were approved by the Animal Institute Committee of the Albert Einstein College of Medicine.

Methotrexate (MTX, USP grade), lipopolysaccharide (LPS) and other chemicals were purchased from Sigma (Saint Louis, MO) unless otherwise stated. Methanol and water (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA). All injected solutions were sterilized by filtering through 0.22 μm syringe filters.

2.2. Intrathecal MTX injection and sample collection

Intrathecal injection was carried out by transcutaneous cisternal magna puncture (Consiglio and Lucion, 2000; Waynforth and Flecknell, 1992). Briefly, 12 week-old male Long Evans rats were anesthetized with inhaled 5% isoflurane/95% oxygen, and positioned in the lateral decubitus position. A 25-gauge butterfly needle with ~2.5 cm tubing was inserted into the cisterna magna. Correct positioning of the needle was verified by outflow of cerebrospinal fluid (CSF). MTX was diluted in artificial CSF (aCSF, concentration in mM, Na^+ 150, K^+ 3, Ca^{2+} 1.4, Mg^{2+} 0.8, P 1.0, and Cl^- 155, in double distilled water). Over a period of 1 min, 0.5 mg/kg MTX was injected via a straight needle inserted into the shortened tubing of a butterfly needle. LPS (100 μg/kg) diluted in aCSF was similarly injected in four rats to induce a robust cytokine response, as positive controls for cytokine studies. Control animals were injected with equal volume of aCSF. For all injections, the dead space of injecting system was taken into consideration. Animals were observed for 1 h after injection for acute toxicity.

CSF was collected by gravity at multiple time points, as indicated in the results. Samples with gross contamination by blood were not analyzed (approximately 20% of all specimens). CSF samples were placed immediately in ice, then centrifuged briefly to remove any cellular elements. Supernatants were stored at −80 °C until further analysis.

Blood was collectected from euthanized rats by cardiac puncture. Serum or plasma were seperated from red blood cells by centrifugation, and stored at −80 °C until further analysis.

2.3. Behavioral tests

All behavioral tests were carried out between day three and day seven after intrathecal injection. The order of tests was counter-balanced between cohorts of animals to prevent potential systematic effects between types and timing of tests.

The open field test was used to assess locomotor, exploratory and anxiety-like behavior, following established procedure (Li et al., 2010). Briefly, rats were free to explore the testing arena (69×69 cm with visual cues) for 6 min. Activities were captured by digital camera and analyzed with a Viewer software (Biobserver, Bonn, Germany). Total activity was assessed by total track length. Thigmotaxis, an indication of anxiety, is inversely related to the time the animal spent in the center zone.

Recognition and spatial memory were tested in the novel object recognition task and the object placement task respectively, and were carried out as previously stated (Li et al., 2010). In these assays, exploration of an object was defined as any physical contact with the object (whisking, sniffing, rearing against or touching). During training, animals were exposed to pairs of identical objects. After spending a certain period of retention interval in their home cages, rats were presented with one new and one old object (object recognition test), or one unmoved and one relocated object (object placement test) in testing trial. Intact memory is demonstrated by preferential exploration of the new or relocated object in the testing trial. Results are reported as exploratory preference scores (100 x exploration duration for the new or relocated object/total exploration time), and as success rates (the proportion of rats in each group with intact memory). Preference score higher than 53 or 55 were defined as 'pass' (see supplementary data for success rate analysis). Total exploration time in the training trial was used to assess levels of novel object exploration.

Motor coordination was assessed by the balance beam test (Stanley et al., 2005). Animals were first pre-trained to readily traverse an open space by walking across a flat plank 7.5 cm wide. In both pre-training and testing, the starting point was brightly lit, and the goal end contained a palatable food (such as Cocoa Krispies®) and a dark "hide" in order to encourage the rat to walk reliably and readily across the beam. Pre-training sessions were repeated until the rat readily traversed the plank, typically 2 to 3 trials. Motor coordination was then assessed as the number of hind leg slips and the beam crossing time on a round beam of similar dimension.

2.4. High performance liquid chromatography

Homocysteine and 5-methyltetrahydrofolate (5-methylTHF; the predominant folate species in rat and human serum and CSF) were measured using a Waters 2695 separation module equipped with a Waters 2475 fluorescene detector (Waters, Milford, MA), and a reversed phase Waters Bondapak C18 analytical column (3.9×150 mm), following our previously published methods (Li et al., 2010).

2.5. Electrochemiluminescence assay

Concentrations of cytokines IL-1β, IL-6 and TNF-α in CSF were measured using electrochemiluminescence detection technology. CSF samples were applied to a custom-made multiplex ultra-sensitive rat cytokine plate, following manufacturer's instruction, and detected by Sector Imager 2400 (Meso Scale Discovery, Gaithersburg, MD). Standard curves for each analyte were generated and concentrations of CSF cytokines were calculated using software supplied by the manufacturer.

2.6. Statistics

Preference scores were compared with GraphPad Prism 5.02 (GraphPad Software, San Diego, CA) using *t*-tests, and the success rates were analyzed by Fisher's Exact Test. Biochemical and cytokine data were analyzed using one-way ANOVA analysis, followed by *post-hoc t*-tests.

3. Results

3.1. MTX induces cognitive deficits

Intrathecal administration of MTX induced deficits in recognition memory, assessed in the object recognition test with a long (2 h) retention interval. Deficits were significant when expressed as preference scores (Fig. 1A), and as success rate (Fig. S1). In contrast, MTX-treated rats showed no deficits in tests with short (2 min) retention intervals (Fig. 1C). MTX also adversely affected spatial memory, assessed by object placement test (Fig. 2A, Fig. S1). MTX did not change total object explorations in trial 1 (training trial) of any tests (Figs. 1B and D, 2B). In summary, MTX caused deficits in recognition and spatial memory, but spared the animal's innate preference for novel objects and sensorimotor abilities.

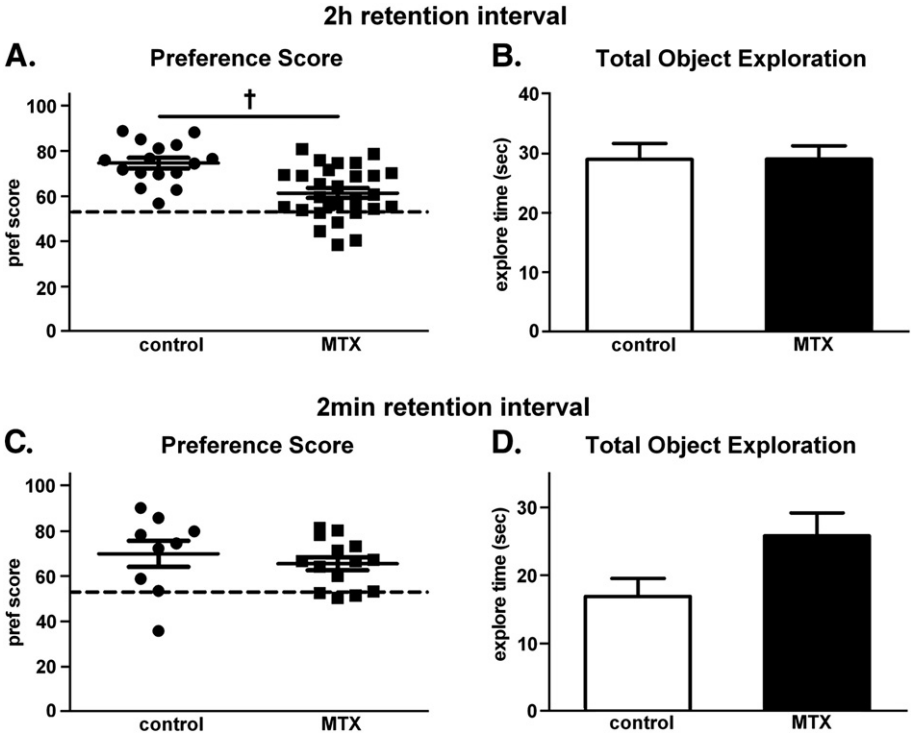

Fig. 1. MTX-induced deficits in recognition memory, assessed by object recognition tests. A. Preference scores in object recognition tests with 2 h retention interval (OR 2 h). Dotted line represents the passing score of 53. Group means: control 74.6 ± 2.3 ($n = 16$), MTX 61.3 ± 2.2 ($n = 28$); †, $p < 0.01$, two-tailed t-test. B. Total exploring time in training trial of OR 2 h test: control 29.04 ± 2.68 s, MTX 29.10 ± 2.20 s; $p = 0.98$, two-tailed t-test. C. Preference scores in object recognition tests with 2 min retention interval (OR 2 min). Broken line represents the passing score of 53. Group means: control 69.8 ± 5.8 ($n = 9$), MTX 65.5 ± 2.9 ($n = 14$); $p = 0.46$, two-tailed t-test. D. Total exploring time in training trial of OR 2 min test: control 16.89 ± 2.66 s, MTX 25.83 ± 3.34 s; $p = 0.07$, two-tailed t-test.

No deficits were found in the remaining behavioral tests. In the open field test, there was no difference in general locomotor activity assessed by total tracklength (control 3186 ± 388 cm, $n = 10$; MTX 3057 ± 230 cm, $n = 15$), or in thigmotaxis assessed as time spent in the center zone (control 16.57 ± 3.41 s, $n = 10$; MTX 19.75 ± 3.59 s, $n = 15$). In the balance beam test, no motor coordination difference was detected in terms of number of slips (control 2.4 ± 0.7, $n = 8$; MTX 3.1 ± 0.5, $n = 12$) or in beam crossing time (control 1.4 ± 1.4 s, $n = 8$; MTX 10.6 ± 0.6 s, $n = 12$).

3.2. MTX perturbed folate homeostasis in CSF and serum

Intrathecal MTX induced significant decreases in folate concentrations (Fig. 3). CSF 5-methylTHF (Fig. 3A) fell to approximately 25% of the baseline level by 24 h post injection and gradually recovered to approximately 75% of baseline by day 7. At each assayed time point, CSF 5-methylTHF levels in the MTX group were significantly lower than those of the control group, and also significantly lower than before-injection level. Serum (Fig. 3B) 5-methylTHF was also significantly lower than that of the control group 7 days post injection.

Intrathecal administration of MTX led to a transient increase in CSF homocysteine (Fig. 4A). One day after injection of MTX, CSF homocysteine increased approximately four-fold relative to baseline, and returned to control level by second day post injection. At 7 days post injection, there was no difference in serum homocysteine level between MTX and control (Fig. 4B), despite a significantly lower serum folate in the MTX group.

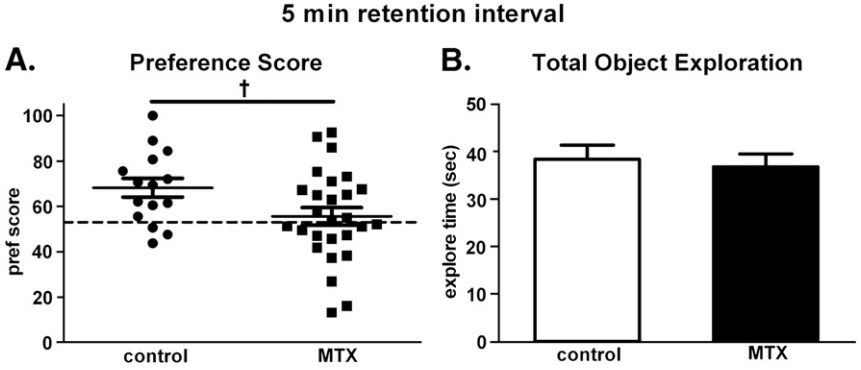

Fig. 2. MTX-induced deficits in spatial memory, assessed by object placement test. A. Preference scores in object placement test of 5 min retention interval (OP 5 min). Dotted line represents the passing score of 53. Group means: control 68.3 ± 4.1 ($n = 15$), MTX 55.7 ± 3.8 ($n = 27$); †, $p = 0.02$, two-tailed t-test. B. Total exploring time in training trial of OP 5 min test. Group means: control 38.80 ± 2.82 s, MTX 36.87 ± 2.67 s. Difference is not significant ($p = 0.71$, two-tailed t-test).

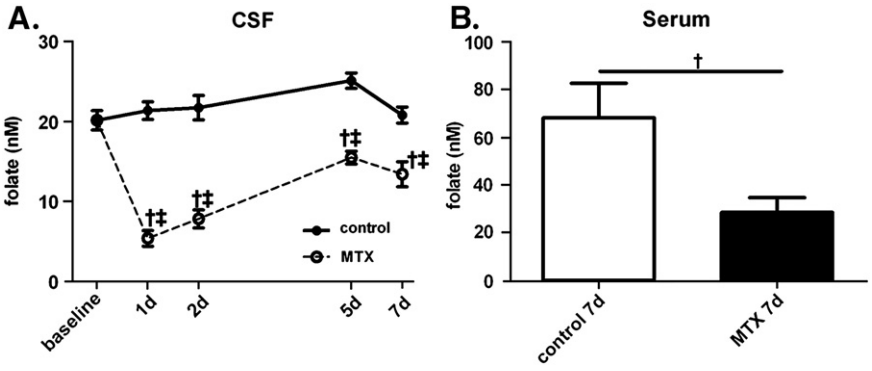

Fig. 3. Intrathecal MTX decreases CSF and serum 5-methylTHF levels. A. Mean CSF 5-methylTHF concentrations (in nM): control 1d 20.2 ± 1.2 (n = 9), MTX 1d 5.3 ± 1.0 (n = 9); control 2d 21.8 ± 1.5 (n = 6), MTX 2d 7.8 ± 1.1 (n = 10); control 5d 25.2 ± 1.0 (n = 3), MTX 5d 15.5 ± 0.8 (n = 11); and control 7d 20.8 ± 1.0 (n = 11), MTX 7d 13.4 ± 1.6 (n = 11). After intrathecal MTX, all CSF 5-methylTHF values were lower than baseline (20.2 ± 1.2 nM, n = 9). Significant differences (p<0.05, two-tailed t-test) comparing to same-time controls were denoted by †, to baseline were denoted by ‡. B. Serum 5-methylTHF level was reduced by intrathecal MTX at 7 days post injection (control 68.1 ± 14.5 nM, n = 11, MTX 28.5 ± 6.2 nM, n = 11; †, p = 0.02, two-tailed t-test).

3.3. Intrathecal MTX induced a nonspecific increase in CSF interleukin-6

CSF concentrations of IL-1β, IL-6 and TNF-α were measured to assess whether intrathecal injection of MTX induces a pro-inflammatory response. As expected, intrathecal injection of the bacterial endotoxin LPS induced robust production of cytokines within 6 h (Fig. 5). Both MTX and control injections induced a significant increase in IL-6 level at 6 h after injection (Fig. 5A), with no increase in IL-1β (Fig. 5B) or TNF-α (Fig. 5C). By 24 h post injection, CSF IL-6 returned to baseline in both groups. No difference in cytokine levels was observed between MTX and control rats at any time point.

4. Discussion

Direct intrathecal administration of MTX is a routine component of therapeutic regimens for children with ALL. Although it effectively reduces the risk of CNS relapse, prophylactic intrathecal chemotherapy has been associated with both acute neurotoxicity and cognitive deficits that persist after therapy (Buizer et al., 2009; Geiser et al., 1975; Harila et al., 2009; Lofstad et al., 2009; Peterson et al., 2008). Here, we established a rodent model of intrathecal MTX administration, demonstrating cognitive deficits in conjunction with folate physiology changes that are directly analogous to what is observed clinically.

Although it is difficult to directly compare dosing across species due to relative differences in compartment volumes, the dose of MTX administered intrathecally in this rat model should result in CSF concentrations similar to those obtained in humans during treatment for ALL. Intrathecal administration of 0.15 mg to a 300 g rat (0.5 mg/kg), which has a total CSF volume of 580 mL (Lai et al., 1983) results in a CSF MTX concentration of 0.26 mg/mL (~570 mM). This is similar to the calculated concentration that would result from an intrathecal dose of 15 mg in a fully-grown male patient with ALL, into CSF volume measured by MRI to a range from 57–287 mL (median 146 mL),(Grant et al., 1987) for a final concentration ranging from 0.05–0.26 mg/mL (115–580 mM).

4.1. Cognitive deficits

We chose the object recognition and object placement tests as our primary cognitive assays due to their similarity to tests used in human subjects (Kessels et al., 2002; Salame et al., 2006). First, they assess similar cognitive domains. Second, they do not require food or water deprivation of subjects, nor application of stressors (such as electric shock). Third, these tests do not require high levels of motor coordination or muscle stamina.

We detected deficits after intrathecal MTX in recognition memory (Fig. 1A) and in spatial memory (Fig. 2A). In contrast, total novel object exploration (Figs. 1B and D, 2B), novel object preferences at shorter retention intervals (Fig. 1C), general activity levels (open field tests) and motor coordination (balance beam tests) were not altered by MTX. These observations indicate that intrathecal MTX induces focal memory deficits while sparing other behavioral domains.

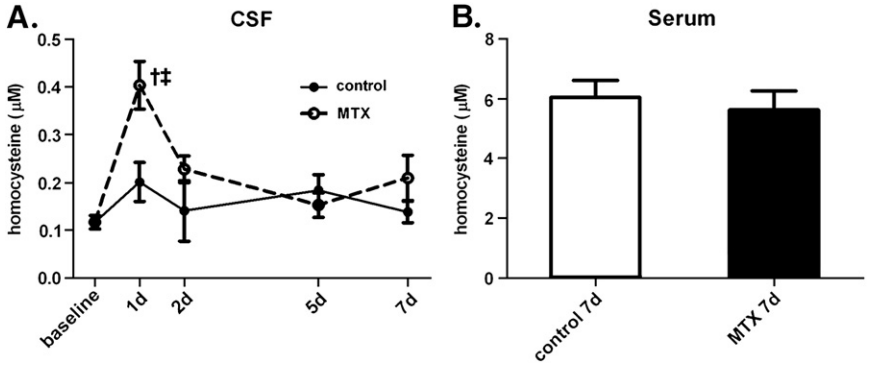

Fig. 4. Intrathecal MTX transiently increased CSF but not serum homocysteine. A. Mean CSF homocysteine concentrations (in μM): control 1d 0.20 ± 0.11 (n = 7), MTX 1d 0.40 ± 0.15 μM (n = 9); control 2d 0.14 ± 0.06 (n = 4), MTX 2d 0.23 ± 0.03 (n = 7); control 5d 0.18 ± 0.03 (n = 5), MTX 5d 0.15 ± 0.03 (n = 7); and control 7d 0.14 ± 0.02 (n = 10), MTX 7d 0.21 ± 0.05 (n = 10). Significant differences (p<0.05 two-tailed t-test) comparing to same-time controls were denoted by †, to baseline (0.12 ± 0.01, n = 9) were denoted by ‡. B. Serum homocysteine was not changed 7 days after injection (control 6.05 ± 0.58 μM, n = 11; MTX 5.63 ± 0.65 μM, n = 11; p = 0.63, two-tailed t-test).

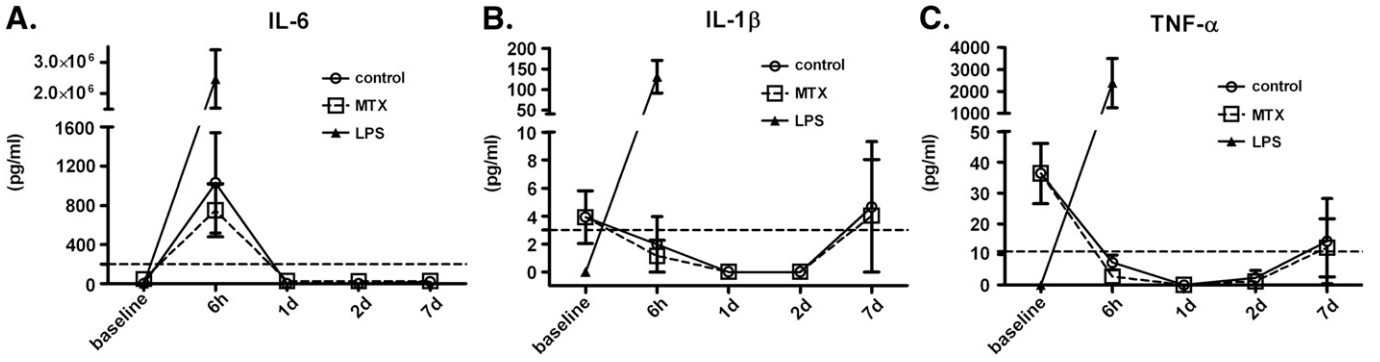

Fig. 5. CSF Cytokines after intrathecal injection. A. CSF IL-6 concentrations. Broken line represents the lower limit for detection. LPS induced more than one million fold increase of IL-6 in CSF 6 h after injection, served as positive control. At 6 h, control 1030 ± 513 pg/ml ($n = 5$), MTX 750 ± 270 pg/ml ($n = 7$). IL-6 levels were below detection at all other time points. Difference not significant between control and MTX at 6 h ($p = 0.61$, two-tailed t-test). B. CSF IL-1β concentrations. Broken line represents the lower limit for detection. Intrathecal LPS induced a 100-fold increase of IL-1β 6 h after injection. Control and MTX levels are mostly below detection. C. Control and MTX CSF TNF-α concentrations are below mostly detection, while LPS induced a 1000-fold increase.

Our model closely replicates the clinical use of intrathecal MTX for children with leukemia. As with children treated for ALL, this dose of MTX (0.5 mg/kg) did not cause severe acute toxicity. No immediate deleterious effects, such as seizures, were observed in any of injected rats. Furthermore, the focal deficits in cognitive function in the present study are consistent with those domains of function reported to be deficient in a meta-analysis of children treated for ALL with multi-agent chemotherapy including intrathecal MTX (Peterson et al., 2008). Lastly, although deficits occurred at a statistically greater rate in rats treated with intrathecal MTX, deficits were not universally observed, in parallel with the observation that significant cognitive deficits are observed among a minority of children treated for ALL.

4.2. MTX alterations in folate homeostasis.

In spite of sufficient dietary folate and methionine supplementation, intrathecal MTX-induced CNS folate deficiency in rats, similar to clinical observations (Cole et al., 2009; Drachtman et al., 2002; Vezmar et al., 2009). A significant reduction in concentration of 5-MethylTHF, the primary reduced folate in CSF, was observed as early as one day post injection. Recovery was not complete 7 days later (Fig. 3A). This reduction in CSF folate was accompanied by a transient increase in CSF homocysteine (Fig. 4A) and a reduction in serum folate 7 days post injection (Fig. 3B).

The systemic reduction in serum folate after intrathecal injection of MTX is likely due to the known active excretion of MTX from CSF into the peripheral circulation (Bleyer et al., 1997; Breen et al., 2004). Once in the systemic circulation, MTX can lower folate stores by increasing renal excretion of folate (Deutsch and Kolhouse, 1989). In addition, serum MTX might further decrease CNS folate uptake by competitively inhibiting folate transport from serum to CSF (Chen and Wagner, 1975; Spector and Lorenzo, 1975). The net result is a reduction in bioactive folate available for neuronal cells, from blood or CSF sources. Neurotoxicity may occur by paralyzing folate-dependent critical reactions, and/or by accumulation of toxic compounds such as homocysteine and its metabolites (Vezmar et al., 2003).

4.3. Cytokines and cognition

Several studies have focused on the influence of pro- and anti-inflammatory cytokines on rodent cognitive function (reviewed in McAfoose and Baune, 2009). We did observe a significant increase in the pro-inflammatory cytokine IL-6 after intrathecal injection of MTX (Fig. 5). However, a similar increase was observed after injection of aCSF. Because cognitive deficits were observed only in the group given intrathecal MTX, we conclude that an increase in CSF IL-6 is not a primary component of the pathophysiology of cognitive dysfunction after intrathecal MTX. This observation is also consistent with a recent report in which systemic administration of 250 mg/kg MTX did not increase cytokine levels in adult rat hippocampus, 5 days post injection (Seigers et al., 2009).

4.4. Pathophysiology of MTX-induced cognitive dysfunction

MTX-induced neurotoxicity is likely caused by multiple sequelae of folate deficiency. First, folate deficiency impairs DNA synthesis, which might suppress hippocampal cell proliferation (Kruman et al., 2005; Seigers et al., 2008). Second, MTX exposure limits additional folate-dependent methylation reactions. Possible consequences include abnormal synapse formation (Igarashi et al., 1989), axon demyelination (Gilbert et al., 1989), and depletion of membrane phosphatidylcholine content (Troen et al., 2008). Third, inhibition of folate metabolism can lead directly to neuronal degeneration (James et al., 2008; Silverstein and Johnston, 1986). Fourth, MTX inhibits folate-dependent neurotransmitter metabolism, leading to a reduction in norepinephrine, dopamine and serotonin (Madhyastha et al., 2002; Silverstein and Johnston, 1986). Fifth, MTX induces accumulation of homocysteine, which is directly vasculopathic, resulting in decreased brain blood flow (Mizusawa et al., 1988). In addition, homocysteine and its metabolites are excitotoxic agonists at the N-methyl-D-aspartate (NMDA) receptor, inducing seizures and neuronal apoptosis (Ho et al., 2002; Parsons et al., 1998; Zieminska and Lazarewicz, 2006). The confluence of all the above mechanisms might manifest as cognitive deterioration. Unfortunately, we were not able to study markers of all the potential mechanisms of MTX-induced toxicity, due to limitations imposed by the small volume of CSF collected after MTX administration. Changes in CSF concentrations of adenosine, SAM/SAH and neurotransmitters such as dopamine and serotonin will be the subject of future studies.

4.5. Comparison of rodent models of intrathecal and systemic MTX

In an earlier study, we reported that systemic MTX administration caused focal cognitive deficits along with disturbances of CNS folate homeostasis (Li et al., 2010). A comparison of these two studies provides additional insights into the cognitive toxicity of MTX. General motor activity and behavior after systemic MTX were also intact after either chronic or acute i.p. treatment. In addition, changes in CSF or serum folate levels were comparable in these studies. However, intrathecal delivery of MTX induced more profound focal cognitive deficits. Recognition memory, which was deficient after intrathecal MTX, was not affected by i.p. MTX. Spatial memory deficits

were evident after both routes of administration, but were arguably more affected by intrathecal MTX, since deficits were evident after shorter retention intervals in object placement test (5 min intrathecal model vs.10 min i.p. model).

Thus it seems that intrathecal MTX was more deleterious than systemic administration despite the dose being only 0.2% of what is used for i.p. treatments. We hypothesize that cognitive deficits induced by i.p. MTX are mostly secondary to decreasing brain folate. In contrast, intrathecal MTX may directly cause neuronal damage (James et al., 2008) and directly alter CNS folate homeostasis, in addition to secondary effects on systemic folate availability, thus causing more profound cognitive deficits.

5. Conclusion

The rat model of intrathecal MTX-induced cognitive dysfunction presented in this report is representative of acute chemotherapy-induced cognitive dysfunction among children treated for ALL without cranial radiation, in terms of the focal cognitive deficits and the associated biochemical changes. This model, therefore, is an appropriate tool to advance research in the pathophysiology of MTX-induced neurotoxicity and for testing preventive interventions.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbb.2010.03.003.

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