Letter

Global chemokine expression in methylmercury-treated mice: methylmercury induces brain-specific expression of CCL3 and CCL4

Min-Seok Kim¹, Tsutomu Takahashi¹, Jin-Yong Lee^{1,2}, Gi-Wook Hwang¹
and Akira Naganuma¹

¹Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences,
Tohoku University, Aoba-ku, Sendai 980-8578, Japan

²Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University,
1-100 Kusumoto, Chikusa-ku, Nagoya 464-8650, Japan

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ABSTRACT — We previously reported that methylmercury induces brain-specific upregulation of a chemokine gene, the CCL4 gene. In this study, we investigated the changes in gene expression levels of all chemokines in various tissues (cerebellum, cerebrum, kidney, liver, and spleen) of methylmercury-treated mice. We found that methylmercury induced upregulation of the CCL4 gene and the CCL3 gene, in a brain-specific manner. This finding suggests that upregulation of these two chemokine genes mediates selective damage by methylmercury in the central nervous system.

Key words: Methylmercury, Chemokine, Induction, Brain, CCL3, CCL4

INTRODUCTION

Methylmercury is an environmental toxicant that selectively damages the central nervous system (CNS), but the mechanism of its toxicity has been unclear (Castoldi *et al.*, 2003). We recently reported that administration of methylmercury induces brain-specific upregulation of a chemokine gene, the CCL4 gene (Hwang *et al.*, 2011; Lee *et al.*, 2012). Chemokines, a family of chemotactic cytokines, are secreted mainly by immune cells and play a role in inflammation (Murdoch and Finn, 2000). To date, 37 chemokines have been identified in mice and are classified into four subfamilies (C, CC, CXC and CX3C) based on their structural characteristics (Giraud *et al.*, 2010; Ito *et al.*, 2006). Recently, it was revealed that chemokines are secreted in several organs, including the brain, kidney, and liver, in addition to immune cells (Gorter *et al.*, 2006; Shimizu *et al.*, 2012; Soria and Ben-Baruch, 2008).

In this study, with the aim of identifying the chemokines that are upregulated in a brain-specific manner in response to methylmercury administration, we employed a mouse model to examine methylmercury-induced changes in comprehensive chemokine expression profiles

in various tissues.

MATERIALS AND METHODS

Animal experiments

C57BL/6 male mice (8 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed in plastic cages (4 animals per cage) at $22 \pm 2^\circ\text{C}$ with a relative humidity of $55 \pm 20\%$ under a 12-hr light/dark cycle. Chow (F-2, Oriental Yeast, Tokyo, Japan) and water were provided *ad libitum*. All experiments were performed in accordance with the Regulations for Animal Experiments and Related Activities at Tohoku University. After an adaptation period, mice were randomly divided into control ($n = 5$) and methylmercury-treated ($n = 5$) groups. Methylmercuric chloride dissolved in physiological saline was administered by subcutaneous injection.

Measurement of chemokine mRNA levels by quantitative real-time PCR

Total RNA was isolated from tissues using the Isogen Kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out using the PrimeScriptTM RT Reagent Kit (Takara,

Shiga, Japan) (Kim *et al.*, 2012). Quantitative real-time PCR was performed using SYBR Premix EX Taq (Takara) and a Thermal Cycler Dice® (Takara) with the oligonucleotides shown in Table 1. Fold changes in mRNA levels were determined from standard curves after calibrating the assay.

RESULTS AND DISCUSSION

Methylmercuric chloride (10 mg/kg) was administered by subcutaneous injection once daily for 7 consecutive days. The cerebellum, cerebrum, kidney, liver, and spleen were removed 24 hr after the final administration, and changes in the gene expression levels of 37 chemokines (see Table 1) were investigated in each of the tissues. Expression levels of the following chemokine genes were significantly upregulated (≥ 5 -fold) by methylmercury: in the cerebellum, CCL2, CCL3, CCL4, CCL5, CCL7, CCL9, CCL12, CXCL5, and CXCL10; in the cerebrum, CCL2, CCL3, CCL4, CCL7, CCL11, CCL12, CCL17, and CXCL10; in the liver, CCL6 and CXCL14; and in the kidney, CCL2, CCL7, CCL9, CCL12, CXCL1, CXCL2, CXCL3, CXCL5, and CXCL17 (Table 2). Methylmercury did not significantly increase the upregulation of any of the tested chemokine genes in the spleen (Table 2). Taken together, the 7-day administration of methylmercury appeared to upregulate CCL4, as well as CCL3 and CXCL10, in a brain-specific manner in mice.

When we examined the time-course changes in the expression levels of the CCL3, CCL4, and CXCL10 genes over the 7-day regimen, on day 5 of administration, CXCL10 gene expression was significantly increased in the kidney and there was evidence of significant brain-specific changes in CCL3 and CCL4 expression levels, but no upregulation was seen in the other organs at any of the time points tested (Fig. 1). Furthermore, when the expression levels of the CCL3 and CCL4 genes were examined 5 days after a single administration of various doses of methylmercury, both were found to be increased in a dose-dependent manner in the cerebellum and cerebrum (Fig. 2). CCL3 and CCL4 are known as macrophage inflammatory protein (MIP) 1 α and MIP 1 β , respectively, and both bind to the chemokine receptor CCR5, thereby activating downstream signal pathways (Gamo *et al.*, 2008; Rollins, 1997). CCL3 and CCL4 are also reported to be upregulated after brain injury (Redell *et al.*, 2013). Therefore, we cannot deny the possibility that excessive activation of signal pathways downstream of CCR5 mediates, via methylmercury-induced brain-specific upregulation of CCL3 and CCL4, methylmercury's toxicity in the CNS.

Table 1. Oligonucleotides primers used for quantitative real-time PCR.

Gene	Sense	Antisense
CCL1	ggatgttgacagcaagagca	tagttgaggcgcagctttct
CCL2	ttgtcaccaagctcaagagaga	gaggtggttggtgaaaaggtag
CCL3	atgaaggtctccaccactgc	cccaggtctctttggagtca
CCL4	caaacctaacccgagcaacac	ggtctcatagtaatccatcacaaagc
CCL5	ccctcaccatcatcctcact	ccttcgagtacaaacacga
CCL6	aggctggcctcatacaagaa	tccctcctgctgataaaga
CCL7	aatgcaccacatgctgcta	ctttggagttggggttttca
CCL8	ttctttgctgctgctcata	tctggaaaaccacagcttcc
CCL9	tgtttcacatgggcttcaa	tttaggtccgtggttgga
CCL11	tccacagcgcttctattcct	ctatggcttcagggtgcat
CCL12	gtcctcaggtattggctgga	gggtcagcacagatctcctt
CCL17	tgcttctggggactttctg	tggcctcttcacatgtttg
CCL19	agactgctgcctgctgtga	gcctttgttcttgagcagaag
CCL20	cgactgttgctctctgtaca	aggaggttcacagccctttt
CCL22	gctctcgtctcttctgctgt	gcagagattttgaggtccaga
CCL24	ctgtgaccatccccatct	tatgtgcctctgaaccaca
CCL25	ctgggttaccagcacaggat	ctcatgccctcttcacatt
CCL26	ttgttctcctggccatcttc	tcactgggtcagctctgttc
CCL27	tgctccaacaagccagaga	gacagcctggaggtgacagt
CCL28	gttcatgcagcatccagaga	cctgtgtgtccacagtgttc
CXCL1	gctgggattcacctcaagaa	tggggacaccttttagcatc
CXCL2	agtgaactgcgctgtcaatg	ttcagggtcaagcgaactct
CXCL3	agtgcctgaacaccctacca	ggacttgccgctcttcagta
CXCL4	agtcctgagctgctgtctt	ggcaaatctctcccttc
CXCL5	tgcccttctcagtcatag	gtgcattccgcttagctttc
CXCL7	tgggcttcagactcagacct	tcctggcctgtacacattca
CXCL9	ttttctcttgggcatcatc	agtccggatctaggcaggtt
CXCL10	aagtgtgcgcgtcattttct	gtggcaatgatctcaacacg
CXCL11	agctgctcaaggcttcctta	ctgcattatgaggcgagctt
CXCL12	gctctgcatcagtgacggta	taatttcgggtcaatgcaca
CXCL13	tctggaagccattacacaa	tttgaaccatttggcacga
CXCL14	aagctgggaatgaagccaaa	cttctcgttcaggcattgt
CXCL15	ccatgggtgaaggctactgt	tagaggtctcccgaattgga
CXCL16	gggaagagtgttcaccacca	ggttggtgtgtctctttgtt
CXCL17	tgtgtctccagtgtgctc	gctgtgtctttctctttgg
XCL1	tgactttctgggagtctgc	catttggcttctggatcagc
CX3CL1	gcagatccccagaaactgag	ggcaccaggacgtatgagtt
GAPDH	atcaccatcttcaggagcga	aggggccatccacagtctt

Brain-specific induction of CCL3 and CCL4 by methylmercury

Table 2. Changes in mRNA levels of chemokines in various organs of methylmercury-treated mice.

		Cerebrum	Cerebellum	Liver	Kidney	Spleen
CC chemokines	CCL1	3.54 ± 2.00	0.71 ± 0.28	ND	1.14 ± 0.76	0.70 ± 0.44
	CCL2	20.57 ± 10.74**	22.31 ± 11.73*	1.25 ± 0.57	39.12 ± 17.77**	0.37 ± 0.13**
	CCL3	51.70 ± 16.65***	75.34 ± 44.42**	1.42 ± 0.30	1.18 ± 0.48	1.53 ± 0.82
	CCL4	72.54 ± 45.02*	70.39 ± 22.63***	2.24 ± 1.17	1.89 ± 0.71	1.58 ± 0.77
	CCL5	2.46 ± 1.09*	7.63 ± 4.24*	4.18 ± 1.06**	4.16 ± 3.07	0.49 ± 0.29*
	CCL6	1.81 ± 0.62	3.26 ± 1.08**	8.56 ± 4.07**	2.17 ± 0.99*	2.35 ± 0.72**
	CCL7	14.03 ± 7.71**	10.13 ± 5.89**	5.51 ± 2.93	8.65 ± 5.23*	0.22 ± 0.09
	CCL8	1.77 ± 1.07	1.62 ± 0.59	4.19 ± 3.04	0.95 ± 0.70	1.33 ± 0.15
	CCL9	3.63 ± 1.99*	6.32 ± 2.91**	1.18 ± 0.51	7.65 ± 2.94**	2.89 ± 1.07*
	CCL11	6.02 ± 3.93*	3.32 ± 1.01**	0.34 ± 0.23	2.27 ± 0.97	0.57 ± 0.29
	CCL12	63.09 ± 34.88**	31.05 ± 19.41**	1.81 ± 1.92	11.36 ± 9.65*	0.02 ± 0.01*
	CCL17	5.68 ± 2.54**	2.82 ± 0.68**	1.03 ± 0.66	0.57 ± 0.36	2.32 ± 1.96
	CCL19	1.57 ± 0.39*	1.38 ± 0.24*	1.20 ± 0.56	0.59 ± 0.23	0.37 ± 0.16**
	CCL20	1.19 ± 0.38	ND	ND	4.47 ± 2.65*	ND
	CCL22	2.22 ± 0.37**	1.95 ± 0.77*	1.13 ± 0.72	1.01 ± 0.79	0.39 ± 0.19**
	CCL24	1.07 ± 0.23	1.17 ± 0.57	2.25 ± 0.87*	0.81 ± 0.36	1.35 ± 0.54
	CCL25	1.17 ± 0.32	1.48 ± 0.44	0.52 ± 0.37	0.62 ± 0.14**	0.31 ± 0.17
	CCL26	0.80 ± 0.33	1.41 ± 0.67	2.63 ± 1.36*	0.71 ± 0.21	ND
	CCL27	0.65 ± 0.15**	1.17 ± 0.87	0.98 ± 0.35	0.50 ± 0.20**	0.36 ± 0.12***
	CCL28	1.17 ± 0.48	1.11 ± 0.36	1.41 ± 0.35	0.54 ± 0.13*	0.09 ± 0.01
CXC chemokines	CXCL1	4.64 ± 2.95	7.60 ± 5.79	3.83 ± 0.51**	86.50 ± 32.02***	ND
	CXCL2	4.37 ± 2.48	4.84 ± 3.23	3.70 ± 2.80	63.07 ± 12.72***	1.11 ± 1.09
	CXCL3	ND	ND	ND	9.19 ± 3.40**	ND
	CXCL4	0.80 ± 0.36	0.82 ± 0.29	2.57 ± 1.59	4.69 ± 1.29***	1.71 ± 0.79
	CXCL5	2.64 ± 1.37*	12.72 ± 5.89**	0.78 ± 0.56	115.06 ± 51.56*	1.47 ± 0.30
	CXCL7	7.50 ± 6.82	2.07 ± 0.58	2.76 ± 1.29*	1.80 ± 0.80	1.92 ± 0.64
	CXCL9	0.86 ± 0.82	5.28 ± 4.35	0.57 ± 0.34	0.86 ± 0.60	0.79 ± 0.48
	CXCL10	50.59 ± 28.52*	20.46 ± 11.76*	0.47 ± 0.21	4.53 ± 3.33	0.69 ± 0.44
	CXCL11	0.62 ± 0.27	1.01 ± 0.18	0.76 ± 0.19	0.88 ± 0.44	0.99 ± 0.61
	CXCL12	0.89 ± 0.17	1.05 ± 0.34	1.67 ± 0.50*	0.90 ± 0.23	1.35 ± 0.48
	CXCL13	ND	0.89 ± 0.16	5.66 ± 4.27	1.87 ± 1.37	0.84 ± 0.21
	CXCL14	0.78 ± 0.25	1.41 ± 0.39	5.12 ± 2.69*	0.95 ± 0.40	0.42 ± 0.38
	CXCL15	ND	ND	ND	1.28 ± 1.09	ND
	CXCL16	2.17 ± 1.00*	2.21 ± 0.52**	1.85 ± 0.60*	2.26 ± 0.35***	ND
	CXCL17	ND	ND	ND	11.69 ± 6.52*	0.71 ± 0.20
CX ₃ C chemokines	CX ₃ CL1	0.84 ± 0.46	1.05 ± 0.17	3.45 ± 1.82	2.20 ± 0.53**	0.63 ± 0.27
C chemokines	XCL1	ND	ND	2.26 ± 1.82	0.94 ± 0.38	0.007 ± 0.002***

Male mice (n = 5) were injected subcutaneously with methylmercuric chloride (10 mg/kg) for 7 consecutive days. Tissues were dissected 1 day after the last injection. Data show fold-changes in mRNA levels (mean ± S.D.). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with controls. Data were analyzed using Student's t-test. ND: not detected.

Fig. 1. Effects of multiple injections of methylmercury on the mRNA levels of CCL3, CCL4, and CXCL10 in various mouse tissues. Male mice ($n = 5$) were injected subcutaneously with methylmercuric chloride (10 mg/kg/day) daily for 7 consecutive days. Selected tissues were dissected 1 day after the first, third, fifth, and seventh injections. Levels of CCL3 (A), CCL4 (B), and CXCL10 (C) mRNA in the cerebellum, cerebrum, kidney, liver, and spleen were measured by quantitative real-time PCR in methylmercury-treated and control mice. mRNA levels were normalized to *GAPDH* levels. Data are presented as means $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control group. Data were analyzed using Student's *t*-test.

Detailed elucidation of the mechanism underlying the methylmercury-induced upregulation of chemokine genes, such as CCL3 and CCL4, in the brain may provide important clues as to the mechanisms mediating the selective toxicity of methylmercury in the CNS.

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(A) CCL3 **(B) CCL4**

Fig. 2. Effects of a single injection of methylmercury on the mRNA levels of CCL3 and CCL4 in the cerebellum of mice. Male mice ($n = 5$) were injected subcutaneously with methylmercuric chloride (10, 15, 20, or 25 mg/kg). Five days later, the cerebellum, cerebrum, kidney, liver, and spleen were dissected, and the levels of CCL3 (A) and CCL4 (B) mRNA were analyzed by quantitative real-time PCR. For further details, see Fig. 1 legend.

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