ORIGINAL

CCL2 level is elevated with metabolic syndrome and CXCL10 level is correlated with visceral fat area in obese children

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Abstract. Recent studies revealed that obesity is a low-grade, chronic inflammatory state that is accompanied by the enhanced production of multiple chemokines. In particular, metabolic syndrome (MS) and visceral adipose tissue (VAT) accumulation are significantly associated with certain chemokines in adults. However, little is known regarding this association in obese children. The aim of this study was to investigate the relationship between circulating chemokine levels and both MS and VAT accumulation in obese children. Forty-four obese schoolchildren (26 boys) with a percentage of overweight (POW) exceeding 20 were evaluated. The median age was 11.4 years (range: 6.8–16.5 years). Blood samples were drawn after overnight fasting, and serum chemokine levels (CCL2, CCL5 and CXCL10) were quantitated. Visceral fat area (VFA) determinations were conducted using computed tomography. The results showed that the median BMI Z-score, POW, waist circumference and VFA of the subjects were 2.24 SD, 49.8%, 88.3 cm and 80.8 cm², respectively. Eighteen were diagnosed with MS. CCL2 was significantly increased in MS subjects compared with non-MS subjects ($p < 0.05$). CXCL10 was positively correlated with VFA ($r = 0.425$, $p < 0.01$). There were no significant correlations between age and chemokine levels. We showed that CCL2 levels were elevated in MS and CXCL10 levels were associated with VFA in obese children. Our results suggest that CCL2 and CXCL10 play important roles in the progression of obesity-related metabolic complications in children.

Key words: Obese children, Metabolic syndrome, Chemokine, Chronic inflammation, Visceral adipose tissue

CHEMOKINES were originally described as a subclass of cytokines that modulate neutrophil and monocyte chemotaxis [1]. To date, more than 50 chemokines and 20 receptors belonging to the seven-transmembrane G protein-coupled receptor family have been identified in humans [2]. Chemokines have four conserved cysteine residues. The motif patterns in which two of these cysteine residues appear are used to classify them into four subfamilies: CXC, CC, C, and CX3C [1, 2]. Most CC chemokines play a role in chronic inflammation and are expressed by monocytes and T cells, whereas CXC chemokines act on neutrophils in acute inflammation [3]. Obesity has been associated with chronic low-grade inflammation [4] and significant infiltra-

tions of adipose tissue by macrophages; notably, the latter phenomenon has been observed in childhood [5]. Enlarged adipocytes in obesity affect the expression or secretion levels of inflammatory cytokines, called adipocytokines, which contribute to the development of obesity-related metabolic derangements and atherosclerosis [6]. In addition to adipocytokines, several chemokines are potential links between inflammation and insulin resistance [7]. Adipose tissue is thought to be a source of chemokines. Notably, Huber *et al.* reported that CC chemokines such as CCL5 (regulated on activation, normal T cell expressed and secreted; RANTES) are high in obese subjects and that their respective receptors are up-regulated in visceral adipose tissue (VAT) [8]. Recent studies revealed that obesity induces significant changes in the circulating levels of adipocytokines such as adiponectin and tumor necrosis factor in children and adolescents [9–11].

In adult, several studies reported the correlation between chemokine and metabolic syndrome (MS)

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which is the co-occurrence of metabolic abnormalities including centrally distributed obesity, hypertension, dyslipidemia and hyperglycemia. Ito *et al.* showed that CC motif ligand 2 (CCL2, monocyte chemoattractant protein; MCP-1)/ CC motif receptor 2 (CCR2) pathway plays a role in macrophage infiltration into obese adipose tissue and obesity-related MS [12]. Previous studies [13-15] reported that the levels of CCL5 are elevated in obese adults with MS. Additionally, Faber *et al.* reported that CXC motif chemokine 10 (CXCL10, interferon gamma-induced protein 10; IP-10) was related to VAT accumulation in adults [16]. Accumulating evidence suggests that these chemokines play important roles in the formation of MS in adult.

In obese children, some reports on circulating chemokine levels have been published. Several reports about chemokine levels showed that CCL2 was elevated in obese children [17-20]. Other study showed that the levels of CXCL10 were significantly higher in obese children than in lean control [21]. There are few reports about other chemokines related obesity. However, there have been few reports on the level of chemokines in obese children with MS. Moreover, little is known regarding the relationship between VAT accumulation and chemokines in obese children.

In this study, we measured CCL2, CCL5 and CXCL10 using cytometric bead array techniques in with and without MS. We selected three chemokines among many chemokines because previous studies reported about importance of these chemokines related obesity and visceral fat accumulation. This study elucidated the relationship between three chemokines and MS and VAT accumulation in obese children.

Materials and Methods

Subjects and anthropometric measurements

Forty-four obese Japanese children (26 boys and 18 girls) who visited the Clinic for Obese Children at the Hospital of the University of Occupational and Environmental Health, Japan, were enrolled in the study from 2006 to 2014. The age of the subjects ranged from 6 to 16 years. The BMI, BMI percentile and BMI standard deviation score (SDS) according to age and gender were calculated from national statistics for Japanese schoolchildren in 2000 (Ministry of Health, Labor and Welfare, Japan). A schoolchild was defined as obese if his/her percentage of overweight (POW) exceeded 20 based on the age- and sex-specific standard body-

weight for the height. POW was calculated as $100 \times (\text{the measured weight} - \text{normal weight}) / \text{normal weight}$ (%). Normal weight data based on age- and sex-specific standard body weights for height were obtained from the Ministry of Education, Culture, Sports, Science and Technology. Theoretically, POW that is unique to Japan is not influenced by height, therefore it is a highly useful index for longitudinal studies and has been widely used in school health checkups to evaluate children's weight periodically [22, 23]. Among 44 obese subject, 22 (14 boys and 8 girls) met the Japan criteria for mild to moderate obesity (POW up to 50%), 22 obese subjects (11 boys and 11 girls) for morbidly obesity (POW $\geq 50\%$). Subjects were defined as having MS if they had at least two of three risk factors (hypertension, dyslipidemia and hyperglycemia) in addition to abdominal obesity according to the criteria for MS in Japanese children and adolescents [24]. The critical values for these risk factors were as follows: hypertension (systolic blood pressure ≥ 125 mmHg or diastolic blood pressure ≥ 70 mmHg), dyslipidemia (HDL-C < 40 mg/dL or TG ≥ 120 mg/dL), hyperglycemia (≥ 100 mg/dL) and abdominal obesity (waist circumference ≥ 80 cm in junior high school students and ≥ 75 cm in elementary school students). Children were excluded if they were taking any oral steroid medication or had any endocrine, metabolic or kidney diseases.

Anthropometric measurements, including height, body weight, and waist circumference, were performed as described previously [25] by some well-trained medical staffs in the pediatric department. In brief, height was measured to nearest 0.1 cm and body weight to nearest 0.1 kg using a stadiometer. The waist circumference was measured at the level of umbilicus to the nearest 0.1 cm wearing no clothes.

The development of puberty was clinically assessed on the basis of Tanner stages. Pre-pubertal stage was equal to Tanner stage 1.

Blood samples obtained for chemokine and adipocytokine analysis were drawn after overnight fasting and immediately processed *via* centrifugation at 3,000 rpm and stored at -80°C .

Visceral fat area (VFA) and subcutaneous fat area (SFA) were determined *via* computed tomography (CT) using previously described methods [25]. Briefly, a single-slice CT scan of the abdomen at the level of the umbilicus was analyzed to obtain the cross-sectional area of adipose tissue. The VFA was measured in cm^2 using DENSITY MASK software assuming a density of

-40 to -140 Hounsfield units for adipose tissue.

The Human Study Committee of the University of Occupational and Environmental Health, Japan, approved this study. Both parental informed consent and the child's assent were obtained as appropriate.

Serum chemokine and biochemical measurements

To reduce inter-assay variance, all of the samples were analyzed simultaneously. The serum concentration of the chemokines CCL2, CCL5 and CXCL10 were measured serially using chemokine cytometric bead array (CBA) reagent kits (BD Bioscience, San Diego, California). The samples were analyzed using a multifuorescence BD flow cytometer (FACSCalibur™) with BD CellQuest™ software and BD™ CBA software. The coefficients of variation for all of the chemokine assays were less than 10%. The serum adiponectin levels were determined *via* ELISA kit (Sekisui Medical Co., Ltd., Tokyo, Japan) as previously described [10].

Serum alanine aminotransferase (ALT), uric acid (UA), triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), insulin and fasting plasma glucose (FPG) were measured in the clinical laboratories of the Hospital of the University of Occupational and Environmental Health, Japan. The homeostasis

model assessment for insulin resistance (HOMA-R) was calculated according to the formula described by Matthews [26].

Data for anthropometric parameters and metabolic parameters are presented as the means $\pm$ standard deviation. And the data for chemokines are presented as medians [range]. Because the data for chemokines were skewed, they were logarithmically transformed prior to statistical analysis. Significance between two groups was examined using the Mann-Whitney U test. Correlations between continuous variables were assessed using Spearman's correlation coefficient. Partial correlations were calculated according to the analysis of covariance. Statistical differences were considered to be significant at $p < 0.05$. Statistical analyses were performed using SPSS version 8.01J (SPSS Inc., Chicago, IL, USA).

Results

All subjects had POW exceeded 20. Fifteen children were defined as obesity by the International Obesity Task Force (IOTF) definition. All of them had POW exceeded 50 and belonged to morbidly obese group.

Eighteen subjects were diagnosed with MS. The clinical characteristics of obese children with and without MS are summarized in Table 1. Age did not

Table 1 Clinical characteristics of obese children, with and without metabolic syndrome

	All	Non-MS	MS	<i>p</i> -value
n (M/F)	44 (26 / 18)	26 (18 / 8)	18 (8 / 10)	
Pubertal stage (pre/pubertal/miss)	44 (13 / 26 / 5)	26 (10 / 15 / 1)	18 (3 / 11 / 4)	
Anthropometric parameters				
Age (year)	11.3 $\pm$ 2.1	11.2 $\pm$ 2.4	11.5 $\pm$ 1.6	n.s.
BMI (kg/m ²)	28.6 $\pm$ 5.0	26.9 $\pm$ 3.7	31.0 $\pm$ 5.8	<0.05
BMI-SDS	2.2 $\pm$ 0.5	2.1 $\pm$ 0.5	2.4 $\pm$ 0.4	<0.05
POW (%)	57.0 $\pm$ 22.8	50.4 $\pm$ 17.6	66.5 $\pm$ 26.5	<0.05
Waist circumference (cm)	91.6 $\pm$ 13.9	85.9 $\pm$ 9.7	99.9 $\pm$ 15.2	<0.05
VFA (cm ²)	88.8 $\pm$ 37.9	75.0 $\pm$ 22.0	108.6 $\pm$ 47.2	<0.05
SFA (cm ²)	302.6 $\pm$ 112.5	269.5 $\pm$ 94.4	350.1 $\pm$ 122.4	<0.05
Metabolic parameters				
FPG (mg/dL)	92.3 $\pm$ 7.1	90.4 $\pm$ 5.5	95.3 $\pm$ 8.1	<0.05
Insulin (IU/L)	22.9 $\pm$ 15.6	16.2 $\pm$ 5.6	32.7 $\pm$ 19.9	<0.001
HOMA-R	5.3 $\pm$ 3.9	3.6 $\pm$ 1.4	7.8 $\pm$ 4.9	<0.001
ALT (IU/L)	50.7 $\pm$ 49.1	35.8 $\pm$ 28.5	72.3 $\pm$ 63.8	<0.05
Uric acid (mg/dL)	5.8 $\pm$ 1.5	5.6 $\pm$ 1.4	6.0 $\pm$ 1.5	n.s.
LDL- cholesterol (mg/dL)	109.8 $\pm$ 33.2	104.4 $\pm$ 26.2	117.9 $\pm$ 43.2	n.s.
HDL- cholesterol (mg/dL)	52.4 $\pm$ 9.9	55.7 $\pm$ 8.4	47.6 $\pm$ 10.2	<0.05
Triglycerides (mg/dL)	121.1 $\pm$ 82.5	83.7 $\pm$ 34.2	175.5 $\pm$ 101.2	<0.001
Adiponectin (μ g/mL)	6.1 $\pm$ 2.2	6.7 $\pm$ 2.2	4.5 $\pm$ 1.2	<0.05

Data are presented as the mean $\pm$ SD. We identified differences between non-MS and MS groups using the Mann-Whitney U test. MS, metabolic syndrome; BMI, body mass index; VFA, visceral fat area; SFA, subcutaneous fat area; HOMA-R, Homeostasis model assessment for insulin resistance; n.s., not significant.

significantly differ between the two groups. Waist circumference, BMI, BMI-SDS and POW in the MS group were significantly higher than those in the non-MS group. VFA and SFA in the MS group were significantly increased compared with those in the non-MS group. The levels of FPG, insulin, HOMA-R, ALT, HDL-C, TG and adiponectin significantly differed between the MS and non-MS groups. According to International Diabetes Foundation (IDF) criteria and the waist circumference percentile for Japanese children which Matsushita *et al.* showed [23], nine subjects were diagnosed with MS and nineteen with non-MS. Other sixteen subject whose ages were between six years old to younger than ten years old could not be diagnosed because there was no definition of MS between six years to younger than ten years old in the IDF criteria.

The circulating levels of CCL2, CCL5 and CXCL10 were 81.2 [13.9-343.3] pg/mL, 6,856.6 [210.2-84,773.0] pg/mL and 212.03 [27.74-522.7] pg/mL, respectively. The CCL2 concentrations in the MS group were significantly higher than those in the non-MS group (138.4 pg/mL vs. 41.86 pg/mL, $p < 0.05$). The serum CCL5 and CXCL10 levels in the MS group were not significantly higher than those in the non-MS group (Fig. 1). Correlations between chemokine levels and anthropometric or metabolic parameters in obese children are shown in Table 2. There were no correlations between age and any of the three chemokines. The circulating CCL2 levels were positively correlated with only FPG. However, the circulating CCL5 levels were positively correlated with UA. The serum levels of CXCL10 were positively correlated with VFA, ALT, UA and LDL-C and negatively correlated with FPG. The SFA levels did not correlate with any of the circulating chemokines. According to IDF criteria, the circulating CCL2 levels in MS were significant higher than those in non-MS obese children and CCL5 and CXCL10 levels had no difference.

As displayed in the scatter plots, there was a significant correlation between circulating CXCL10 levels and VFA (Fig. 2). The CCL2 and CCL5 levels did not correlate with VFA.

The circulating levels of CCL2, CCL5 and CXCL10 in the mild to moderate obese group were 77.4 [13.9-343.3] pg/mL, 6,879.9 [210.2-42,026.0] pg/mL and 201.7 [89.8-426.5] pg/mL respectively and in morbidly obese group 89.0 [14.5-256.1] pg/mL, 5,924.4 [240.3-

Fig. 1 Circulating levels of chemokines in MS (n=18) and non-MS (n=26) children (a) CCL2, (b) CCL5 and (c) CXCL10. Significance was evaluated using the Mann-Whitney U test. Data are presented as quartiles $\pm$ SEM.

Table 2 Correlation of chemokines levels with anthropometric, metabolic parameters in children

	CCL2	CCL5	CXCL10
Age	-0.246	-0.106	0.224
Waist circumference	0.070	-0.206	0.110
BMI	0.010	-0.043	0.188
BMI-SDS	0.297	0.033	0.116
POW (%)	0.079	0.012	0.135
VFA	-0.062	-0.021	0.425 *
SFA	0.014	0.146	0.170
FPG (mg/dL)	0.362 *	-0.041	-0.428 *
Insulin	0.257	-0.061	-0.012
HOMA-R	0.276	-0.048	-0.062
ALT	-0.100	-0.103	0.365 *
UA	-0.267	0.319 *	0.435 *
LDL-C	-0.295	0.296	0.461 *
HDL-C	0.018	-0.188	-0.115
TG	0.269	-0.002	0.049
Adiponectin	0.045	-0.273	-0.211

Correlations between continuous variables were assessed by Spearman's correlation coefficient. * $p < 0.05$

25,561.1] pg/mL and 234.5 [27.7-522.7] pg/mL respectively (Fig. 3). In the mild to moderate obese group, VFA levels were positive correlated with CXCL10. On the other hand, in the morbidly obese group, VFA levels were positive correlated with CCL2. In the obese group defined by the IOTF definition, VFA levels were positive correlated with CCL2.

The circulating levels of CCL2, CCL5 and CXCL10 in pre-pubertal group were 115.2 [21.9-260.0] pg/mL, 9,631.1 [814.14-84,773.0] pg/mL and 163.1 [27.7-443.4] pg/mL respectively and in pubertal group 68.7 [14.5-343.3] pg/mL, 6,856.4 [313.04-40,274.2] pg/mL and 233.7 [87.0-522.7] pg/mL respectively (Fig. 4). There were no significant differences in the levels of three chemokines between pre-pubertal group and pubertal group. CCL2 was significantly increased in MS compared with non-MS in pre-pubertal group (143.2 pg/mL vs. 31.62 pg/mL, $p < 0.01$) and pubertal group (183.1 pg/mL vs. 64.75 pg/mL, $p < 0.05$). CXCL10 was positively correlated with VFA in pre-pubertal group and pubertal group. CCL5 was not significantly increased in MS compared with non-MS and was not positively correlated with VFA in pre-pubertal group and pubertal group.

Fig. 2 Relationship between VFA levels and (a) CCL2, (b) CCL5 and (c) CXCL10 levels (n=44). Spearman's rank correlation coefficient and probability are shown. Logarithmic transformation was performed on the chemokine values for analysis. VFA, visceral fat area.

Fig. 3 Circulating levels of chemokines in the mild to moderate obese group (n=22) and the morbidly obese group (n=22) (a) CCL2, (b) CCL5 and (c) CXCL10. Significance was evaluated using the Mann-Whitney U test. Data are presented as quartiles $\pm$ SEM.

Fig. 4 Circulating levels of chemokines in the pre-pubertal group (n=13) and the pubertal group (n=26) (a) CCL2, (b) CCL5 and (c) CXCL10. Significance was evaluated using the Mann-Whitney U test. Data are presented as quartiles $\pm$ SEM.

Discussion

The present study demonstrated that obese children with MS had higher serum CCL2 levels than non-MS obese children. Several studies have reported that circulating CCL2 levels are higher in obese subjects than lean controls and are correlated with BMI percentile [18, 27-29]. Samaan *et al.* proposed that circulating CCL2 levels could be a potential biomarker of fitness based on an observed correlation between the BMI percentile and CCL2 [18]. However, Roth *et al.* reported that the changes in BMI-SDS after intervention were not correlated with CCL2 levels [28]. Therefore, further studies are needed to confirm that CCL2 levels may serve as a potential biomarker to monitor the effects of treatment on childhood obesity.

Several reports revealed that mice with targeted deletions in the genes for *Mcp-1/Ccl2* and *Ccr2* have decreases in macrophage content and inflammation in adipose tissue and are protected from high-fat diet-induced insulin resistance [30, 31]. Therefore, the interaction between CCL2 and its receptor CCR2 is considered to be pivotal in obesity-induced insulin resistance [32].

In this study, we showed that circulating CXCL10 levels were associated with VAT accumulation. These findings were consistent with those in previous reports in adults [13, 16]. Moreover, the serum concentrations of CXCL10 were significantly correlated with obesity-related metabolic abnormalities. VAT accumulation is a well-known cause of MS. We first demonstrated a relationship between fat accumulation and circulating chemokine levels in obese children.

In the mild to moderate obese group, VFA levels were correlated with CXCL10. On the other hand, VFA levels were correlated with CCL2 in the morbidly obese group. These results may suggest that the importance of chemokines differs according to the phase of obesity onset. Hypertrophic adipose tissues released proinflammatory cytokines and chemokines. Released CCL2 caused an influx of monocytes to adipose tissue [33]. Recent studies showed that CD8⁺ T cells and neutrophils which have receptors for CXCL10 [34, 35] infiltrated into adipose cells prior to an influx of monocytes [2, 36-38]. Thus, CXCL10 may attract CD8⁺ T cells and neutrophils before CCL2 causing the influx of monocytes. These results suggest that CXCL10 may be an important chemokine in early stage of obesity. The biochemical and func-

tional study will be needed to clarify roles of CCL2 and CXCL10 in the phase of obesity.

In our study, circulating CCL5 levels in MS were higher than those in non-MS obese children but with no statistically significant difference. Previous studies reported CCL5 levels to be significantly higher in obese adults with MS than those without MS [14, 15]. The cause of this age-related difference is currently unclear.

The variation of CCL5 levels is quite huge in the present study. The reason of this huge variation is unclear at this point. In the previous study, the variation of CCL5 was reported to be quite huge with near 0.25 pg/mL to near 256,000 pg/mL [39]. The range of CCL5 may be huge in childhood. Further studies with large samples are needed to clarify this point.

Interestingly, our results suggest that VFA differs from SFA regarding its effect on chemokine levels. We previously reported that plasma leptin levels were more closely linked to SAT than VAT, whereas plasma visfatin was a specific marker for VAT [40]. In our study, circulating CXCL10 levels were correlated with VFA and not SFA. No chemokine levels were correlated with SFA. These results collectively suggest that the specific area of fat accumulation could be an important determinant contributing to the circulating chemokine levels in obese children.

Various groups have proposed several definition of obesity and criteria of MS in children by consensus among experts. Among those, the definition of obesity of IOTF has been worldwide used [41]. In this obese group defined by the IOTF definition, VFA levels were positive correlated with CCL2 as morbidly obese group. In regard to MS, other definitions for childhood MS employ the percentiles of waist circumference. In particular, the criteria of MS defined by IDF has been worldwide used in research [42]. According to IDF criteria, circulating CCL2 levels in MS were significant higher than those in non-MS obese children and CCL5 and CXCL10 levels had no difference as the criteria for MS in Japanese children and adolescents. Therefore, our results would be accepted by international definition.

The potential limitations of the present study include its relatively small sample size and lack of lean controls. Another limitation is that the differences in subject sex and pubertal stage may have introduced background variation in chemokine levels.

As for sexual dimorphism, Lamason *et al.* reported that post-pubertal female mice produced significantly

higher levels of CCL2 than do post-pubertal males [43]. On the other hand, Thorsen *et al.* reported that there were no significant differences in the levels of CCL2 and CCL5 between male and female in children [39]. Uchi *et al.* showed that the levels of CCL2, CCL5 and CXCL10 did not differ significantly between male and female in healthy Japanese adult [44]. We suggested that there might be no significant difference in these chemokine levels between male and female about human subject. Additional studies about the sexual dimorphism with larger samples are needed.

In regards to pubertal stage, there were no significant differences in the levels of three chemokines between pre-puberty and puberty in our study. In addition, the levels of CCL2 were significantly higher in MS and levels of CXCL10 were positively correlated with VFA in both pre-puberty and puberty in the present study. CCL5 had no difference in MS and had no correlation with VFA in both stage. Therefore we considered our analyzing subjects constituted with both pre-puberty and puberty as uninfluenced to our result. However, Thorsen *et al.* reported that CCL5 levels were significantly higher in 5-10 years than < 10 years [39]. There were no reports about the CXCL10 levels in regard to pubertal stage. Further investigation is needed including longitudinal observation during puberty in regard to the effect of puberty on the levels of chemokines.

In summary, we first showed that circulating CCL2 levels were significantly higher in a group of MS children than a group of non-MS children. Furthermore,

VFA was associated with CXCL10 levels in obese children. Our studies suggest that these chemokines may be associated (one similar to that of adipocytokines) with the pathophysiology of obesity-related metabolic complications during childhood

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M. I. and S. A. conceived and carried out the experiments. S. A. and Y. Y. conceived the experiments and analyzed the data. M. I. and M. G. carried out the experiments. All of the authors were involved in writing the paper and gave final approval of the submitted and published versions.

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Disclosure

None of authors have any potential conflicts of interest associated with this research.

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