Neuroscience Research 113 (2016) 63–70

Neuroscience Research

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

A possible serologic biomarker for maternal immune activation-associated neurodevelopmental disorders found in the rat models

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ARTICLE INFO

Article history:
Received 9 February 2016
Received in revised form 21 June 2016
Accepted 7 July 2016
Available online 15 July 2016

Keywords:
Maternal immune activation
Serologic biomarker
Ig light chain
Schizophrenia
Autism
Neurodevelopmental disorders

ABSTRACT

Epidemiological studies have shown that maternal infection during early pregnancy increases the risk of neurodevelopmental disorders (i.e., schizophrenia or autism) in offspring. Recently, diagnostic/stratification biomarkers for the maternal immune activation background in patients with neurodevelopmental disorders have been energetically searched for in the patient blood. Here, we report a novel serologic marker candidate for the disorders found in the maternal immune activation (MIA) rat model. Serum proteome analysis of the MIA rat showed that the immunoglobulin (Ig) light chain is reproducibly augmented. The Ig light chain in sera takes two forms — free form or bound to the Ig heavy chain. Only the former is an inflammatory disease marker, but pro-inflammatory cytokine levels in the sera of the MIA rats were below detectable limits of the ELISA protocol we used. We thereby carried out serum assays of Ig light chains and pro-inflammatory cytokines of commercially available schizophrenia patient sera for research. Although the number of samples was limited, we found augmentation of free Ig light chains but not pro-inflammatory cytokines in sporadic schizophrenia patient sera. Our findings suggest that Ig light chain assay of the schizophrenia/autism patient sera would be worthy to be validated in larger scale.

1. Introduction

Emerging literature has provided epidemiological evidence that maternal immune activation (MIA) during the first half of gestation increases the risks of neurodevelopmental disorders including schizophrenia and autism in offspring, based on the fact that these increased risks are correlated not only with influenza infection at pregnancy (Barr et al., 1990; Brown et al., 2004; Kendell and Kemp, 1989) but also with other maternal infectious diseases (Blomstrom et al., 2012; Brown and Susser, 2002; Lee et al., 2015; Suvisaari et al., 1999) such as *poliovirus*, *rubella* and *T. gondii*. From this, we can infer that not a specific viral infection but MIA itself would increase the risks in offspring. It is estimated that 30% of schizophrenia cases could be prevented if certain prenatal infections were entirely eliminated from the pregnant population (Brown and Derkits, 2010; Cannon et al., 2014). MIA would be a common immune-related background for neurodevelopmental disorders in offspring. In fact, a number of studies have been published that searched for clues to diagnostic/stratification biomarkers of such disorders (Blomstrom et al., 2015; Tomasik et al., 2012; Vargas, 2014), but candidates for related diseases with immune-related background have not yet been fully found.

MIA of pregnant rodents can be induced by injection of synthetic polyriboinosinic-polyribocytidilic acid (Poly I:C), which mimics viral infections through Toll-like receptor 3 stimulation and causes their offspring to have permanent immune system disturbances and behavioral abnormalities (Hsiao et al., 2012, 2013; Meyer,

Abbreviations: MIA, maternal immune activation; Ig, immunoglobulin; Poly I:C, synthetic polyriboinosinic-polyribocytidilic acid; GD, gestation day; I.P., intraperitoneally; 2-DE, two-dimensional electrophoresis; CHAPS, 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate; DTT, dithiothreitol; IPG, immobilized pH gradient; BPB, bromophenol blue; KLH, keyhole-limpet hemocyanin; HRP, horseradish-peroxidase; IL-6, interleukin 6; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α; κFLC, free immunoglobulin κ light chain; λFLC, free immunoglobulin λ light chain; FLCs, free Ig light chains; rFLC, κ-to-λ FLC ratio; MHC, major histocompatibility complex.

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http://dx.doi.org/10.1016/j.neures.2016.07.003

2013a,b; Meyer et al., 2005; Patterson, 2009). A non-human primate Poly I:C complex-induced MIA model has also presented alteration of social attention, abnormal behavioral development and neuropathology (Bauman et al., 2014; Machado et al., 2015; Weir et al., 2015). Although the total picture of the MIA model animal phenotype has still to be fully clarified, we herein suggest its usefulness in our quest for diagnostic/stratification markers of MIA-associated neurodevelopmental disorders.

In the present study, we explored a novel serologic MIA-associated marker candidates in the serum proteome of the rat model. To see whether we can find corresponding markers in human samples, we carried out a pilot study using sporadic schizophrenia patient sera for research.

2. Materials and methods

2.1. Experimental animal care

All experiments were performed in accordance with international guidelines on the ethical use of animals (NIH Guide for the Care and Use of Laboratory Animals) and the guidelines of the National Institute of Radiological Sciences in Chiba, Japan, and all efforts were made to minimize suffering and the number of animals used. The present study was approved by the Animal Ethics Committee of the National Institute of Radiological Sciences, Chiba, Japan. The rats were housed under controlled temperature and at a 12-h light/dark cycle with food and water ad libitum until the experiments.

2.2. Preparation of MIA rat

Female Wistar rats (Japan SLC, Shizuoka, Japan) were mated at 12–15 weeks of age. The first day after copulation was defined as gestation day 1 (GD1). Poly I:C potassium salt (Product No. P9582, Lot No. 012M4032V, Sigma–Aldrich, Tokyo, Japan) (4 mg/kg) dissolved in saline was injected into the pregnant Wistar rats intraperitoneally (I.P.) daily for 4 consecutive days from GD15 to GD18. Their neonatal- or mature-male offspring (postnatal days 3 or postnatal weeks 9–16) were used as MIA rats. We also injected saline (vehicle) into pregnant Wistar rats by the same procedures and their male offspring were used as controls. In the present study, all pregnant Wistar rats having been injected with Poly I:C potassium salt or with only saline could give birth to living pups (Poly IC treated dams: $N = 9$ and saline treated dams: $N = 7$). The number of offspring per dam were not significantly different between the two groups of dams (Poly I:C treated dam: 9 ± 1.11 and saline treated dam 9.5 ± 0.75 (mean $\pm$ SEM), $t(14) = 0.39$, $P = 0.69$ (Student's t-test)). All offspring were culled to 6–8 pups on postnatal day 3. Offspring were separated from their mothers 21 days after birth. We prepared 1–3 male offspring from each dam for each serum analyses.

2.3. Antipsychotic drug injection

The MIA rats (9 weeks postnatal) were injected intramuscularly (I.M.) with haldol-decanoate (Sumitomo Dainippon Pharma, Tokyo, Japan) (0.5 mg/kg) or propylene glycol (Vehicle) (Vincent et al., 1994). Sera were collected 3 weeks later.

2.4. Rat serum sampling

Blood was collected from deeply anesthetized rats with an overdose of pentobarbital (Kyoritsu Seiyaku Co., Tokyo, Japan) by cardiac puncture with 23-gauge needles attached to 2.5 ml syringes. Blood was incubated for 3 h at 4 °C and centrifuged for 40 min at $1000 \times g$ at 4 °C. Supernatant sera were collected and stored at −80 °C until use.

2.5. Two-dimensional electrophoresis (2-DE) and image analysis

We carried out standard 2-DE as described previously (Koga and Minohata, 2011). An aliquot of a serum sample was mixed at a volume ratio of 1:1 with an extraction medium (7 M urea, 2 M thiourea, 2% 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS), 0.1 M dithiothreitol (DTT), and 2.5% Pharmalyte (pH 3–10)), and was homogenized using an ultrasonic disruptor (UR-20P; Tomy Seiko, Tokyo, Japan), followed by 20-min centrifugation at 15,000 rpm, 4 °C, using Centrifuge 3500 (Kubota, Gunma, Japan). The supernatant was collected and stored at −80 °C until use. An 18-cm-long dry immobilized pH gradient (IPG) gel strip (GE Healthcare, Tokyo, Japan) was rehydrated for 8–24 h at 20 °C in rehydration buffer (7 M urea, 2 M thiourea, 2% triton X-100, 13 mM DTT, 1% Pharmalyte (pH 3–10), 25 mM acetic acid, and 0.025% bromophenol blue (BPB)). The sample solution (0.054 ml) was applied to filter paper, which was placed on the cathodic edge of an immobilized pH gradient (IPG) gel strip. IPG gel strips were subjected to 1st dimension isoelectric focusing (IEF) electrophoresis in CoolPhoreStar IPG IEF Type PX (Anatech, Tokyo, Japan). After the 1st dimension IEF, the IPG gel strips were rinsed briefly in distilled water to remove silicon oil and were then equilibrated for 40 min at room temperature under gentle shaking in SDS treatment buffer (6 M urea, 32.5 mM DTT, 25% glycerol, 25 mM Tris–HCl (pH 6.8), 0.0025% BPB, and 2% SDS). The IPG gel strips were then placed on top of 12% SDS-polyacrylamide slab gels for 2nd dimension SDS-PAGE.

The 2nd dimension SDS-PAGE was carried out with CoolPhoreStar SDS-PAGE Dual 200 K (Anatech), after which the slab gels were stained with Flamingo fluorescent gel stain (Bio-Rad). FluoroPhoreStar 3000 (Anatech) was used for capturing fluorescent images of the Flamingo-stained slab gels. These fluorescent images were captured three times per gel, and were analyzed by Progenesis PG220 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). The integrated fluorescent intensity of a spot in a gel image was calculated after subtracting the lowest-on-boundary pixel intensity around the spot from the pixel intensity within the spot.

2.6. Identification of proteins by LC–MS/MS

We identified proteins in 2-DE gels by following a standard protocol with several modifications (Shevchenko et al., 2006). Flamingo-stained spots were excised from a 2-DE slab gel using FluoroPhoreStar3000 (Anatech). Excised gel pieces were submitted for protein identification by mass spectrometry, which were processed using a robot (ProGest, DigiLab, MA, USA) by the following protocol: the gel pieces were washed with 25 mM ammonium bicarbonate followed by acetonitrile, reduced with 10 mM DTT at 60 °C followed by alkylation with 50 mM 2-iodoacetamide at room temperature, and trypsin digestion (Promega) at 37 °C for 4 h. Finally, the samples were quenched with formic acid and the supernatant containing digested peptides was used for protein identification by mass-spectrometry without further processing. The experiments were performed by RelyOn Ltd. (Tokyo, Japan).

Our nano LC/MS/MS system was equipped with a Waters NanoAcquity HPLC system (Waters Corp., MA, USA) interfaced to a ThermoFisher Orbitrap Velos Pro (Thermo Fisher Scientific K.K., Kanagawa, Japan). Peptides were loaded on a trapping column of the nano LC and eluted over a 75-μm-diameter nano LC column at a flow rate of 350 nL/min; both columns were packed with Jupiter Proteo resin (Phenomenex, CA, USA). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 FWHM resolution and MS/MS performed in LTQ™

(Thermo Fisher Scientific K.K.). The 15 most abundant ions were selected for MS/MS. The nano LC–MS/MS experiments were performed by RelyOn Ltd.

Database search concerning MS/MS peptide data was carried out at Uniprot DB (http://www.uniprot.org/) by using Mascot, a mass spectrum analysis software. The Mascot program was given database query arguments as follows. Enzyme: Trypsin, Database: Uniprot Rat (concatenated forward and reverse plus common contaminants), Fixed modification: Carbamidomethyl (C), Variable modifications: Oxidation (M), Acetyl (Protein N-term), Deamidation (NQ), Pyro Glu (N-term Q), Mass values: Monoisotopic, Peptide Mass Tolerance: 10 ppm, Fragment Mass Tolerance: 0.8 Da, Max Missed Cleavages: 2. Mascot DAT files obtained by the database query were parsed by Scaffold, a MS/MS data validation software for creating a non-redundant peptide list (Proteome Software, Inc., OR, USA). Database query results were filtered using a minimum protein value of 90%, a minimum peptide value of 50% in Prophet scores, and at least two unique peptides were required for successful identification of a protein. The LC–MS/MS identification of proteins explained above was done by RelyOn Ltd.

2.7. Western blotting using anti-rat immunoglobulin κ (Ig-κ) light chain antibody

We prepared polyclonal rabbit anti-rat Ig-κ light chain antibodies using an immunizing peptide conjugated with the keyhole-limpet hemocyanin (KLH) protein. The immunizing peptide had a partial sequence from the 54th to the 73rd amino acid of rat Ig-κ (SVTDQDSKDSTYSMSSTLSL) (Sheppard and Gutman, 1981) plus a cysteine residue at the N terminus where KLH was conjugated. Two rabbits were immunized twice by subcutaneous injections of 4 mg of the peptide. Crude antisera of both rabbits were affinity-purified against the immobilized immunizing peptide. Preparation and purification of the polyclonal rabbit anti-rat Ig-κ antibodies were performed by RESVO Inc., Tokyo, Japan. The specificity of anti-sera was confirmed by dot blot.

Sera of the control and MIA rats were respectively mixed at a volume ratio of 1:1 with protein extracting buffer solutions including 100 mM Tris–HCl, pH 8.8, 2% SDS, 20% sucrose, 0.06% BPB, 7 M urea, 2 M thiourea and 0.1 M DTT. The mixture was subjected to 10–20% gradient SDS-PAGE (ATTO, Tokyo Japan). Electrophoresed proteins were transferred from the SDS-PAGE gel to a PVDF membrane (ATTO), which was blocked for 1 h at room temperature in EzBlock BSA (ATTO), a blocking buffer solution. The PVDF membrane was then incubated overnight at 4 °C in a blocking buffer solution containing 1:1000-diluted rabbit anti-rat Ig-κ light chain primary antibody or 1:20,000-diluted rabbit anti-rat albumin primary antibody (Assaypro, MO, USA) and finally transferred into a blocking buffer solution containing a horseradish-peroxidase (HRP) conjugated anti-rabbit IgG (GE Healthcare). Chemiluminescence from HRP elicited by ImmunoStar Zeta or ImmunoStar Basic (Wako, Tokyo, Japan) was detected by ChemiDoc XRS.

Optical density (OD) of protein bands was determined by using Image J (NIH, Bethesda, MD, USA), followed by normalization to the albumin OD for correcting variations in loading. Finally, the OD ratio values were scaled to the average values of the control rats.

2.8. Measurement of cytokines in serum of rat

Rat serum Interleukin 6 (IL-6) concentration was measured by rat IL-6 colorimetric ELISA kit (Thermo Scientific, IL, USA), and rat Tumor Necrosis Factor α (TNF-α) and Interleukin 1β (IL-1β) by Quantikine® ELISA kits (R&D Systems Inc., MN, USA).

2.9. Human sera sample

Anonymized sera of sporadic schizophrenia patients and healthy controls were purchased from PrecisionMed. Inc. (CA, USA; see details at http://www.precisionmed.com/) and stored at −80 °C until use. The present human sample study was approved by clinical ethics committees of the National Institute of Radiological Sciences, Chiba, Japan. Free Ig-κ light chain (κFLC) and free Ig-λ light chain (λFLC) (κFLC isoform) production are augmented under chronic inflammatory diseases (van der Heijden et al., 2006) and clonal diseases (Katzmann et al., 2002; Kuku et al., 2005; Tsirakis et al., 2011). To exclude false-positive or -negative donors, we first inspected anonymized medical charts of those donors supplied by PrecisionMed. Inc. in terms of whether their clinical data satisfied the following conditions: (1) Donors must be free of medical diseases. (2) The pro-inflammatory cytokine concentrations in serum samples must be below the ELISA detection limits (IL-6 < 10 pg/ml; IL-1β < 3 pg/ml; TNF-α < 10 pg/ml). (3) To exclude donors with clonal diseases such as myeloma, the κ-to-λ ratio values in sera samples must be within the reference range (0.26–1.65) (Katzmann et al., 2002). (4) The mean ages of the two groups must match closely. (5) Finally, the gender of donors was limited to males. The numbers of sera samples that satisfied the selection criteria were 8 patients (mean age 43.8 ± 3.5 y.o., duration of illness 23.2 ± 3.3 years, PANSS positive score 17.8 ± 1.8, negative score 16.7 ± 1.5, general score 37.5 ± 3.3, chlorpromazine equivalent antipsychotic dose 995.7 ± 262.2 mg/day) and 9 healthy controls. We measured FLCs in sera samples of the patients with sporadic schizophrenia ($N = 8$) and healthy control ($N = 9$) donors purchased from PrecisionMed, Inc., USA.

2.10. Measurement of free immunoglobulin κ light chain (κFLC) and free immunoglobulin λ light chain (λFLC) in human sera

κFLC and λFLC in human sera were measured using Freelight κ® and Freelight λ® (The Binding Site, Burmingham, UK). The measurements were done by LSI Medience Co., Ltd.

2.11. Data analysis and statistical analysis

All results were expressed as mean ± SD. Comparisons of the two groups were performed by multiple *t*-test, two-tailed Student's *t*-test or Mann–Whitney *U*-test. In all cases, differences were considered significant when $P < 0.05$. As the number of samples was limited, we carried out power analysis using G* power, a free statistical analysis software package available for OS X and Windows (see details at http://www.gpower.hhu.de/).

3. Results

3.1. Exploration for serum markers in the MIA rat

We used standard methods of proteomics to search for serum biomarkers in the mature MIA rat (postnatal weeks 16). First, we separated serum proteins of the MIA and control rats individually by 2-DE, followed by comparison of the 2-DE patterns between the MIA model and control samples ($N = 3$ each). Using Progenesis PG220 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK), we matched 482 spot images with each other in the 2-DE patterns of the MIA and the control samples (Supplementary Fig. 1). Among the 482 spots in the 2-DE images, 3 spots showed difference in volume between MIA and control samples with uncollected *P* value of less than 0.01, although any of the differences did not reach significance after adjusting for multiple comparisons. The summary statistics for the 3 spots were as follows: Spot 1: $t(4) = 25.98$, $P = 0.006$ (uncorrected), effect size = 4.16, α error probability: 0.05,

Power ($1-\beta$ error probability): 0.99, and the volume ratio of the spots in 2-DE images of the 6 MIA:control pairs: 4.04; Spot 2: $t(4) = 6.32$, $P = 0.003$ (uncorrected), effect size = 5.17, α error probability: 0.05, Power ($1-\beta$ error probability): 0.999, and the volume ratio of the spots in 2-DE images of the 6 MIA:control pairs: 2.18; Spot 3: $t(4) = -12.77$, $P = 0.0002$ (uncorrected), effect size = 10.43, α error probability: 0.05, Power ($1-\beta$ error probability): 1, and the volume ratio of the spots in 2-DE images of the 6 MIA:control pairs: 0.5. Although protein extractions from spots 2 and 3 (Supplementary Fig. 1B) were failed probably due to insufficient amount of proteins, proteins from spot 1 (Fig. 1A) was successfully extracted and subjected to the LC–MS/MS protein identification.

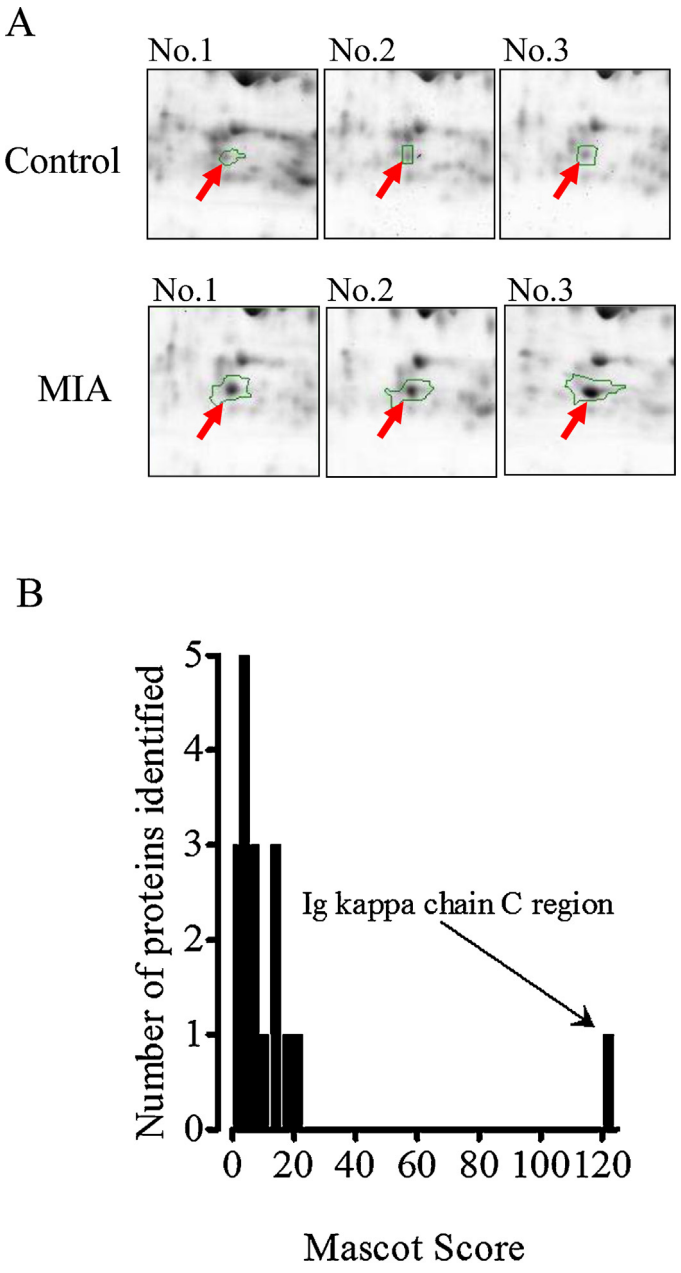

Fig. 1. Serum proteome analysis of mature MIA rat (postnatal weeks 16). (A) Locally expanded 2-DE patterns in the vicinities of spot 1 in each of the three control and three MIA model rat sera. Red arrows in the panels indicate the spots chosen as marker candidates. Green lines in the panels indicate spot boundaries. (B) Mascot score distribution by MS/MS identification of the protein extracted from the candidate 2-DE spots surrounded by the green lines in (A). As Mascot scores higher than 67 are considered significant, we considered the top-ranked protein with a Mascot score of 122 in (B) to be the Ig-κ chain C region.

We identified the protein in the spot by the following procedure: excision of the 2-DE spot, in-gel trypsin digestion of the protein in the spot, separation of peptides of the digested protein by liquid chromatography, and peptide identification by tandem mass spectrometry (LC–MS/MS). As shown in Fig. 1B, the top-ranked peptide identified by Mascot search was the Ig-κ light chain C region, a constitutive part of the Ig-κ light chain. The apparent molecular mass of the protein spot estimated from electrophoretic mobility in the 2-DE gel was approximately 25k, also conforming to the reported molecular mass of 22.5k of the Ig-κ light chain (Davies et al., 1975).

The rabbit anti-rat Ig-κ light chain antibody was produced in our laboratory by immunizing a rabbit with a synthetic peptide representing an amino acid sequence (54–73) of rat Ig-κ light chain. Fig. 2A shows an example Western blotting image of SDS-PAGE of a control rat serum using the polyclonal anti-rat Ig-κ light chain antibody. The antibody stained a single band in SDS-PAGE gel at about 25k. Upper panels in Fig. 2B are example Western blotting patterns that compare the MIA and control sera Ig-κ of mature rats (postnatal weeks 16) using the antibody. Western blotting images were quantified by Image-J and normalized by the albumin levels, followed by scaling to the mean of the control sera. Filled and open dots in the lower panel of Fig. 2B are Ig-κ light chain levels of the MIA (filled dots) and control sera (open dots), respectively. The Ig-κ light chain level in the sera of the mature MIA rats was about 2.1 times as high as that of the control one ($t(12) = 3.52$, $P = 0.004$, $N = 7$ each, Fig. 2B). Similarly to Fig. 2B, Fig. 2C shows neonatal rat data; example Western blotting patterns are in the upper panel and dot charts in the lower graph, showing that the serum Ig-κ light chain level in neonatal MIA rat (postnatal days 3) was about 1.6 times as high as that of the control rats ($t(10) = 5.791$, $P = 0.0005$, $N = 6$ each, Fig. 2C).

Augmentation of Ig-κ light chain in the serum is a result of B-cell activation (Davies et al., 1975). We therefore conjectured that augmentation of Ig-κ light chain in mature MIA rat sera would presumably be a result of B lymphocyte cell activation. Free Ig-light chains (FLCs) including Ig-κ light chain in sera are chronic inflammation markers (van der Heijden et al., 2006), but in the sera of mature MIA rats ($N = 7$) three pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, were all undetectable with ELISA detection limits of 62.5 pg/ml, 62.5 pg/ml and 25 pg/ml, respectively.

We investigated the effect of antipsychotic drug (haloperidol-decanoate) long-acting injection on Ig-κ light chain in sera of the MIA rat by Western blotting. The Ig-κ light chain bands of the haloperidol-decanoate-injected MIA rat sera were not significantly different (data now shown) in staining intensities from the vehicle-injected sera ($t(8) = 0.51$, $P = 0.61$, $N = 5$ each).

Our rat model study showed augmentation of Ig-κ light chains but not of the three pro-inflammatory cytokines, IL-6, IL-1β and TNF-α, in mature MIA rat sera. These findings suggest that the augmentation of Ig-κ light chain was caused by immune B-cell activation in the rat, accompanied by elevation of polyclonal FLCs.

3.2. Free Ig light chains (FLC) in sporadic schizophrenia patient sera — a pilot study

Protein syntheses of Ig heavy and light chains in mammalian immune systems are asynchronously carried out on different ribosomes in immune B cells, Ig light chain production is 10% to 40% higher than Ig heavy chain production, and excess light chains are secreted as free Ig light chains (FLCs) into the blood stream of the host (Nakano et al., 2011). Two isoforms of Ig light chains, Ig-κ and Ig-λ light chains, are produced in the human immune system, and the κ-to-λ ratio (rFLC) of the two light chains is species-dependent (Redegeld and Nijkamp, 2003; Redegeld et al., 2002).

If B-cell activation, found in the serum proteome of the MIA model rat, also takes place in patients with schizophrenia, it would

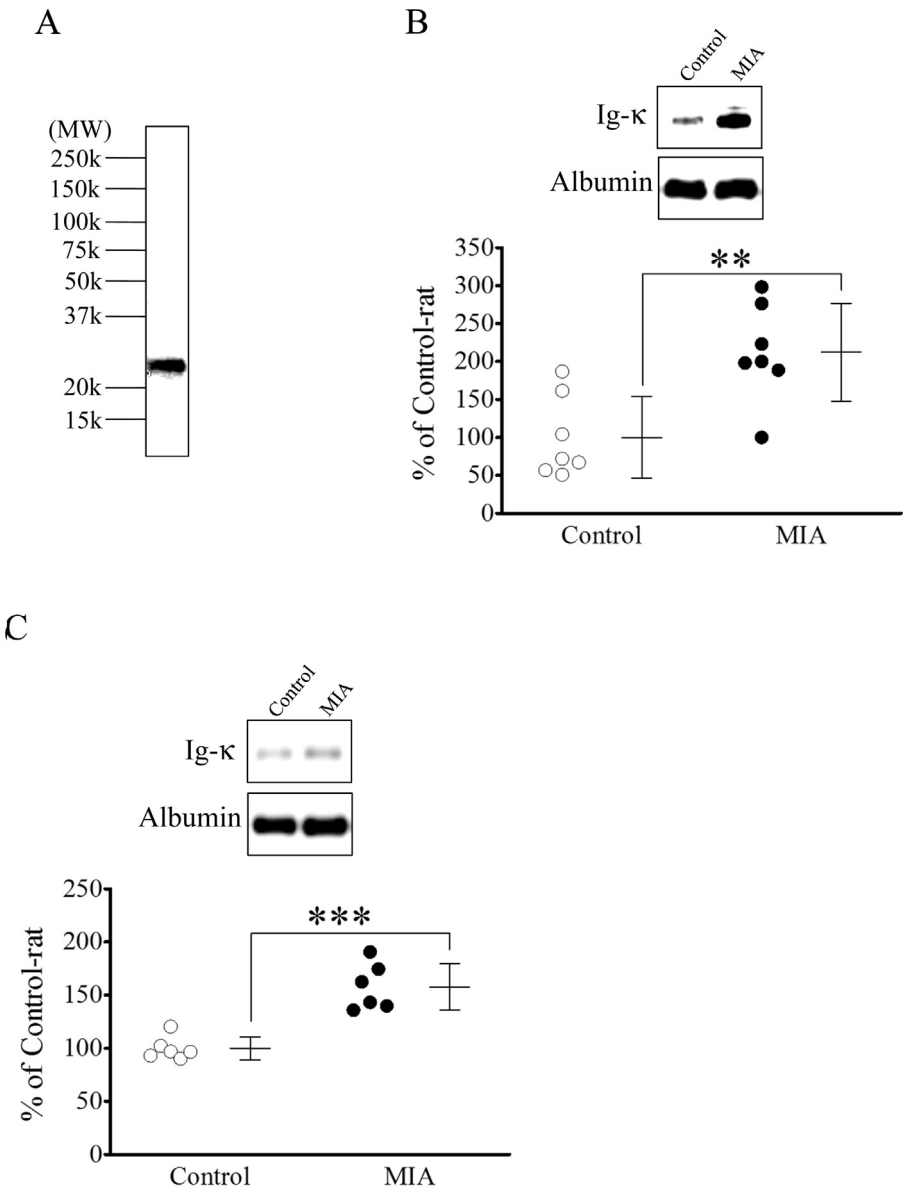

Fig. 2. Serum Ig-κ light chain Western blot analysis of MIA rat. (A) An example Western blotting image of a control rat serum using polyclonal rabbit anti-rat Ig-κ antibody. The antibody stained a single band at about 25k. (B) Serum Ig-κ light chain Western blot analysis of mature MIA rat (postnatal weeks 16). (C) Serum Ig-κ light chain Western blot analysis of neonatal MIA rat (postnatal days 3). (B) and (C) Upper four panels show Western blots of Ig-κ light chain proteins together with albumin as controls in serum of the control and MIA samples. The Ig-κ light chain and albumin specific bands were at 25k and 65k, respectively, on SDS-PAGE gel. The Ig-κ light chain levels were normalized by albumin of the MIA sera. Dot charts indicate the normalized serum Ig-κ light chain levels of control- and MIA-rat. Concentration ratios of Ig-κ light chain to albumin indexed to control show increase in the serum of both mature- and neonatal-MIA rats. Data are expressed as percentage of the value of the control rats. Asterisks **, *** in the figures indicate $P < 0.01$, $P < 0.001$ respectively. Error bars denote mean ± SD.

increase FLC concentrations in the patient sera. We thereby measured FLCs and pro-inflammatory cytokines in serum samples of patients with sporadic schizophrenia and healthy control donors purchased from PrecisionMed, Inc., USA.

Fig. 3A and B shows that both free Ig-κ light chain (κFLC) and free Ig-λ light chain (λFLC) concentrations in the patient sera were significantly higher than those in the sera of healthy control. Summary statistics by Student's t-test for κFLC ($t(15) = 2.22$, $P = 0.041$, effect size: $d = 1.08$, α error probability: 0.05, Power ($1-\beta$ error probability): 0.547); and for λFLC ($t(15) = 2.7$, $P = 0.016$, effect size: $d = 1.93$, α error probability: 0.05, Power ($1-\beta$ error probability): 0.959). Fig. 3C shows that κFLC-to-λFLC ratio (rFLC) of the patient group sera and healthy control sera were not significantly different between the two groups.

4. Discussion

The present study showed the following two kinds of experimental data. (1) Reproducible specific augmentation of Ig-κ light chain was observed in serum proteome of MIA rat, but pro-inflammatory cytokines in sera were too low to be detected by our ELISA protocol. The augmentation of Ig-κ light chain was also observed in serum of neonatal MIA rat. Antipsychotic drug (haloperidol-decanoate) long-acting injection had little effect on the Ig-κ light chain level in sera of MIA rat. (2) FLCs but not pro-inflammatory cytokines were augmented in sporadic schizophrenia patient sera for research.

Maternal immune activation GD15 to GD21 produce neuronal and behavioral abnormality in the offspring and maternal immune

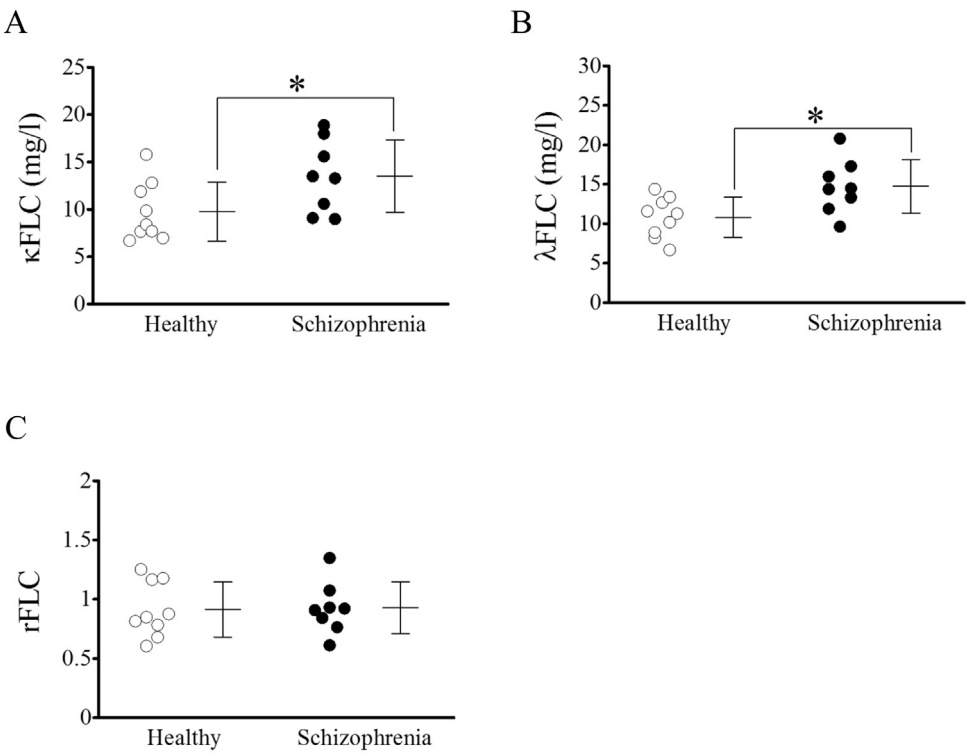

Fig. 3. Free light chain (FLC) analyses of the sera from healthy control and schizophrenia patient groups. (A) Dot chart of κFLC concentrations in mg/l, and (B) that of λFLC in mg/l of healthy and schizophrenia groups. (C) Dot chart of the κ-to-λ FLC ratio (rFLC) of the two groups. Numbers of sera samples of patients and healthy controls are $N = 8$ and $N = 9$, respectively. Asterisks "*" stand for $P < 0.05$. Horizontal lines and error bars denote mean ± SD.

activation induced by Poly I:C doses ranging from 4 to 20 mg/kg led to a number of abnormal behaviors in the offspring at adulthood (Bilbo and Schwarz, 2009; Macedo et al., 2012; Meyer et al., 2005; Ozawa et al., 2006; Smith et al., 2007; Wolff and Bilkey, 2008; Zuckerman et al., 2003a, 2003b; Zuckerman and Weiner, 2005). In the present study, Poly I:C potassium salt (4 mg/kg) dissolved in saline was injected into the pregnant Wistar rats intraperitoneally (I.P.) daily for 4 consecutive days from GD15 to GD18. The injection protocol in the present study is therefore different from those used in previous MIA rat model studies (Dickerson et al., 2010; Wolff and Bilkey, 2008; Zuckerman et al., 2003a, 2003b; Zuckerman and Weiner, 2005), but our injection protocol seems to be adequate having produced neuronal and behavioral abnormality in the Wistar rat offspring born from Poly I:C-injected rats similar to the one seen in another MIA rodent model (Oh-Nishi et al., 2011).

In our study, we found more than two-fold augmentation of Ig-κ light chain in serum proteome of the MIA model rat. It should be remembered that we had observed Ig light chains in rat sera with no distinction whether they were free or not, but it is not unreasonable to hypothesize that the augmentation of Ig-κ light chain was caused by immune B-cell activation in the rat, accompanied by elevation of polyclonal FLCs, ending up with abnormal T cell responses. A recent work showed that MIA leads to permanently hyper-responsive $CD4^+$ T cells in offspring of mice, and the hypersensitive immune system in offspring and also behavioral abnormality of rodents could in part be normalized by bone marrow transplant (Hsiao et al., 2012).

Free Ig-κ light chain have long been considered simply to be spillover remnants of the regular Ig production in B cells, but actually induce mast-cell-driven hypersensitivity (Redegeld and Nijkamp, 2003; Redegeld et al., 2002). Although the causal relationship between augmentation of serum Ig-κ light chain and behavioral dysfunction in the MIA animal model is not yet known, we can at least infer the implication of the augmentation of Ig-κ light chain in the MIA rat model with reference to the behavior-modulating role of mast cell activation, which extends beyond several behaviors in rodents. Pretreatment of rats with mast cell degranulator inhibitor before a restraint stress protected them from exhibiting social behavior post stress (Manchanda et al., 2011), suggesting that mast cell activation modulates emotional and social behaviors.

Relative proportions of Ig-κ and Ig-λ light chains vary considerably with species (Davies et al., 1975) and rFLC is therefore species-dependent. Ig-κ light chains level are much more than Ig-λ light chains in rodent serum (Davies et al., 1975). rFLC in rodent serum seems not to be useful for immunological indicator. As both Ig-κ and Ig-λ light chains are richly present in human serum, rFLC in human sera is used for clinical diagnostics index (Katzmann et al., 2002). The reference range of rFLC for human sera is 0.26–1.65 (Katzmann et al., 2002). Outlier rFLC values are observed with an elevation in at least one of κFLC or λFLC by clonal disorders in plasma cells such as MGUS (monoclonal gammopathy of undetermined significance) (Dispenzieri et al., 2012). The mean rFLC value of sera of the patients with schizophrenia was 0.926 in the present study, which is in the reference range.

Serum IL-6 increase has been reported in patients with schizophrenia (Potvin et al., 2008), but IL-6 in MIA rat sera was undetectable by the ELISA protocol. MIA leads to permanently hypersensitive $CD4^+$ T-cells in offspring, which indicates a chronic pro-inflammatory phenotype (Hsiao et al., 2012), and hypersensitive $CD4^+$ T-cells release more IL-6 after in vitro stimulation. If MIA rats raised in the SPF (specific pathogen-free) laboratory of our institute had been exposed to a lot of various immune stimulators (i.e., virus, bacteria, etc.), augmentation of IL-6 in the serum would have been detected.

There have been a number of studies on schizophrenia-linked genomic variations. Genome-wide association studies have revealed that the major histocompatibility complex (MHC) region

on the short arm of chromosome 6 is involved in schizophrenia susceptibility (Irish Schizophrenia Genomics Consortium and the Wellcome Trust Case Control Consortium 2, 2012; Corvin and Morris, 2013). Considering the multi-role of the MHC region in immunity, its involvement in schizophrenia susceptibility is not surprising. Our finding of FLC elevation as a biomarker candidate of the disease also suggests that patients' immune systems are involved in the disease. As Ig-κ light chain gene transcription is enhanced by NF-κB in B cells (Kaileh and Sen, 2012), NF-κB-related gene alterations in patients with schizophrenia (Begni et al., 2003; Hashimoto et al., 2011; Liou et al., 2012) might be related to the elevation of FLCs.

Recently it is suggested that mild chronic inflammation could cause multiple endophenotypes similar to patients with schizophrenia. Deficiency of schnurri-2 induced mild chronic inflammation to produce neuronal and behavioral phenotypes related schizophrenia (Choi et al., 2015; Takao et al., 2013). Our MIA rats reveal parvalbumin positive interneuronal loss in medial prefrontal cortex and reduction of pre-pulse inhibition (Oh-Nishi et al., 2011) which is similar to schnurri-2 knockout mouse. Schnurri-2 knockout mouse reveal constitutive NF-κB activation in $CD4^+$ cell (Kimura et al., 2007). The findings indicate that NF-κB related mild chronic inflammation might cause neuronal and behavioral deficits. Augmentation of Ig-κ light chain in the MIA rat sera shown in the present paper might be re-examined in the future in the context of mild chronic inflammation caused by NF-κB activation.

Our study has four limitations. First comes from 2-DE-based proteomics, i.e., not all high-molecular-mass and/or low-abundance proteins and low molecular-mass peptides in sera of MIA rats are separated by and detected in 2-DE gels. In the context of our present study, immunological factors other than FLCs would naturally be expected to be biomarker candidates, but 2-DE-based proteomics that we had used made it difficult for us to detect such important immunological factors as cytokines and chemokines. Second is the number of the rodent model sera used in the present study; we compared 2-DE gel images of three MIA and three control rat sera for exploring biomarker candidate proteins, and we identified Ig-κ in the MIA rat sera. Although the number of comparisons was limited, the rodent models being homogeneous genetically and having been raised in a controlled environment, we successfully detected variations of Ig-κ abundance in six 2-DE images of the MIA and control rat sera. Third is the uncontrollable heterogeneity of the human sera we used; the donors are heterogeneous in their life and medical histories, such as smoking, antipsychotic agent treatments, duration of medication and weight gain potentially, which would influence their immune systems (Hinze-Selch and Pollmacher, 2001; Pollmacher et al., 1997). Moreover, it was not known whether or not the patient donors had experienced maternal infection during their fetal lives. Despite of the donor heterogeneities noted in the above we observed augmentation of serum FLCs in the sera of patients with sporadic schizophrenia. Those donor heterogeneities must be taken into consideration in any clinical study in the future. Fourth is the specificity of FLCs for the MIA-associated neurodevelopmental disorder. Augmentation of serum FLCs itself occurs under various conditions, such as chronic inflammatory diseases, oncological and B-cell related disorders, to name a few, and is not specific enough for the MIA-associated neurodevelopmenatal disorders. Our rodent model experiment indicates that MIA-associated neurodevelopmental disorders have characteristics different from chronic inflammatory diseases (i.e., augmentation of serum FLCs but not pro-inflammatory cytokines). In this context, the combined assay of serum FLCs and other immunological factors would provide higher specificity for MIA associated neurodevelopmental disorders than serum FLC alone.

5. Conclusion

We found a novel serologic biomarker candidate for MIA associated neurodevelopmental disorders in MIA rat model. We propose a future research direction in which combined assay of serum FLCs and other immunological factors would provide a clue toward realization of a serodiagnostic test for neurodevelopmental disorders with MIA background. Our proposal surely needs to be scrutinized more closely than examined in the present study, but it is still worthy of being validated for our developing novel serodiagnostic tools, preventive methods and treatment strategy of the organic mental disorders with MIA background.

Author contribution

Conceived and designed the experiments: A.O.-N. Performed the experiments: A.O.-N. and K.K. Analyzed the data: A.O.-N. and K.K. Wrote the paper: A.O.-N., T.M., K.K., and T.S.

Conflict of interest

The National Institute of Radiological Sciences has submitted a patent application (PCT/JP2014/57227) on the use of the biomarker candidate proteins described in this paper. A.O.-N. and T.S. are listed as co-inventors of this patent. Anatech Corporation provided support in the form of salary for K.K., but had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. This does not alter our adherence to Neuroscience research policies. Other authors have no Competing Interests to declare.

Acknowledgments

We are grateful to Dr. Y. Kodera (Kitasato Univ.) for giving us advice, and to Dr. H. Tomizawa (Chiba Univ.) for technical assistance. This work was supported by KAKENHI 15K08633 (to A.O.-N.), *Japan Advanced Molecular Imaging Program* from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (to T.S.), and Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS) (to A.O.-N. and T.S.) from the Japan Agency for Medical Research and Development, AMED Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neures.2016.07.003.

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