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Matrix metalloproteinases-2 and -9 in *Campylobacter jejuni*-induced paralytic neuropathy resembling Guillain-Barré syndrome in chickens

Kishan Kumar Nyati [a, **, 1, 2], Kashi Nath Prasad [a, *, 1], Vinita Agrawal [b], Nuzhat Husain [c]

[a] Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226 014, India
[b] Department of Pathology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226 014, India
[c] Department of Pathology, Dr. RML Institute of Medical Sciences, Lucknow 226 010, India

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ABSTRACT

Inflammation in Guillain-Barré syndrome (GBS) is manifested by changes in matrix metalloproteinase (MMP) and pro-inflammatory cytokine expression. We investigated the expression of MMP-2, -9 and TNF-α and correlated it with pathological changes in sciatic nerve tissue from *Campylobacter jejuni*-induced chicken model for GBS. *Campylobacter jejuni* and placebo were fed to chickens and assessed for disease symptoms. Sciatic nerves were examined by histopathology and immunohistochemistry. Expressions of MMPs and TNF-α, were determined by real-time PCR, and activities of MMPs by zymography. Diarrhea developed in 73.3% chickens after infection and 60.0% of them developed GBS like neuropathy. Pathology in sciatic nerves showed perinodal and/or patchy demyelination, perivascular focal lymphocytic infiltration and myelin swelling on 10th- 20th post infection day (PID). MMP-2, -9 and TNF-α were up-regulated in progressive phase of the disease. Enhanced MMP-2, -9 and TNF-α production in progressive phase correlated with sciatic nerve pathology in *C. jejuni*-induced GBS chicken model.

1. Introduction

Guillain-Barré syndrome (GBS) is an autoimmune heterogeneous disorder of the peripheral nervous system (PNS) comprising several clinical subtypes [1,2]. It is most often triggered by an aberrant immune response to an infectious pathogen. *Campylobacter jejuni* (*C. jejuni*) is most commonly implicated in the development of GBS. Numerous studies subsequently confirmed the association between *C. jejuni* and GBS [3–5]. *Campylobacter jejuni*-associated cases of GBS are more severe and may result in more irreversible neurological damage. Matrix metalloproteinases (MMPs) are believed to participate in the development of peripheral neuropathy in GBS patients; however, there are no data available on the role of MMPs in *C. jejuni*-associated GBS due to the lack of appropriate animal model.

MMPs comprise a family of calcium-dependent zinc proteolytic enzymes that enhance T-cell migration or adhesion and degrade components of the extra-cellular matrix (ECM) proteins [6]. It can be divided into four subclasses based on substrate specificity and domain structure: gelatinases, collagenases, stromelysins, and membrane-type MMPs [7]. Their involvement in various physiological and pathological conditions is not yet completely understood. However, several studies suggested their role in numerous pathological conditions including breakdown of the blood-brain barrier (BBB), demyelination, facilitation of macrophages, T-cell adhesion to matrices and endothelial cells, propagation of an inflammatory response, exit of lymphoid cells from the circulation of tissue [8–11]. Evidences are emerging that MMPs might play a role in the pathogenesis of inflammatory demyelinating diseases of central nervous system (CNS) [12–16]. On the other hand, the role of MMPs in the PNS and their participation in the pathogenesis of inflammatory demyelinating disorders has not yet been completely understood. Several studies reported that both MMP-2 and -9 can augment the levels of active tumor necrosis factor-α (TNF-α), which is a potent pro-inflammatory cytokine [17–19]. MMP-9 appears to

* Corresponding author. Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Rae-bareli Road, Lucknow 226 014, Uttar Pradesh, India.
** Corresponding author. Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Rae-bareli Road, Lucknow 226 014, Uttar Pradesh, India.
E-mail addresses: nyati15@ifrec.osaka-u.ac.jp (K.K. Nyati), kashinprasad@gmail.com (K.N. Prasad).
[1] Equal corresponding authors.
[2] Present address: Laboratory of Immune Regulation, Immunology Frontier Research Center, Osaka University, Osaka-5650871, Japan.

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be involved in the pathogenesis of multiple sclerosis (MS) since increased MMP-9 levels is found to be associated with leaky BBB and also correlated with disease severity in GBS [20–22]. Earlier, the role of MMP-2 and -9 had been reported in destruction of basement membranes in the salt-induced experimental ascites syndrome in broiler chickens [23]. Previously, we have also investigated the role of MMP-2 and -9 in patients with GBS and have correlated with the levels of pro-inflammatory cytokines (TNF-α and IL-1β) in progressive phase of the disease [21,24]. Li et al. (1996) had attempted to establish chickens as an animal model for the disease and tried to develop a GBS like paralytic neuropathy induced by *C. jejuni* [25]. We had also reported that GBS-like neuropathy resembling both axonal and AIDP variants of pathological spectrum can be developed in chickens following *C. jejuni* infection suggesting that chickens may be useful as an experimental animal model to study the immunopathogenesis of *C. jejuni*-associated GBS [26,27]; however, further studies on this model are elusive. In the present study, sciatic nerves from chickens that developed GBS like neuropathy after *C. jejuni* infection were used to investigate the possible role of MMP-2 and -9 with TNF-α in the pathology of GBS during clinical course of the disease.

2. Materials and methods

2.1. Animals

Two to four weeks old chickens (white feathered, *Gallus gallus domesticus*; N = 60), procured from a nearby government poultry farm were included in the study. Animals were housed in separate cages and given free access to food and water at animal care facility. To determine the prior infection of *C. jejuni*, stool samples from all the chickens were cultured on blood free charcoal cefaperazone deoxycholate agar (CCDA; Himedia, Mumbai, India) and subjected to polymerase chain reaction (PCR) following standard protocol [3,28]. Chickens negative for *C. jejuni* were included in the study. The study was approved by institutional ethical committee of Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India.

2.2. Chicken inoculation and sacrifice schedule

Chickens were randomly assigned to two different groups (30 chickens/group). Experimental group was fed with 5 ml *C. jejuni* suspension [4] isolated from a patient with GBS, containing 5×10^9 CFU/ml directly into the gizzard. Control chickens received only sterile phosphate buffer saline. For the detection of *C. jejuni* infection, stool samples from all chickens were subjected to culture and PCR using *C. jejuni*-specific *fla* and *hip* gene primers [3,28]. All the animals were monitored regularly up to 30 days for the development of diarrhea, and weakness of the hind limbs, difficulty in standing or walking, wing droop, difficulty in feeding etc. Five animals from each group were euthanized by cervical dislocation periodically at 5, 10, 15, 20, 25 and 30 post inoculation days (PIDs) and sciatic nerve tissue was excised aseptically. The nerve tissue was cut in four parts-first and second was stored in liquid N_2 for real-time PCR and immunohistochemistry, third part in 10% formalin for histopathology and, fourth part was kept for zymography.

2.3. Histopathology

Nerve tissues were fixed with 10% buffered formalin, paraffin embedded, sectioned at 4–5 μm and stained with hematoxylin and eosin. Histopathological evaluation was performed blinded to the experimental group by the competent pathologist. The extent of inflammation and nerve fiber damage was categorized as normal, mild, moderate or severe.

2.4. RNA extraction and cDNA synthesis

The sciatic nerve from both groups of chickens was excised and kept in liquid N_2. Total RNA was extracted from immediately 'snap-frozen' tissue with Trizol LS reagent as per the manufacturer's instruction (Invitrogen, USA) and stored at −80 °C till further use. RNA was quantified by spectrometry and 100 ng RNA was reverse transcribed using Revert aid™ H minus first strand cDNA synthesis kit (Fermantas life sciences, USA) to make complementary DNA (cDNA) and stored at −20 °C. Primers were designed corresponding to sequences from GenBank using Applied Biosystems primer express software v3.0 (Applied Biosystems, Carlsbad, CA). The primers were synthesized by Sigma (Sigma, USA). Previously published primer sequences for MMP-2, -9 and GAPDH were used in the study are mentioned in Table 1 [29,30]. GAPDH was used as housekeeping gene. The specificity for each primer set was tested by analyzing the melting curve following real-time PCR.

2.5. Quantitative real-time PCR

The analysis of real-time PCR data and relative quantification of MMPs and TNF-α gene was carried out by the ABI Prism 7300 system (Applied Biosystems, Foster City, CA). PCR was performed in a 20 μl volume containing 1 μl cDNA, 10 μl 2X power SYBR Green PCR master mix (Applied Biosystems, Forster City, CA), 300 nM of each gene-specific primer. Thermal cycling parameters for MMP-2 and -9 genes were as follows: a 95 °C denaturation step for 15 min followed by 40 cycles of 95 °C denaturation (15 s), 60 °C annealing (30 s), and 72 °C extension (30 s); for GAPDH gene amplification the PCR parameters were initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C denaturation (15 s) and 60 °C annealing (1 min) and 72 °C extension (30 s). The PCR parameters for TNF-α were same as in MMPs gene amplification except that the annealing temperature was 56 °C. The final step was to obtain a melt curve for the PCR products to determine the specificity of amplification. All experiments were carried out in triplicate and GAPDH was utilized as the reference gene. The quantification of gene expression by real-time PCR was conducted as detailed elsewhere [31,32]. Expression levels of genes were calculated relative to the expression of the GAPDH gene.

2.6. Zymography

The gelatinolytic activity of MMPs was measured in the homogenates of sciatic nerve tissues as described elsewhere [21,33]. In brief, the concentration of protein was measured in the homogenate using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Two fold diluted supernatant after centrifuging homogenate having 20 μg of proteins was separated on the 10% precast zymogram (Novex, San Diego, CA) at 125 V for 1.5 h under non-reducing conditions of 5X non-reducing loading buffer, 0.4 M Tris-HCl (pH 6.8), 20% glycerol, 5% sodium dodecyl sulfate, and 0.1% bromophenol blue, followed by 1 h of incubation (three times, 20 min each) in 2.5% Triton X-100 (Sigma, St. Louis, MO) and incubation for 16 h in 1X zymogram developing buffer (Novex) for the development of enzyme activity bands. After incubation, the gels were stained with 0.5% Coomassie G-250 (Hi-Media, Mumbai, India) in a mixture of methanol:acetic acid:H_2O (30:10:60) and destained in methanol:acetic acid:H_2O (50:10:40). The gelatinolytic activities were detected as transparent bands against the background of Coomassie brilliant blue-stained gelatin and bands densities were analyzed by densitometric scanning software ImageQuant TL (GE-