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Short Communication

Strain Difference of DNA-Binding Activity of NF-κB and Cytokine Gene Expression in Spleen Dendritic Cells of Lewis and Fischer Rats

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Abstract

To investigate the mechanisms of different responses to inflammatory stimuli between Lewis and Fischer rats, the DNA-binding activity of the nuclear factor kappa B (NF-κB) and cytokine production of spleen dendritic cells (SDC) in Lewis and Fischer rats after peptidoglycan-polysaccharide (PG-PS) treatment were determined. The results show that the DNA-binding activities of NF-κB in SDC were higher in Lewis rats than in Fischer rats. Furthermore, in Lewis rats, the increase in NF-κB DNA-binding activities was dose-dependent. However, there is no significant change in SDC of Fischer rats. In Lewis rats, the levels of IL-2 and IL-4 were decreased along with the increase of the concentration of PG-PS while TNF-α was increased. However, there was no obvious change of cytokine expression in Fischer rats in the presence of PG-PS. In conclusion, these findings indicate that the differences in the DNA-binding activity of NF-κB and cytokine production might mediate strain-specific differences of susceptibility to chronic inflammatory stimuli in Lewis and Fischer rats.

Key Words: dendritic cell, NF-κB, cytokines

Introduction

Lewis and Fischer rats exhibit different responses to inflammatory stimuli of carrageenan, streptococcal cell walls and lipopolysaccharide (LPS) (4). For example, Lewis rats are susceptible to developing peptidoglycan-polysaccharide (PG-PS)-induced inflammatory bowel disease (IBD), a potential model of Crohn's disease, whereas Fischer rats are not. There is substantial evidence to indicate that the strain-specific differences in inflammatory and disease susceptibility are attributed to environmental, genetic and neuroendocrinal factors (7).

Dendritic cells (DC) are regarded as potential antigen-presenting cells (APC) involved in T-cell activation. They also directly or indirectly affect B-cell functions, antibody synthesis and isotype switching (3). Spleen is one of the most important immune organs in which DC must have a key role in the regulation of immune reactions (11). A previous study showed that rat spleen DC (SDC) was heterogeneous in population and could be subdivided into two groups based on the expression of surface CD4 and SIRPα: CD4/SIRPα$^+$ and CD4/SIRPα$^-$ (14). We postulate that the difference in susceptibility to inflammatry stimuli may partly be mediated by SDC. In the present study, the DNA-binding activity and expression of cytokines of SDCs from Lewis and

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Fischer rats were analyzed to determine if there were genetic differences in the two rat strains.

Materials and Methods

Animals

Lewis and Fischer rats of 6-8-week were obtained from Nanjing Medical University and were housed in a temperature- and humidity-controlled room with a 12-h light-dark cycle. Food and water were available and rats were maintained in our animal facilities for at least one week before being used in the following experiments. The research ethics committee of Nanjing University gave approval for the animal experiments.

Reagents

Collagenase D, peptidoglycan-polysaccharose (PG-PS) and fetal calf serum (FCS) were purchased from Sigma Chemical Co. (St., Louis, Mo, USA). RPMI 1640 was purchased from HyClone Laboratories (Inc., Logan, UT, USA). Rat Fc Block^{TM}, Cytofix/Cytoperm^{TM} (with GolgiStop^{TM}), R-phycoerythrin (PE)-conjugated antibodies of IgG2a, IL-2, IL-4, IL-10, IFN-γ, TNF-α and OX-62 were bought from Becton, Dickinson and Company (San Jose, CA, USA). Rat OX-62 microbeads were purchased from Miltenvi Biotec (Bergisch Gladbach, Germany). [γ-^{32}P]ATP and the oligonucleotide 5'-CAAGGGGACTTTCCATGGATCCAAGGGGACTTTCCATG-3' were obtained from Promega (Madison, Wisconsin, USA).

Preparation of SDCs

Rats were euthanatized by cervical dislocation and then kept in 70% ethanol for 10 min. The spleens were teased apart and digested with 2 mg/ml collagenase D for 30 min at 37°C in the presence of 10 μM EDTA in the last 5 min. The cells were passed through a steel mash and the erythocytes were depleted by ACK solution (0.83% NH_4Cl, 0.1% $KHCO_3$ and 0.004% EDTA, pH 7.2-7.5). Cells were then passed through a filter, collected in a 50 ml tube, washed and counted. 20 μl of rat OX62 microbeads per 10^7 total cells were added after supernatant was removed completely and cells were resuspended in 80 μl of buffer per 10^7 cells at 4°C for 15 min. OX62- positive cells were enriched and harvested by immunomagnetic bead selection with MACS columns and resuspended in RPMI1640 media (10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin and 0.1 mg/ml streptomycin). The purity of SDCs obtained in this manner was up to 95% through staining of PE-OX62 antibodies and followed by analysis by flow cytometry. Cells obtained in this manner consistently contained 95% live cells in trypan blue staining.

Electrophoretic Mobility Shift Assay (EMSA)

To monitor the presence of NF-κB DNA-binding activity in the nuclear extracts, we performed electrophoretic mobility shift assays (EMSA). A double-stranded DNA fragment generated by annealing of the oligonucleitide 5'-CAAGGGGACTTTCCATGGATCCAAGGGGACTTTCCATG-3' containing two NF-κB-binding sites was end-labeled with [γ-^{32}P]ATP using polynucleotide kinase and was used as the probe. EMSA reactions consisted of: 12 μl of 2 × gel-shift reaction buffer (12% glycerol, 24 mM HEPES, 8 mM Tris-HCl, 2 mM EDTA, 1 mM dithiothreitol), 1 μl of bovine serum albumin (3 μg/μl), 2 μl of poly (dI-dC) (0.5 μg/μl), and 20,000-50,000 cpm DNA probe. Lastly, 3 μg of nuclear extract and a sufficient volume of extract in buffer were added up to 5 μl in total. Samples were incubated on ice for 20 min, loaded on a 4% polyacrylamide gel and electrophoresed at 200 V for 1-1.5 h at 4°C. Proteins were transferred to 3 MM Whatman paper, dried under vacuum at 80°C for 1 h, and autoradiographed using Kodak X-omat films. The assay is based on the observation that protein-DNA complexes migrate through a polyacrylamide gel more slowly than free DNA fragments.

Intracellular Cytokine Assay

Cytokine production was measured by flow cytometry. SDCs were cultured with 0, 5, 50 μg/ml PG-PS and GolgiStop^{TM} for 12 h followed by treatment with Fc Block^{TM} for 15 min at 4°C. The cells were then washed, fixed and permeabilized with a Cytofix/Cytoperm solution. SDCs were incubated with R-PE-labeled monoclonal antibodies of IL-2, IL-4, IL-10, IFN-γ and TNF-α for 30 min. SDCs were washed twice in perm/wash buffer and once in FACS staining buffer (PBS, 1% BSA, and 0.09% NaN_3) before analysis on the FACS can Becton-Dickson, Immunocytometry System (San Jose, CA, USA). For each experiment, the cells were stained with isotype control antibodies to establish background staining and to set the quadrants before calculating the percentage of positive cells. 10,000 cells total events were collected.

Statistical Analysis

Data are expressed as means ± SD. Significance was assessed by one way analysis of variance (ANOVA) and Student's *t*-test. For all statistical

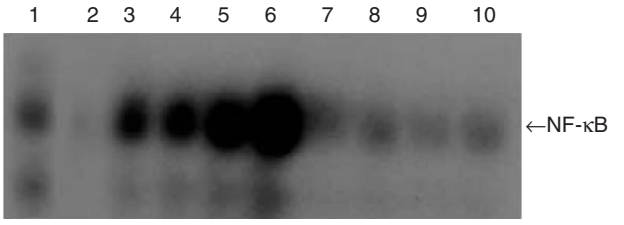

Fig. 1. Effects of PG-PS on the DNA-binding activities of NF-κB in SDCs from Lewis and Fischer rats. Nuclear extracts were incubated with radio-labeled probes for NF-κB and were analyzed on a 6% polyacrylamide gel. The figure is a representative autoradiogragh with the numbers representing positive control (1) and negative control (2). Lewis SDCs were treated with PG-PS in 0 ng/ml (3), 10 ng/ml (4), 100 ng/ml (5) and 1000 ng/ml (6). Fischer SDCs were treated with PG-PS in 0 ng/ml (7), 10 ng/ml (8), 100 ng/ml (9) and 1000 ng/ml (10). The presented data represent three independent experiments.

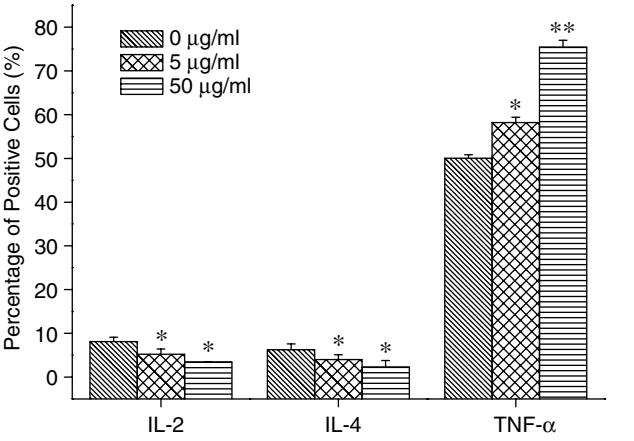

Fig. 2. Effects of PG-PS on cytokine expression of SDCs from Lewis rats. After treatment with PG-PS for 12 h, SDCs were stained with phycoerythrin (PE)-labeled polyclonal IL-2, IL-4 or TNFα antibodies, respectively. Fluorescence was analyzed by flow cytometry. Representative histograms of three consistent experiments are shown. *: $P < 0.05$, **: $P < 0.01$ versus control.

analyses, the level of significance was set at a probability of 0.05 and 0.01 to be considered significant and notably significant, respectively.

Results

Comparison of NF-κB DNA-Binding Activity of SDCs in Lewis and Fischer Rats

The results show that NF-κB DNA-binding activities of SDCs were notably lower in the controls (no PG-PS stimulation) than those treated with PG-PS. In the presence of all concentrations of PG-PS, the DNA-binding activities of NF-κB in SDCs from Lewis rats were higher than those of Fischer rats and all had notably statistical difference. The increase of DNA-binding activities of NF-κB was dose- dependent in Lewis rats. However, there is no significant change of the DNA-binding activity of NF-κB in SDC of Fischer rats (Fig. 1).

Comparison of Intracellular Cytokines of SDCs between Lewis and Fischer Rats

The results show that the levels of IL-2 and IL-4 in SDCs significantly decreased with PG-PS treatment compared with the Lewis control rats. The IL-2 and IL-4 significantly decreased in the 50 μg/ml PG-PS group compared with the 5 μg/ml PG-PS group ($P < 0.05$). The TNF-α level significantly increased after PG-PS treatment compared with the control in Lewis rats. In the 50 μg/ml PG-PS group, TNF-α significantly increased relative to the 5 μg/ml PG-PS group ($P < 0.05$) (Fig. 2). There were no obvious changes in IL-10, IL-12 and IFN-γ after PG-PS treatment in Lewis rats. However, all the detected cytokines were not significantly changed in SDCs of Fischer rats.

Discussion

Fischer and Lewis rats have been characterized by their differential reactivities to a variety of pro-inflammatory stimuli (4). In addition, numerous studies have compared a number of biochemical, physiological and behavioral endpoints of these inbred strain rats (7). Previous studies also showed that PS-PG-induced IBD model could be built in Lewis rats but not in Fischer. The present study aimed to investigate possible differences in the DNA-binding activity of NF-κB and cytokine production of SDCs between these two source rats which might be related to strain difference in IBD induction.

DNA-binding activity of NF-κB in SDCs from Lewis rats was notably higher compared with that of Fischer rats. The nuclear transcription factor NF-κB is important in regulating the expression of many genes proteins of which are involved in the control of apoptosis (cell suicide), the development of B- and T-cells, anti-viral and bacterial responses, responses to multiple stresses, embryonic development and inflammatory responses. In most untransformed cells, NF-κB complexes are largely cytoplasmic and remain transcriptionally inactive until the cell is stimulated (8). Once the cell is activated, NF-κB is liberated and accumulated in the nucleus where it activates the expression of specific genes involved in immunity, inflammation and proliferation (15). Recently, NOD2

(nucleotide-binding oligomerization domain), the first susceptibility gene linked with Crohn's disease, was highly restricted to monocytes, macrophages and DCs (10). NOD2 has been proposed to directly bind bacterial LPS or PG-PS and subsequently acts as an activator of NF-κB which plays an important role in inflammation (12). The lower DNA-binding activity of NF-κB in SDCs from Fischer rats may reflect the blocking of NOD2 signaling pathway and impaired immune responses.

Our results show that IL-2 and IL-4 in SDCs were significantly decreased compared with the control in Lewis rats. TNF-α significantly increased in SDCs of Lewis rats than in the control. However, there were no significantly differences in IL-2, IL-4 and TNF-α in SDCs from Fischer rats. In previous reports, when exposed to muramyl dipeptide, human DCs secreted IL-1β, IL-6, IL-12 and TNF (6, 9). IL-2, produced by T-cells or DCs, could induce proliferation and activation of both $CD4^+$ and $CD8^+$ T cells (5). TNF-α is regarded as a classical pro-inflammatory cytokines (2). IL-4 is an anti-inflammatory cytokine which downregulates both the innate and adaptive immune responses (1). Therefore, the results suggest that the greater susceptibility to PG-PS-induced inflammation in Lewis but not in Fischer rats might attribute to low levels of IL-4 and IL-10 and high levels of TNF-α in Lewis rat SDCs.

In the present study, the effects of PG-PS might be mediated by TLR4 (Toll-like Receptor 4) on the SDCs. Studies of Qian *et al*. showed that enhanced mRNA expression of TLR-4 was found in HLA-B27 transgenic rats compared to wild-type littermates which resulted in exaggerated proinflammatory responses to symbiotic bacteria and uncontrolled inflammation in this colitis model (13). We thought that the differences in the DNA-binding activity of NF-κB and cytokine production of SDCs were induced by different expression of TLR-4 in Lewis and Fischer rats.

In conclusion, SDCs from Lewis and Fischer rats showed obvious differences in the DNA-binding activity of NF-κB and cytokine production. These findings suggested that the difference in PG-PS-induced IBD might have contributed to the differences in the DCs in these two rat strains.

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