Correlation between NF-κB signal pathway-mediated caspase-4 activation and Kawasaki disease

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Abstract. The aim of the study was to investigate the role and mechanisms of action of nuclear factor-κB (NF-κB)-mediated caspase-4 activation in the induction of inflammatory cytokines during Kawasaki disease (KD) and coronary artery endothelial cell injury. Peripheral blood mononuclear cells (PBMCs) were isolated from KD patients and healthy controls and cultured. Double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was applied to detect tumor necrosis factor (TNF)-α levels in activated PBMC-conditioned culture media. To establish a culture model for human coronary artery endothelial cells (HCAECs), we employed KD patient-origin PBMC culture-conditioned media to induce HCAEC transformation and detected the nuclear activation of NF-κB p65 and intracellular caspase-4 protein concentrations using western blot analysis. We also investigated the nuclear transfer of NF-κB p65 using immunofluorescence, as well as HCAEC interleukin (IL)-6 and IL-1β secretion using ELISA. Finally, we investigated HCAEC apoptosis using using Annexin V/PI double staining. After PBMCs were stimulated *in vitro*, TNF-α secretion was significantly higher in the KD group versus controls (P<0.01). HCAEC cells treated with supernatant conditioned by cells from KD patients showed a significant elevation of NF-κB p65 and caspase-4 protein expression versus HCAEC cells treated with supernatant conditioned by control cells (P<0.01). Similarly, IL-6 and IL-1β secretion, as well as apoptotic rate, were significantly elevated (P<0.01). SN50, an NF-κB inhibitor, significantly attenuated caspase-4 expression, secretion of IL-6, IL-1β, and TNF-α, as well as HCAEC apoptosis in cells treated with KD patient PBMC-conditioned media. NF-κB can induce the generation of various inflammatory factors including IL-6 and IL-1β, mediate the expression of caspase-4 in HCAEC cells, and affect apoptosis and injury of HCAEC cells. Therefore, the expression of caspase-4, mediated by NF-κB signal pathway, plays a critical role in KD.

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Introduction

As a type of common acute pediatric vasculitis syndrome, Kawasaki disease (KD), with an increasing prevalence on a year-by-year basis, mainly injures the middle-sized general muscle arteries, and, in some severe cases, can cause various complications, including dilation, stenosis, or even atresia of coronary arteries, as well as coronary artery aneurysms (1-3). Currently, it has been estimated that KD has become a common cause for pediatric acquired heart disease (4). The pathogenesis of KD is very complicated, in which multiple causes and factors are involved, such as immune activation with the participation of mononuclear macrophages, and vascular injury with the participation of nitric oxide (NO) and matrix metalloproteinases (5).

Studies have revealed that nuclear factor-κB (NF-κB), through regulating the expression of inflammatory factors, can play an important role in KD-induced vasculitis (6). Among the caspase family, caspase-1, -4 and -5 are closely associated with inflammatory factors, in which caspase-4 can promote the maturation of inflammatory cytokines through trimming the precursor component from interleukin (IL)-1β (7). Caspase-4, in addition to a correlation with the generation of cytokines, can also participate in cellular apoptosis. It has been shown to be activated in Fas-induced HeLa cell apoptosis (8).

It has found that NF-κB in neuroblastoma mediates the protein expression of caspase-4, through which it can participate in cell apoptosis (9). NF-κB can be activated by various cytokines; most studies have focused on tumor necrosis factor (TNF)-α, a member of the TNF family. Findings of those studies have confirmed that TNF-α can activate NF-κB (10).

In this study, we established a human coronary artery endothelial cell (HCAEC) injury model, aimed to investigate the role of NF-κB signal pathway-mediated caspase-4 expression in coronary artery endothelial cell injury in pediatric KD patients.

Materials and methods

Materials. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-1β, and TNF-α were purchased from Nanjing Jiancheng Bioengineering Co., Ltd. (Nanjing, China). NF-κB p65, caspase-4, and GAPDH primary antibodies, as well as

HRP- and FITC-labeled secondary antibodies were purchased from Wuhan Sanying Biotechnology Co., Ltd. (Wuhan, China). The inhibitor of NF-κB SN50 was purchased from Merck KGaA (Darmstadt, Germany), PMA was purchased from Sigma-Aldrich (St. Louis, MO, USA), while human fibronectin was purchased from Millipore (Billerica, MA, USA). HCAECs and HCAEC cell culture medium was purchased from ScienCell™ Research Laboratories (Carlsbad, CA, USA). Peripheral blood mononuclear cells (PBMCs) were isolated and acquired in this laboratory.

All of the clinical cases were collected from the Xuzhou Children's Hospital, consisting of 15 pediatric patients who had not previously received any KD-specific therapies in the KD group and 15 randomly selected children who received physical examinations at the Pediatric Department. This study was approved by the Ethics Committee of the Xuzhou Children's Hospital. Signed written informed consent was obtained from the parents of the participants before the study.

Isolation and purification of PBMCs. Peripheral venous blood (5 ml) was drawn aseptically from patients in the KD and control groups. Heparin was then added and a Ficoll (Q=11077) density gradient centrifugation was applied, facilitating the separation and isolation of PBMCs. PBMCs were cultured using RPMI-1640 medium containing 10% FBS at 37˚C in an incubator containing 5% CO_2. PBMCs were activated using PMA (10 ng/ml).

ELISA detection of TNF-α levels in activated PBMC conditioned media. PBMCs in the logarithmic phase were inoculated onto a 96-well culture plate at a density of $1x10^5$ cells/well. Culture conditions were as given above. After 24 h of culture, the supernatant was taken for analysis of TNF-α levels. For each experimental group, the cells from six separate wells were used, and the assay was repeated three times. A commercially available ELISA kit was used for detection in accordance with the manufacturer's instructions, and the $OD_{450\ nm}$ was measured in a microplate reader (Victor; Perkin-Elmer, Waltham, MA, USA).

HCAEC cell culture and establishment of injury models. HCAEC cells were regularly inoculated into culture flasks internally coated with human fibronectin containing endothelial cell medium with 10% FBS. Culture flasks were placed into the incubator containing 5% CO_2 for culture at 37˚C, and the culture solution was replaced every day. When cell confluence reached 80-90%, they were digested using 0.25% trypsin for passaging.

Culture supernatant of PBMCs extracted from KD patients was added to HCAEC cells in the logarithmic phase, facilitating the establishment of HCAEC injury models.

Western blot detection of NF-κB p65 and caspase-4 in HCAEC cells. Single-cell suspensions were prepared using HCAECs in the logarithmic phase and added to 6-well plates coated with human fibronectin and divided into 3 groups: Control (HCAEC cells + normal PBMC culture supernatant), model (HCAEC cells + KD patient PBMC culture supernatant), and NF-κB blocking (HCAEC cells + PBMC culture supernatant + SN50 5 ng/ml). After the cells were treated, cell lysis buffer was added for protein extraction according to the manufacturer's instructions for commercially available total protein extraction and nuclear protein extraction kits. Protein concentration was assayed using the BCA method, followed by gel electrophoresis, membrane transfer, and blocking using 5% BSA. Then antibodies for NF-κB p65, caspase-4, and GAPDH were added onto the membrane for incubation overnight at 4˚C. The membrane was then washed using TBST and incubated using HRP-labeled secondary antibody for 2 h. Color development was carried out in the dark using ECL and the results were scanned. GAPDH served as the internal loading reference.

Table I. TNF-α concentrations in PBMC-conditioned supernatant (mean ± SD, n=15).

Groups	TNF-α (ng/ml)
Control	95±23
KD	486±46[a]

[a]P<0.01 vs. control group. TNF-α, tumor necrosis factor-α; PBMC, peripheral blood mononuclear cell; KD, Kawasaki disease.

Immunofluorescence detection of NF-κB p65 nuclear transfer in HCAEC cells. Cells, after being treated in accordance to the protocol (see Material and methods), were fixed using formaldehyde (4%), permeabilized using Triton X-100 (0.2%), and blocked using BSA (2%). Then rabbit monoclonal NF-κB p65 antibody (dilution, 1:50; cat. no. ab32536; Abcam, Cambridge, MA, USA) was added into the cells for incubation overnight at 4˚C. The cells were washed using PBS 3 times, followed by 1 h of incubation using goat anti-rabbit FITC-IgG secondary antibody (dilution, 1:100; cat.no. ab7086; Abcam) at room temperature in the dark. Finally, the cells were incubated with DAPI (5 μg/ml) for 5 min, and, after a PBS rinse, were observed using a laser scanning confocal microscope.

ELISA detection of IL-6 and IL-1β levels in HCAEC culture supernatant. Following treatment of different groups in accordance to the protocol, the cells were incubated for 24 h. IL-6 and IL-1β levels in the conditioned supernatant were detected using the double antibody sandwich ELISA method, following the manufacturer's instructions.

Detection of the apoptotic rate of HCAEC cells using AV/PI double staining method. Following treatment of different groups in accordance to the protocol, the cells were incubated for 24 h, rinsed in PBS 3 times, digested using trypsin, and collected for staining. Collected cells were re-suspended in 0.3 ml binding buffer, to which 5 μl Annexin V and 5 μl PI were added. After 15-min incubation at room temperature, an additional 0.2 ml binding buffer was added, followed by the automatic detection procedure.

Statistical analysis. Data were presented as mean ± standard deviation (mean ± SD). SPSS 17.0 software (Chicago, IL, USA) was used to process data and single-factor ANOVA was carried out. $P \leq 0.05$ indicates statistically significant difference.

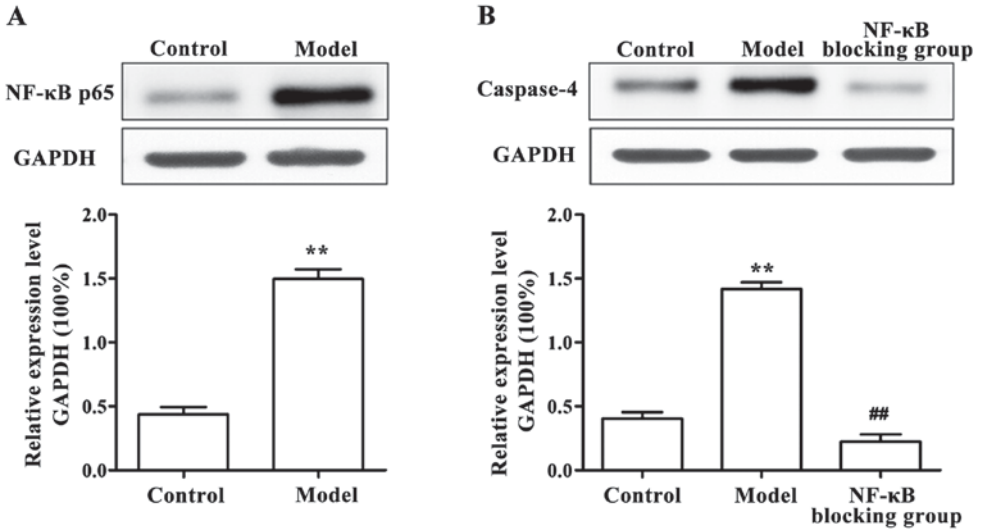

Figure 1. Nuclear factor-κB (NF-κB) p65 and caspase-4 protein expression in human coronary artery endothelial cells (HCAECs). **P<0.001 and $^{\#\#}$P<0.01.

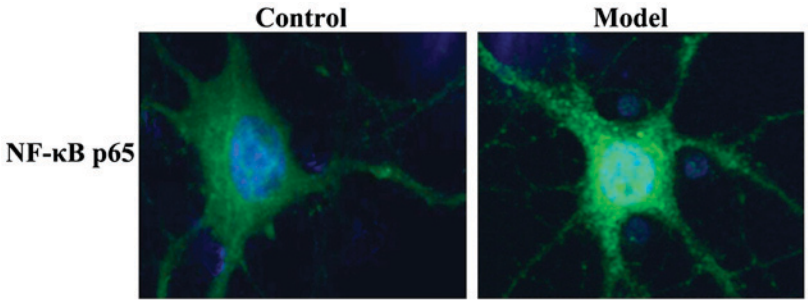

Figure 2. Nuclear factor-κB (NF-κB) p65 in human coronary artery endothelial cells (HCAECs) using immunofluorescence method (magnification, x800).

Table II. HCAEC culture supernatant levels of IL-6 and IL-1β (mean ± SD, n=6).

Groups	IL-6 (pg/ml)	IL-1β (ng/ml)
Control	205±53	1,692±225
Model	528±89[a]	3,967±472[a]
NF-κB blocking	182±37[b]	1,138±105[b]

[a]P<0.01 vs. control group; [b]P<0.01 vs. model group. HCAEC, human coronary artery endothelial cell; IL, interleukin; NF-κB, nuclear factor-κB.

Table III. HCAEC apoptosis induced by KD patient-extracted PBMC-conditioned supernatant (mean ± SD, n=3).

Groups	Apoptotic rate (%)
Control	3.6±0.4
Model	42.7±4.9[a]
NF-κB blocking	7.4±0.6[b]

[a]P<0.01 vs. control group; [b]P<0.01 vs. model group. HCAEC, human coronary artery endothelial cell; KD, Kawasaki disease; PBMC, peripheral blood mononuclear cell; NF-κB, nuclear factor-κB.

Results

Secretion of TNF-α in activated PBMC-conditioned supernatant. ELISA detection results for TNF-α in the PBMC culture supernatant are shown in Table I. We found that TNF-α levels were significantly higher in supernatant conditioned by KD group PBMCs compared to the control after *in vitro* stimulation (P<0.01).

NF-κB p65 and caspase-4 expression in HCAECs. Western blot analysis and immunofluorescence were, respectively, applied to measure nuclear NF-κB p65 and cellular caspase-4 levels in HCAECs. Nuclear protein expression of NF-κB p65 in HCAECs stimulated by KD patient-extracted PBMC-conditioned supernatant was significantly higher than in HCAECs stimulated by control PBMC-conditioned supernatant, indicating the presence of nuclear transfer (Figs. 1A and 2). The cell expression of caspase-4 in HCAECs, stimulated KD patient-extracted PBMC-conditioned supernatant, was significantly inhibited by the NF-κB inhibitor SN50 (Fig. 1B).

IL-6 and IL-1β levels in HCAEC culture supernatant. We detected IL-6 and IL-1β levels in HCAEC culture supernatant using ELISA (Table II). IL-6 and IL-1β levels in HCAECs treated with KD patient-extracted PBMC-conditioned supernatant were significantly higher than those treated with control

PBMC-conditioned supernatant. This phenomenon was significantly inhibited using the NF-κB inhibitor SN50.

Apoptosis in HCAEC cells induced by KD patient-extracted PBMC-conditioned supernatant. Control HCAECs experienced good growth, with apoptotic cells only accounting for 3.6±0.4% of cells after 24 h. However, after the addition of KD patient-extracted PBMC-conditioned supernatant, the proportion of apoptotic cells increased to 42.7±4.9%. This phenomenon was attenuated by addition of the NF-κB inhibitor SN50 (Table III).

Discussion

Activation of NF-κB can regulate the expression of various inflammatory factors, growth factors, and adhesion molecules, and participate in inflammatory processes, immunologic reactions and cell apoptosis (11). Research has revealed that activation of NF-κB is critical in the pathological development of vasculitis during KD through regulation of inflammatory factor expression (12,13).

It has been confirmed that NF-κB is activated in $CD14^+$ mononuclear/macrophages and $CD3^+$ T cells in the peripheral blood of pediatric acute phase KD patients. Intravenous infusion of immunoglobulin, due to its inhibitory effect on the activation of NF-κB, has now become the preferred therapeutic procedure for KD (14).

Within the caspase family, caspase-1, -4 and -5 have all been correlated with inflammation (15). In particular, caspase-4, located on the outer membrane of the endoplasmic reticulum, has been found to be involved in stress-related apoptosis of the endoplasmic reticulum (16-18). In addition, some studies also indicated that caspase-4 plays an important role in the TRAIL-induced apoptosis (19,20).

In this study, we found that levels of TNF-α were elevated in KD patient-extracted PBMC-conditioned supernatants. We established an HCAEC injury model using KD patient-extracted PBMC-conditioned supernatant, and confirmed that nuclear NF-κB p65 and cellular caspase-4 protein expression, IL-6 and IL-1β levels, and apoptosis were elevated in HCAECs treated with KD patient-extracted PBMC-conditioned supernatant versus controls. The phenomena were attenuated by the addition of the NF-κB inhibitor SN50. Therefore, activated NF-κB can mediate caspase-4 expression, which participates in a series of inflammatory reactions and apoptotic processes culminating in HCAEC injury. This study established a critical role for NF-κB-mediated caspase-4 activation in KD.

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