Analytical Biochemistry 557 (2018) 69–76

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Development and characterization of stable reporter cells for fast and sensitive detection of pyrogen

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ARTICLE INFO

Keywords:
Pyrogen
Endotoxin
Zymosan
NF-κB
TLR4
TLR2

ABSTRACT

Pyrogens are a class of heterogeneous compounds that cause fever and induce inflammatory responses in the host. Lipopolysaccharides (LPS, also known as endotoxin) is the major pyrogen in the category of drug quality control. Accurate and fast quantification of pyrogens is crucial for drug safety. In the present study, we aimed to develop a sensitive and reliable method for rapid detection of pyrogens using luciferase reporter assay. Stable human A549 luciferase reporter cells were constructed under the control of a NF-κB-responsive element or IFN-β promoter. Our results showed that several monoclonal stable cell clones responded to 0.1 EU/ml endotoxin, which was less than human fever threshold at 0.3 EU/ml of endotoxin. Further, compared with original A549 cells, TLR4 expression on the reporter cells were significantly increased after low amount LPS stimulation. In addition, reporter cells also responded to zymosan stimulation. Therefore, these results indicated that the stable luciferase reporter cells respond to endotoxin and non-endotoxin pyrogens and have the potential to further develop into a sensitive and fast pyrogen evaluation method.

Introduction

Contamination of medicines and medical devices with pyrogens is the most common cause of systemic inflammation and, in worst cases, of septic shock [1,2]. Most known pyrogens are of microbial origin, including bacteria, yeasts, fungi, viruses or their components and environmental particles [3]. The best studied are components of bacterial and yeast cell walls, such as lipopolysaccharides (LPS, also known as endotoxin) of Gram-negative bacteria, or lipoteichoic acid (LTA) and peptidoglycan (PGN) of Gram-positive bacteria [3,4], or zymosan of yeast [5], which stimulate host leukocytes to release inflammatory cytokines and lead to severe reactions such as multiple organ failure, septic shock, and even death [4,6,7]. Therefore, pyrogen detection is a prerequisite for pharmaceutical products. Currently, several different methods for detection of pyrogens are available, the rabbit pyrogen test (RPT), a bacterial endotoxin test (BET, also called the limulus amebocyte lysate assay (LAL), only can be used to detect endotoxin pyrogens) and monocyte activation test (MAT) [4].

The rabbit pyrogen test (RPT), a golden standard for pyrogen detection, is based on the rise of temperature in rabbits after intravenously injection of a testing solution or implantation of alternatively the medical device [4,8]. At the beginning of the 20th century, RPT was introduced to detect various types of contaminants [1] because rabbits' sensitivity to pyrogen is similar to that of humans [9]. However, the sensitivity of rabbits is influenced by many factors, such as the strain used, the age and gender of the rabbit, and housing conditions [4]. Furthermore, the RPT is costly and time-consuming. In addition, the RPT is only qualitative rather than quantitative [6].

An established in vitro quality control was the bacterial endotoxin test (BET), which detects endotoxins from Gram-negative bacteria based on the coagulation enzymatic cascade reaction in the horseshoe crab amebocyte lysate, and therefore it is also called the Limulus Amebocyte Lysate (LAL) test [10]. Compared with RPT, the LAL test can perform the semi-quantitative or quantitative measurement of endotoxins, even at femtogramme levels, which is a great development as it is possible for the first time to quantify endotoxin. However, LAL

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https://doi.org/10.1016/j.ab.2018.07.016
Received 8 February 2018; Received in revised form 18 June 2018; Accepted 17 July 2018
Available online 18 July 2018
0003-2697/

results are affected by protease. LAL also has shown reactivity to some polymeric forms of glucose, for example, β-(1,3) -D-glucan, existing in the cells of fungi, algae, and yeast. Although β-(1,3) -D-glucan is not pyrogenic, it can trigger the coagulation cascade, interfering with the LAL response to LPS [8,11]. Moreover, this test reacts extremely differently to LPS in comparison to the human immune responses [6].

Recently, the monocyte activation test (MAT), an alternative method to the RPT or LAL test, has been developed [12]. The MAT is based on the activation of human monocytes by pyrogens and on the measurement of cytokine release, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) by the Enzyme-Linked Immunosorbent Assay (ELISA), and which is similar to human fever reaction [2]. Currently, the MAT is able to detect all types of pyrogens and is also more accurate, time-efficient than other methods [13]. However, in addition to economic considerations, it may still be a challenge to produce a large quantity of pooled human whole blood with uniform quality for use in the MAT because of differences in lifestyle and genetic background between donors [12]. Therefore, it is an urgent need to seeking novel pyrogen test methods.

Endotoxins are among the most potent bacterial inducers of cytokines [14]. During infectious processes, pyrogens stimulate human monocytes to synthesize or release inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) [14–16]. Nuclear factor-kappa B (NF-κB) is a major transcription factor that regulates genes encoding these proinflammatory cytokines [15,17]. Moreover, evidences have demonstrated that blocking NF-κB activation may be an effective strategy in fever therapy [15]. Meanwhile, human beta interferon (IFN-β) dramatically augments the febrile response to endotoxin in normal rabbits by the enhancement of TNF-dependent TNF production [18–20], and restores the normal febrile response to endotoxin in pryogen-tolerant rabbits [21–23]. Therefore, detection of the activation of NF-κB or type I IFN mimics the physiological response to pathogens at an upstream step, respect to the production of cytokines. Recently, human leukemia cells with a luciferase reporter gene under the control of a NF-κB responsive element has shown potential suitability for pyrogen determination [1].

Previous reports have shown that bacterial flagellin can stimulate lung epithelial cells to produce cytokine IL-8 through Toll-like receptor TLR5 [24,25]. However, how other pyrogens induce activation of lung epithelial cells has not been fully clarified. In the present study, we aimed to develop a stable and sensitive reporter cell-based assay for fast detection of pyrogens by using human alveolar epithelial cell line A549 cells.

Materials and methods

Cell lines and cell culture

A549, 293 T, HeLa, and THP-1 (ATCC® number: CCL-185™, CRL-3216™, CCL-2™, TIB-202™, respectively. A549 and HeLa cells were purchased from Peking Union Medical College Hospital, 293 T and THP-1 cells were obtained from Dr. Luis Sigal, Thomas Jefferson University) cells were grown at 37 °C with 5% CO_2, using complete DMEM or 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 1 × penicillin or streptomycin, 1 M β-mercaptoethanol.

Transfection and dual-luciferase reporter gene assay

A549, 293 T and HeLa cells were seeded on a 48-well plate and cultured for 24 h (hs). Then, the cells were transiently transfected with 300 ng/well pNF-κB-Luc (Beyotime Biotechnology) or pGL3-IFN-β-Luc (from Dr. HongbingShu, Wuhan University) together with 10 ng/well pRL-TK vector (Promega, USA) using serum- and antibiotics-free DMEM with 1 mg/ml polyetherimide reagent (0.5μL/well) for 3 h s and then cultured with complete DMEM medium. Next day, the cells were stimulated with different doses of LPS for 6 h s and lysed according to the manufacturer's instructions, and the plate was read for luminescence in a plate luminometer (Promega, USA). The relative activity of the NF-κB/IFN-β was determined by the ratio of firefly luciferase activity to Renilla luciferase activity.

Lentiviral vector construct and selection of stable monoclonal cell clones

Lentiviral vector pCDH-CMV-MCS-EF1-Puro (Cat. Number CD510B-1, System Biosciences) was used to construct the reporter gene. The CMV coding sequence was excised with enzymes and replaced with the firefly luciferase coding sequence, driven by NF-κB binding site or IFN-β promoter. The constructed lentiviral virus plasmids (pCDH-Puro-NF-κB or pCDH-Puro-IFN-β) were sequenced and transfected together with packaging plasmids into 293 T cells.

Collected lentiviral particles were added to A549 cells along with 10 μg/ml polybrene. 48 h s after infection, puromycin was added to the cell culture to select the resistant cells. Monoclonal cell clones were obtained by single cell sorting with Flow Cytometry of the resistant cells. Most selected monoclonal cell clones were stable, exhibited similar performance after at least 20 generation of passages.

Stimulation with pyrogens

The LPS (Sigma-Aldrich, from *E. coli* serotype 0111:B4) was used as ng/ml. The concentration of LPS means the final concentration gained by adding stock solution of LPS to the cells. Standard endotoxin (RSE) (10EU/ml from *Escherichia coli* serotype 0111:B4 was purchased from National Institute for the Control of Pharmaceutical and Biological Products, China) was used as EU/ml. Zymosan (Sigma-Aldrich, number:Z4250 from *Saccharomyces cerevisiae*) was used as μg/ml.

Pyrogen treatments were performed as indicated for each experiment with the time and doses. The indicated cells were washed with PBS, and replaced with different diluted concentrations of pyrogens solutions. After stimulation, the cells were lysed and performed luciferase assay.

High throughput single luciferase assay

All sorted monoclonal A549 cell clones were seeded at 2×10^5 cells/ml on a 96-well plate and cultured for 24 h s. The cells were then stimulated with LPS (RSE) or zymosan. After 6 h s stimulation, the cells were lysed with lysis buffer (Promega) and then the supernatant was transferred to a 96-well microplate read for luminescence in a Glomax 96-well luminometer (Promega, USA) according to the manufacturer's instructions. Finally, the activity of the NF-κB/IFN-β promoter is determined by the firefly luciferase value.

Flow cytometry

The indicated cell clones were respectively stained with human anti-TLR4 (clone: HTA125, eBioscience™), or anti-TLR2 (clone:W15145C, Biolegend), or an isotype antibody conjugated with phycoerythrin or APC on 4 °C for 30 min. After incubation, the cells were washed three times with PBS containing 2% FBS, and fixed with 0.5% paraformaldehyde at 4 °C for 15 min or overnight. The expression of TLR4 or TLR2 was measured using a flow cytometer (FACS Calibur) with CellQuest software (BD Biosciences, San Jose, CA, USA). Flow cytometric data were analyzed by using FlowJo software (TreeStar, San Carlos, CA, USA).

LAL assay

Quantification of endotoxin content was determined by LAL assay using different concentrations of Limulus Amebocyte Lysate (National Institute for the Control of Pharmaceutical and Biological Products,