Herpes simplex virus–1 infection or Simian virus 40–mediated immortalization of corneal cells causes permanent translocation of NLRP3 to the nuclei

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Abstract

- AIM: To investigate into the potential involvement of pyrin containing 3 gene (NLRP3), a member of the nucleotide–binding oligomerization domain–like receptors with cytosolic pattern recognition, in the host defense of corneas against viruses.

- METHODS: The herpes viral keratitis model was utilized in BALB/c mice with inoculation of herpes simplex virus–1 (HSV–1). Corneal tissues removed during therapy of patients with viral keratitis as well as a Simian vacuolating virus 40 (SV40)–immortalized human corneal epithelial cell line were also examined. Immunohistochemistry was used to detect NLRP3 in these subjects, focusing on their distribution in tissue or cells. Western blot was used to measure the level of NLRP3 and another two related molecules in NLRP3 inflammasome, namely caspase–1 and IL–1β.

- RESULTS: The NLRP3 activation induced by HSV–1 infection in corneas was accompanied with redistribution of NLRP3 from the cytoplasm to the nucleus in both murine and human corneal epithelial cells. Furthermore, in the SV40–immortalized human corneal epithelial cells, NLRP3 was exclusively located in the nucleus, and treatment of the cells with high concentration of extracellular potassium (known as an inhibitor of NLRP3 activation) effectively drove NLRP3 back to the cytoplasm as reflected by both immunohistochemistry and Western blot.

- CONCLUSION: It is proposed that herpes virus infection activates and causes redistribution of NLRP3 to nuclei. Whether this NLRP3 translocation occurs with other viral infections and in other cell types merit further study.

- KEYWORDS: pyrin containing 3 gene; inflammasome; translocation; herpes simplex virus–1; keratitis; human corneal epithelial cell; Simian vacuolating virus 40; immortalization

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INTRODUCTION

During the last two decades, the field of knowledge concerning recognition of, and response to, antigenic stimuli by the immune system has quickly expanded. Specifically, as the immune system's first-line response to pathogens, the innate immune compartment recognizes various pathogen-associated molecular patterns or danger-associated molecular patterns. Among the pattern-recognition receptors in the host cells, the most-studied membrane-bound receptors to date have been the Toll-like receptors, and the most-studied cytoplasmic receptors have been the nucleotide-binding oligomerization domain-like receptors (NLRs). NLRs are comprised of 22 members of the protein family in humans and 34 members in mice; of these, nucleotide-binding domain, leucine rich family, pyrin containing 3 gene (NLRP3) is most extensively characterized. NLRP3 inflammasome activation is observed in infections caused by fungi, bacteria, viruses, or microbial components. Specifically, NLRP3 is highly expressed in myeloid immune cells such as monocytes, macrophages, and neutrophils. When the cells are activated by diverse stimuli,
including pathogen infection and endogenous damage, NLRP3 proteins polymerize, leading to the formation of large inflammosomes that bind to the apoptosis speck protein adaptor and promote the recruitment of procaspase-1, which in turn induces the maturation of interleukin IL-1β and IL-18[8]. Accommodation of the cellular metabolism is necessary to activate the NLRP3 inflammasome, and potassium efflux and reactive oxygen species production is a requirement for activation of the NLRP3 pathway, hence blocking potassium efflux by providing a high concentration of potassium to the cells effectively blocks inflammation [4,8,10]. While many related studies detected the expression level of NLRP3 expression by using Western blot to measure NLRP3 protein or using polymerase chain reaction (PCR) for NLRP3 messengers, only several of them addressed the location of NLRP3 in the studied cells. In specific, most studies detailing the location of NLRP3 showed that this molecule resides and transports in cytoplasm as exemplified by a recent paper [12]. Recently, Ichinohe team reported that, upon infection with encephalomyocarditis virus or, NLRP3 in bone-marrow derived macrophages translocated to perinuclear spaces [13]. Transfection of cells with virus encoding the structure protein 2B of encephalomyocarditis virus or other picornaviruses, poliovirus, enterovirus 71, measles virus, or influenza virus M2 protein all caused similar NLRP3 redistribution [13,14]. This study was carried out to detect whether the NLRP3 pathway is involved in the pathogenesis of corneal infection with herpes simplex virus (HSV)-1.

SUBJECTS AND METHODS

Human Tissues, Cells, and HSV−1 Strain Use of human corneal tissues was approved by the institution review board and the Helsinki Tenet was observed. Diseased corneal buttons were removed from patients during therapeutic surgery, and normal buttons were from healthy eyes enucleated for other reasons. SV40-T Ag-immortalized human corneal epithelial cell (HCEC) line (ATCC CRL-11135) was cultured in Dulbecco’s modified Eagle medium (DMEM)/F12 medium with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C. The HSV-1 McKrae strain was multiplied in Vero cells and freshly prepared while adhering to our institution’s biosafety code [15]. The viral preparation was titrated in a routine fashion and adjusted to 3×10⁵ pfu/mL in DMEM.

Cytoplasmic and Nuclear Protein Extraction from Human Corneal Epithelial Cells About ten million HCECs were seeded in 6-well plates and allowed to grow to exponential phase. Potassium chloride was then added to 130 mmol/L KCl in the medium for 6h before harvest [16]. Untreated cells were used as the control. The cells were washed with phosphate-buffered saline (PBS) and harvested into a 1.5-mL microcentrifuge tube. After centrifugation at 500 g for 3 min, the supernatant was removed and the cytoplasmic and nuclear protein extractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA) following the instructions of the manufacturer. The cytoplasmic and nuclear extracts were recovered and used for immunoblotting as described below.

Animal Model of HSV−1 Keratitis Male, 8-10-week-old BALB/c mice were purchased from the Academy of Military Medical Sciences (Beijing, China). All animal experimental protocols adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institution in accordance with the Guidelines on the Humane Treatment of Laboratory Animals (Chinese Ministry of Science and Technology, 2006). Only the left corneas were used for manipulation; the right eyes were used as untreated controls. Mice were anesthetized by intraperitoneal injection with ketamine (50 mg/kg) and chlorpromazine hydrochloride (10 mg/kg). Under anesthesia with topical application of 0.5% proparacaine hydrochloride (Alcon-Couvreur, Puurs, Belgium), each cornea was pierced near the center with a 30-gauge needle to the depth of the stroma. A 33-gauge needle (Hamilton, Reno, NV, USA) was used to inject 1 μL of HSV-1 suspension into the center of the cornea. In the control arm of the study, buffer was injected in place of the HSV-1 preparation. At day 7 post-infection, the animals were sacrificed under anesthesia and the central part of corneas were excised using a 2-mm-diameter trephine, and the corneal buttons were either frozen in optimal cutting temperature (OCT) compound for immunofluorescence (as below), or minced for whole-tissue protein extraction for immunoblotting. For the latter purpose, the buttons were homogenized in protein lysates with 1% protease inhibitor by a glass pestle in a 1.5-mL microcentrifuge tube, sonicated for 30s, and cleared by centrifugation at 12,000 g for 10 min.

Western Blot Expression of NLRP3 in HCECs and of several other proteins in infected corneas were measured using immunoblotting. In brief, protein extracts from HCECs or corneal buttons were loaded onto and resolved in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. After blotting onto polyvinylidene difluoride membranes, the membranes were blocked with 5% dry milk in Tris-buffered saline containing 0.1% Tween-20 for 1h, and then incubated overnight with primary antibodies (2 μg/mL in blocking buffer at 4°C). After wash with gentle agitation for 30 min, the blots were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies at room temperature for 1h. After extensive washing, hybridized bands were detected with an enhanced chemiluminescence kit (Perkin Elmer Life Sciences, Boston, MA, USA) as recommended by the manufacturer. The primary antibodies used for immunoblotting
were anti-NLRP3 antibody (ab91525); anti-caspase-1 antibody (ab17820), and anti-IL-1β antibody (ab9722), all from Abcam (Cambridge Science Park, Cambridge, UK). The secondary antibodies were peroxidase-conjugated affinipure goat anti-rabbit IgG antibody from ZSGB-BIO Biosciences (Beijing, China) and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) from Kangchen (Shanghai, China).

Immunofluorescence Location of NLRP3 in HCECs or murine corneas was monitored using the immunofluorescence method. For HCECs, about 1 x 10^6 cells/mL were grown in 96-well plates and treated with 130 mmol/L KCl for about 6h as detailed above. Untreated cells were used as controls. The cells were fixed with 5% paraformaldehyde for 5min, washed twice with PBS, and permeabilized with 1% Triton X-100 in PBS. After blocking with 1% fetal bovine serum in PBS for 1h, the cells were incubated with anti-NLRP3 antibody for 1h at room temperature. Cells were washed 3 times, and fluorescein isothiocyanate- (FITC)-conjugated bovine anti-rabbit IgG was added at room temperature for 2h. After 3 washes with PBS, propidium iodide (PI) was added to 2 μg/mL for 5min. Cells were observed with a C1-plus confocal microscope (Nikon, Japan). For immunofluorescence assay in corneas, frozen corneal buttons were cut into continuous 6-μm sections and mounted on poly-L-lysine-coated slides (Polysciences, Warrington, PA, USA). After air drying, the sections were fixed in cold acetone for 10min and then stained for NLRP3 as described above.

RESULTS

HSV-1 Activates the NLRP3 Pathway in Murine Corneas As a typical immune-mediated disease, the pathogenesis of HSV-1 keratitis has been studied extensively, especially from the perspective of initiation of innate or acquired immune responses [17]. When we looked at the newly identified NLRP3 inflammasome pathway in the murine models of HSV-1 keratitis, it was found that the NLRP3, caspase-1, and IL-1β expression were increased in the corneal homogenates as measured by Western blot at day 7 after infection (Figure 1A). However, immunofluorescence staining for NLRP3 in the corneas revealed obvious redistribution of NLRP3 in the infected corneal epithelial cells. In detail, NLRP3 protein is exclusively expressed in the cytoplasm of normal corneal epithelial cells and not in the nucleus. Upon the development of keratitis, NLRP3 was partially redistributed to the nucleus of corneal epithelial cells at day 7 (Figure 1B), a time point at which typical keratitis is present (not shown). On the contrary, one day after acute infection, NLRP3 location was not significantly changed when compared with controls.

Our next step was to determine translocation of NLRP3 from the cytoplasm to the nucleus also occurred in human corneas with HSV-1 infection. As with the murine case previously described, NLRP3 protein was expressed mainly in cytoplasm of epithelial cells of normal donor corneas. In contrast, NLRP3 protein density in corneal buttons from patients with acute HSV-1 keratitis was much greater in the nuclei of cells as compared to the cytoplasm (Figure 1C), highly suggestive of translocation of NLRP3 in the corneal epithelial cells of the infected human corneas.
Figure 2 Reverse translocation of NLRP3 from nucleus to cytoplasm in SV40–immortalized human corneal epithelium cells treated with potassium. HCEC were cultured in medium supplemented with 130 mmol/L KCl for 6 h and then stained for NLRP3 (green) with PI counterstain (red) (A). Scale bars: 50 μm. Alternatively, after culture the cells were harvested and proteins were prepared from either whole cells or from cytoplasm and the nucleus separately; the contents of NLRP3 in each preparation were analyzed by Western blot analysis, with GAPDH as internal control (B). PI: Propidium iodide.

Location of NLRP3 Protein in SV40 Immortalized Human Corneal Epithelial Cells

Our above findings with murine and human corneas showed that NLRP3 resides in cytoplasm of normal epithelial cells but translocates to the nucleus at certain stages upon HSV-1 infection. This was unique because few of the dozens of studies concerning NLRP3 addressed the location of NLRP3. It was thus plausible that the nuclear translocation of NLRP3 was due to viral DNA integration rather than to just activation of signaling pathways by external stimuli. To verify this possibility, we monitored the location of NLRP3 in the SV40 large T antigen-transfected HCEC using both the immunofluorescence and Western blot tests. Results showed that NLRP3 proteins in HCEC were mainly present in the nuclei; only a small percentage of cells also stained for NLRP3 in cytoplasm area (Figure 2A), and the NLRP3 level in cytoplasm was undetectable in untreated HCEC (Figure 2B). It is known that the efflux of cellular potassium is a requirement for activation of NLRP3 inflammasome, and that elevating potassium concentration in a cellular environment will block NLRP3 activation. To check whether the location of NLRP3 in the nucleus in HCEC was due to consistent activation of this pathway, KCl was added to culture media to 130 mmol/L, a concentration shown to block NLRP3 activation in previous studies [16]. After 6 h of culture, some NLRP3 was found to return from the nucleus to the cytoplasm (Figure 2A). Correspondingly, the Western blot assay demonstrated that KCl treatment significantly decreased the amount of NLRP3 in total cells or in nuclei, but increased the amount of NLRP3 in cytoplasm compared to control (Figure 2B).

DISCUSSION

Beside NLRP3, absent in melanoma 2 (AIM2) and interferon gamma-inducible protein 16 (IFI16) of PYHIN (i.e. PYrin and haematopoietic interferon-inducible nuclear antigens with 200 amino-acid repeats domain-containing protein) family are another two important molecules that detect viral DNA and initiate cellular response via inflammasome formation [1, 18-20]. While AIM2 and NLRP3 exist in cytoplasm, the IFI16-inflammasome mechanism functions both in cytoplasm and in nucleus [21-23]. Previous to the current study and by using a human skin transplantation model in severe
combined immunodeficiency mice, Nour et al. [20] showed that NLRP3 could be detected in the nucleus as well as in cytoplasm at day 7 of infection with alpha herpes virus. On the contrary, all studies that claimed cytoplasmic distribution for NLRP3-inflammasome were carried out in early phase (that is, in hours) after the infection. Without much experiment-based explanation for this novel finding, we propose that prolonged stimulation of cells by viral component, either in the HSV-1 keratitis model (7d) or in the immortalized corneal cells, alters the overall status of NLRP3 compartment concerning redistribution to nuclear. Thus during viral latency in host cells or in viral-immortalized cells, NLRP3 might be trapped in the nuclei, and when a high level of extracellular potassium (i.e. the NLRP3-inflammasome inhibitor) is present, NLRP3 is released into cytoplasm (Figure 2).

One unfortunate limitation of this study is that we did not check other molecules involved in the NLRP3-inflammasome pathway or other inflammasome pathways like AIM2- or IFI16-inflammasome, thus making it impossible to provide a plausible explanation on how NLRP3 was activated in cytoplasm, prompted to nuclear, and trapped there. Future studies should measure other components relating to or paralleling with NLRP3-inflammasome pathway so that an overall view of inflammasome networking should be built in the context of constant viral infection. While this study was in progress, Chandran team reported their findings that help to understand our work. In brief, differential activation of inflammasome pathways depends on the species of virus, so that IFI16-inflammasome and not AIM2-inflammasome or NLRP3-inflammasome was activated by KSHV infection, while HSV-1 activated both IFI16- and NLRP3-inflammasomes [23,26]. Furthermore, IFI16- or NLRP3-inflammasome activation was self-limited in HSV-1 infection but not in KSHV infection model [30]. Again, like other investigators, Chandran and colleagues only looked at distribution of NLRP3 in cells at 4 and 8h after HSV-1 infection and did not catch toward-nuclear relocation of NLRP3, but they were able to monitor persistent activation and formation of IFI16-inflammasome in nuclear during Epstein-Barr virus latency in B and epithelial cells in another study [26,27].

The findings described in this study have some implications. Most significantly, immortalization of primary cells for long-term and easy maintenance has been a well-accepted practice, and quite a few SV40-immortalized or other virus-immortalized epithelial cell lines from various tissues had been established in attempts to solve inflammation mediation/immune system questions. The validity of this common practice was challenged by current study. Since the change of NLRP3 location in the cells caused by viral immortalization would inevitably alter the biological processes related to the function of this molecule, great caution should be taken when trying to apply the observations obtained using such cell lines to situations in normal cells, especially when the status of the NLRP3-inflammasome pathway is likely to be involved. For example, it has been reported that primary and SV40 immortalized human corneal epithelial cells manifest differential responses to IL1 treatment [20]. We hypothesize that the differential status of NLRP3 in these two types of corneal epithelial cells is at least partially responsible for their differential responses to external stimuli.

In summary, though lacking extensive mechanistic studies, the findings in HSV-1 infected murine or human corneas and in SV40-immortalized corneal epithelial cells imply that viral infection of corneas results in nuclear translocation of NLRP3 in these cells. Similar translocation of NLRP3 has not been often noted in other cell types or with infection by other pathogens. Besides, evidence are coming out to show that NLRP3 is involved in non-infectious processes as well, such as ischaemia-reperfusion injury, atherosclerotic lesions, cancer, diabetes, nerve disorders [29,30], etc. Thus more effort is desirable to investigate into the universal significance of this trafficking of NLRP3 in cells or tissues in healthy and disease conditions.

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