RESEARCH ARTICLE

Caveolin-1, Through its Ability to Negatively Regulate TLR4, is a Crucial Determinant of MAPK Activation in LPS-challenged Mammary Epithelial Cells

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Abstract

Background: To explore the role of caveolin-1(CAV-1) gene silencing on MAPK activation in lipopolysaccharide (LPS)-challenged human mammary epithelial cells. Methods: We established a MCF-10ACE of CAV-1 gene silencing from human mammary epithelial cell line MCF-10A by RNAi technology. DNA Microarray were used to detect the expression of inflammation-associated genes in MCF10ACE. Western blotting was used to examine the activation of MAPK in lipopolysaccharide(LPS)-challenged MCF-10A and MCF-10ACE. Moreover, immunofluorescence and Western bloting were performed to detect the co-localization of CAV-1 and toll-like receptor 4 (TLR4) in human mammary epithelial cells. Results: MCF-10ACE exhibited significant increases in inflammation-associated gene expression, especially IL-6 (~7-fold) and IL6R (~17-fold). In addition, LPS-induced p38 MAPK and JNK MAPK activation was significantly increased in MCF-10ACE. Furthermore, CAV-1 co-localized with TLR4 and appeared a negative correlation trend. Conclusion: CAV-1 gene silencing promotes MAPK activation via TLR4 signaling in human mammary epithelial cells response to LPS.

Keywords: CAV-1 - LPS-MAPK pathway - TLR4 - mastitis - breast cancer

Asian Pacific J Cancer Prev, **14 (4)**, 2295-2299

Introduction

Mastitis is an inflammation of the mammary gland commonly caused by bacterial infection (Aitken et al., 2011). Mammary epithelial cells mount defense against invading pathogens by detecting their respective danger signals or ligands and initiating appropriate immune responses (Ibeagha-Awemu et al., 2008). Caveolaes are a subset of lipid rafts that are rich in glycol-phospholipide and cholesterol, mediate nonclathrin-dependent endocytosis, and regulate the internalization of particles such as bacteria (Bastiani et al., 2010). CAV-1, component of caveolae membranes, may function as a tumor suppressor that exert functions including the regulation of vesicular transport, cholesterol homeostasis, proliferation, and apoptosis (Nidda et al., 2010; Nidda et al., 2010; Jin et al., 2011).

CAV-1 has also been implicated as a modulator of innate immunity and inflammation. Some studies reported that it regulates LPS-induced cytokines production by involving the MKK3/p38MAPK pathway (Wang et al., 2006) and eNOS-derived NO production to activate NF-κB pathway (Garrean et al., 2011). In atherogenesis, it could promote monocyte to macrophage differentiation through the regulation of EGR-1 transcriptional activity (Fu et al., 2012). What's more, it also affects STAT5 and Akt activity in host immunity against Klebsiella pneumonia (Guo et al., 2012). However, whether CAV-1 is a positive adaptation by mammary epithelial cells to effectively respond to mastitis pathogens is largely still unknown.

Toll-like receptors(TLRs), play a central role in the regulation of the host immune system and each TLR recognizes specific pathogen-associated molecular patterns. TLR4 is one of the well characterized pathogen recognition receptors that recognizes the LPS of Gram-negative bacteria (Lu et al., 2008).

Recently, more and more studies have shown that CAV-1/TLR4 interaction plays a vital role in inflammation response. Some researches reported that CAV-1 deficiency dampens TLR4 signaling through eNOS activation (Mirza et al., 2010), while others showed it attenuates TLR4 expression and NF-κB activation to produce inflammatory cytokines (Tsai et al., 2011). Wang et al., also found that HO/CO pathway suppresses TLR4 signaling by regulating CAV-1/TLR4 interaction to reveal an anti-inflammatory effect (Wang et al., 2009).

The aim of the present study was to address the role of CAV-1 in human mammary epithelial cells inflammatory response induced by LPS. It was hypothesized that CAV-1, may through its ability to negatively regulate TLR4, is a

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crucial determinant of MAPK activation and inflammatory lesions in LPS-challenged human mammary epithelial cells.

Materials and Methods

Materials

The Olingo Ge Array of inflammation signaling pathway was purchased from Kangcheng Corporation, Shanghai (Catalog No. HS-020 (human)). Primary antibodies against CAV-1, TLR4, p-JNK, p-38 and β-actin were obtained from Santa Cruz, CA, USA. ECL reagent was from Amersham Biosciences. The RNA purification kit and DAB kit were purchased from Tiangen Corporation. Other reagents were from corporations in China. MTT reagent were obtained from Sigma Chemicals, St. Louis, MO, USA. Dimethylsulfoxide (DMSO) was Sigma Chemicals. Enzyme linked immunosorbent assay (ELISA) reader was obtained from Tecan Sunrise Company. Other reagents were from corporations in China.

Cell lines and cell culture

A mammary epithelial cell line MCF-10A was purchased from ATCC. Stable clones (designated as MCF-10ACE) which were CAV-1 down-regulated that derived from MCF-10A by RNAi technology. Culture mediated Dulbecco's modified Eagle's medium-F12 (DMEM/F12) charcoal-stripped horse serum was obtained from Hyclone Biotechnology. Hydrocortisone, cholera toxin, insulin, epithelial growth factor were purchased from Sigma. OPTI-MEMI was purchased from GBICO Corporation. Zeocin, was obtained from Invitrogen Corporation. Seakem LE agarose was from Cambrex Corporation.

DNA Microarray

Total RNA was extracted from MCF-10A and MCF-10ACE cells with TRIZOL and RNA purification kit. Agarose gel electrophoresis was used to check the quality of the RNA. We used the Olingo GE Array to observe altered gene expression associated with inflammation in human mammary epithelial cells. The DNA micro-array was performed according to the manufacturer's instructions. The final signals were acquired by a Typhoon 9000 scanner (Perkin-Elmer) and exposed to X-ray film (Kodak). Image Quant software (Perkin-Elmer) and the GE Array Expression Analysis Suite (Super Array) were used for quantification.

MTT assay for cell proliferation

Each groups of MCF-10A and MCF-10ACE cell lines were seeded at 4×10^4 per well in 96-well plates and treated with LPS (10 μg/mL, 20 μg/mL, 50 μg/mL and 100 μg/mL) and cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO_2. The MTT reagent (5mg/mL) was added to the maintenance cell medium after LPS-challenged for 24 hours and incubated at 37°C for an additional 4 hours. The reaction was terminated with 150μL DMSO per well and the cells were lysed for 15 minutes, and the plates were agitated every 5 minutes. Absorbance values were determined using the ELISA reader at 492 nm.

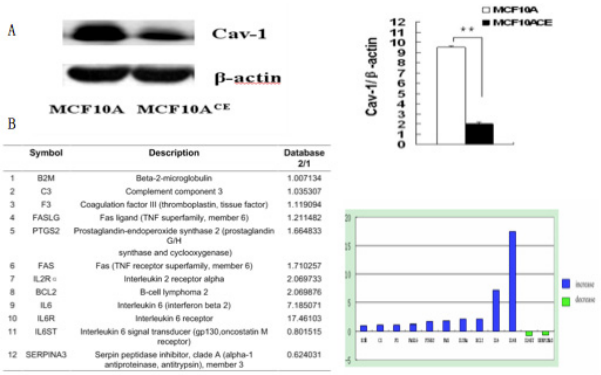

Figure 1. CAV-1 Downregulation Promotes Inflammation-associated Genes Expression in Human Mammary Epithelial Cells. (A) Expression of CAV-1 in human mammary epithelial cell line MCF-10A and MCF-10A^{CE} by Western blot. Results are representative of three independent experiments. **vs MCF-10A ($p<0.01$). (B) The Oligo GE Array was used to observe altered gene expression associated with inflammation in human mammary epithelial cells

Western blot analysis

MCF-10A and MCF-10ACE cells were washed with PBS and lysed with lysis buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 0.25mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100, 50mM NaF and protease inhibitor cocktail). Equal amount of cell lysates were analyzed by Western blot analysis. 20μg proteins were boiled for 5 minutes in SDS gel loading buffer and separated on a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane. The membranes were probed with primary antibodies (CAV-1, TLR4, p-JNK, and p-38). Western blots were visualized with ECL detection reagents. The same membranes were stripped and reprobed with an antibody against β-actin to confirm equal loading.

Immunofluorescence

MCF-10A and MCF-10ACE cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes. After being permeabilized with 0.4% Triton X-100 for 10 minutes at room temperature, cells were blocked in 4% bovine serum albumin (BSA)-supplemented PBS for 1 hour and incubated overnight at 4°C with antibody. After 3 washes in PBS, the cells were labeled with TRITC-conjugated secondary antibody.

Statistical Analysis

Results were reported as the mean±SD for typical experiments done in three replicate samples and compared by the Student's T-Test. Results were considered significantly different for $P<0.05$.

Results

CAV-1 down-regulation promotes inflammation-associated genes expression in human mammary epithelial cells. (NOTE: Simplify 2 results to 1, which is more direct to clarify our point.)

Previously, we have established a CAV-1 gene silencing human mammary epithelial cell line MCF-10ACE from normal human mammary epithelial cell line MCF-10A

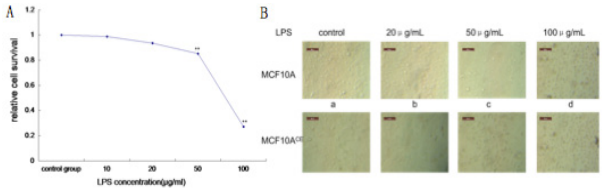

Figure 2. CAV-1 Downregulation Promotes Inflammation-associated Genes Expression in Human Mammary Epithelial Cells. (A) MTT assay for cell proliferation in human mammary epithelial cell line MCF-10A after LPS challenge. Results are representative of three independent experiments. **vs control (p<0.01). (B) Inflammatory lesions in LPS-challenged human mammary epithelial cell line MCF-10A and MCF-10A^{CE}

by RNAi technology. To verify CAV-1 expression was down-regulated in MCF-10ACE, Western blot analysis was performed. As shown in Figure 1A, compared with MCF-10A, MCF-10ACE showed significant decrease of CAV-1 by 70% (p<0.01), illustrating that the CAV-1 gene silencing human mammary epithelial cell line MCF-10ACE we established could be used for the succeeding research.

As is known that CAV-1 is implicate as a modulator of inflammation. To address its precise effect on inflammation-associated genes expression in MCF-10A and MCF-10ACE, DNA Microarray was performed. As shown in Figure 1B, MCF-10ACE exhibited significant increases in Prostaglandin-endoperoxide synthase 2 (PTGS2), B-cell lymphoma 2 (BCL2), Fas ligand (FAS), Interleukin 2 receptor alpha (IL2Rα) by >1.5-fold, especially Interleukin 6 (IL-6) by 7-fold and Interleukin 6 receptor (IL6R) by 17-fold. The above showed that CAV-1 gene silencing promoted inflammation-associated genes expression, which indicates that it probably act as a pro-inflammatory effector in human mammary epithelial cells response to LPS.

CAV-1 down-regulation aggravates inflammatory lesions in LPS-challenged human mammary epithelial cells

To verify our hypothesis, human mammary epithelial cells were induced by LPS. MTT assay was used to detect human mammary epithelial cells proliferation after LPS challenge. As shown in Figure 2A, cells proliferation exhibited significant decreases after LPS (10 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL) challenge, and it reached the lowest point at dose of 100μg/mL (p<0.01). The above showed that LPS could inhibit human mammary epithelial cells proliferation in a dose-dependent manner. Then, inflammatory lesions were observed in the two cells after LPS challenge for 24 hours under microscope. As shown in Figure 2B, neither of them appeared any inflammatory lesions at 20 μg/mL. However, when LPS was up to 50μg/mL, MCF-10ACE became shriveled and even occurred necrosis, while it could not be seen in MCF-10A. Furthermore, both of them occurred necrosis when at 100 μg/mL, and even MCF-10ACE exhibited more serious inflammatory lesions. The above showed that CAV-1 gene silencing intensified LPS-induced cell necrosis and inhibited cell proliferation, indicating that

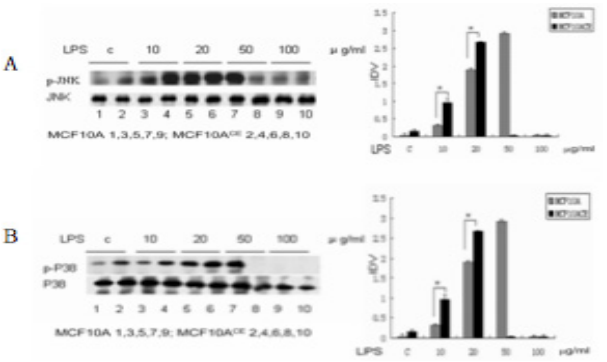

Figure 3. JNK MAPK and P38 MAPK Activation in Human Mammary Epithelial Cell Line MCF-10A and MCF-10A^{CE} after LPS Challenge. (A) JNK MAPK activation in human mammary epithelial cell line MCF-10A and MCF-10ACE after LPS challenge. Results are representative of three independent experiments. **vs MCF-10A (p<0.01). (B) P38 MAPK activation in human mammary epithelial cell line MCF-10A and MCF-10A^{CE} after LPS challenge. Results are representative of three independent experiments. **vs MCF-10A (p<0.01)s

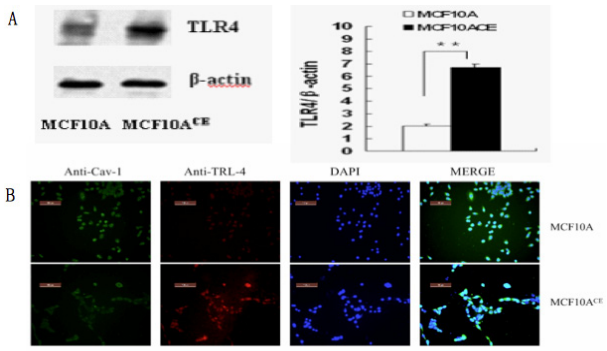

Figure 4. Co-localization of CAV-1 and TLR4 in Human Mammary Epithelial Cell Line MCF-10A and MCF-10A^{CE}. (A) Expression of TLR4 in human mammary epithelial cell line MCF-10A and MCF-10ACE. Results are representative of three independent experiments. ** vs MCF-10A (p<0.01). (B) Co-localization of CAV-1 and TLR4 in human mammary epithelial cell line MCF-10A and MCF-10A^{CE}

it could exert as a pro-inflammatory effector after LPS challenge in human mammary epithelial cells.

JNK MAPK and P38 MAPK activation in human mammary epithelial cell line MCF-10A and MCF-10ACE after LPS challenge

We attempted to delineate the possible mechanism by which CAV-1 gene silencing exerted its pro-inflammatory effect on human mammary epithelial cells. Given that MAPK pathways mediate cytokines production and are regulated by CAV-1 in fibroblasts and endothelial cells (Sasai et al., 2007; Jagielska et al., 2012), we examined whether MAPK pathways were modulated in CAV-1 gene silencing human mammary epithelial cells. As shown in Figure 3, compared with MCF-10A, LPS-induced both p38 MAPK and JNK MAPK activation were significantly increased in MCF-10ACE, indicating that CAV-1 gene silencing probably exerted its pro-inflammatory effect via activating MAPK pathway in human mammary epithelial cells response to LPS.

Co-localization of CAV-1 and TLR4 in human mammary epithelial cell line MCF-10A and MCF-10ACE

TLR4 is identified as the principle membrane receptor for LPS. Recently, more and more studies have shown that CAV-1/TLR4 interaction plays a vital role in inflammatory response. To explore whether there existed a CAV-1/TLR4 interaction in human mammary epithelial cells, we detected the relationship between CAV-1 and TLR4 by immunofluorescence. As shown in Figure 4A, CAV-1 and TLR4 co-localizated in human mammary epithelial cells as our expected. Furthermore, we measured TLR4 expression in both MCF-10A and MCF-10ACE by Western blot analysis. As shown in Figure 4B, compared with MCF-10A, MCF-10ACE exhibited a marked increase in TLR-4 expression ($p<0.01$), indicating that regulation of TLR4 function may occur within caveolae or lipid raft micro-domains in human mammary epithelial cells.

In summary, CAV-1 gene silencing, may through its ability to negatively regulate TLR4 signaling, is a crucial determinant of MAPK activation and inflammatory lesions in human mammary epithelial cells response to LPS.

Discussion

Previously, we have showed that CAV-1 gene silencing activates estrogen receptor alpha expression and leads to 17 beta-estradiol-stimulated mammary tumorigenesis (Zhang et al., 2005). Downregulation of CAV1 increases cell apoptosis in vitro (Xuening et al., 2011). In this study, our results demonstrated an important regulatory role of CAV-1, the principal signaling and structural protein of caveolae, in the mechanism of LPS-induced mammary epithelial cells that imitating mastitis. CAV-1 has also been shown to participate in innate immunity and inflammation. It is unclear, however, whether CAV-1 plays a role in the organization of inflammatory signaling.

First, we used human mammary epithelial cell line MCF-10A and MCF-10ACE to address the role of CAV-1 in the regulation of human mammary epithelial cells response to LPS. Among the inflammation-associated genes, PTGS2, BCL2, FAS, IL2Rα, IL6 and IL6R were significantly increased, and even IL6 and IL6R after CAV-1 silencing. As CAV-1 was a crucial negative regulator of inflammation-associated genes expression, we addressed the possibility that it participated in the mechanism of mammary epithelial cells inflammatory lesions induced by LPS.

In this study, we also observed obvious increases in LPS-induced phosphorylation JNK expression and phosphorylation p38 expression in MCF-10ACE relative to MCF-10A, and found that CAV-1 gene silencing interfered with LPS-induced MAPK activation to regulate the consequent expression of inflammation-associated genes.

Caveolins serve as a platform in plasma membrane associated caveolae to orchestrate various signaling molecules to effectively communicate extracellular signals into the interior of cell. Key molecules involved in inflammatory pathways include TLRs, NF-ϰB, cytokines, growth factors (Jagielska et al., 2012). TLR4 is now recognized as a pattern recognition receptor against a diverse array of ligands including endogenous stress ligands or damage-associated molecular patterns such as heat-shock proteins and fibronectin (Zhang et al., 2005). Lipid rafts and caveolae play a pivotal role in organization of signaling by TLR4 and several other immune receptors (Akira et al., 2006).

At last, we examined TLR4 expression in MCF-10A and MCF-10ACE, and found that TLR4 indeed existed in them. Besides, TLR4 expression was increased more significantly ($p<0.01$) in MCF-10ACE, indicating that CAV-1 gene silencing could promote TLR4 expression in human mammary epithelial cells.

Recently, several studies have shown that CAV-1 bounds to TLR4 and inhibits LPS-induced pro-inflammatory cytokines production (TNF-α and IL-6) in murine macrophages (Fessler et al., 2011). Therefore we hypothesized that CAV-1 bonded to TLR4, and consequently influenced the downstream regulation of inflammation-associated genes expression. This novel interaction was detected by immunofluorescence in MCF-10A and MCF-10ACE. The results showed that CAV-1 and TLR4 colocated in human mammary epithelial cells, as well as appeared a negative correlation trend.

As we known, inflammatory signaling initiates when LPS binds to the acute-phase protein LPS-binding protein, which is recognized by TLR4 and by CD14, a glycosyl-phosphatidyl-inositol-anchored protein lacking a cytoplasmic domain. Following ligand binding, a TLR4 complex is assembled, composed of CD14, TLR4, MD-2, MyD88, and other adapters (Wang et al., 2009). So we thought that complex formation was followed sequentially by the intracellular activation of signaling mediators that include IL-1R-associated kinase, Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF), p38 MAPK and p-JNK MAPK (Aksoy et al., 2011).

In conclusion, CAV-1 gene silencing, through its ability to regulate TLR4 signaling promotes MAPK activation and pro-inflammatory lesions in human mammary epithelial cells response to LPS.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30570225; No. 30970353).

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