

Role of Hypothiocyanous Acid on the Cytokine Production in Airway Epithelial Cells

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Abstract

When influenza viruses infect to airway, it is thought that hypothiocyanous acid (HOSCN), an oxidant with anti-influenza virus activity, is produced by the catalysis of peroxidases such as myeloperoxidase (MPO) and lactoperoxidase (LPO). However, the actions of HOSCN on host cells are poorly understood. In this study, we examined the effects of HOSCN on the expression of cytokines in airway epithelial cells. Human airway epithelial (NCI-H292) cells were exposed to HOSCN that generated in LPO/glucose oxidase/SCN⁻ culture system. The gene expression of cytokines was determined by quantitative polymerase chain reaction and the concentration of cytokines also was measured by ELISA method. We found that HOSCN induced the expression of pro-inflammatory cytokines such as IL-6, IL-8, TNF- α , IL-1 β and M-CSF, but it did not do GM-CSF. The results suggest that HOSCN may contribute to the production of pro-inflammatory cytokines during influenza viral infection. And also it may impact on the balance of M1/M2 development of macrophages, because M-CSF plays an important role for the differentiation of macrophages toward M2 phenotype.

Introduction

Influenza virus infection leads to recruit neutrophils and monocytes to the infectious sites and produce inflammatory mediators including hydrogen peroxide (H₂O₂) to response against influenza virus infection^{1,2,3}. Previously we showed that MPO, which is an enzyme released by activated neutrophils and monocytes, accumulated in the lung in the early phase after influenza virus infection⁴. In plasma, MPO is able to generate equal amounts of hypochlorous acid (HOCl) and HOSCN by catalytic oxidation of chloride (Cl⁻) and thiocyanate (SCN⁻), respectively, with H₂O₂⁵. In the airway, HOSCN is thought to be a major product by the MPO-catalytic oxidation, because airway surface liquid (ASL) covering the airway epithelium contains higher concentrations of SCN⁻ than plasma⁵. In the presence of H₂O₂, it is also possible that SCN⁻ may be oxidized to HOSCN by LPO, which is secreted into ASL by submucosal glands and goblet cells⁶. Therefore, we are focusing on roles of

HOSCN during influenza virus infection.

As well as HOCl, HOSCN is an oxidant with anti-influenza virus activity^{7,8}. However, the reactivity of individual oxidants is markedly different. HOCl reacts with the majority of functional groups in amino acids. In contrast, HOSCN is a much milder oxidant that reacts specifically with thiol and selenol residues, suggesting that HOSCN may act as a second messenger in redox signaling⁹.

So far, effects of HOSCN on airway epithelial cells are poorly understood. A study showed that HOSCN activated NF- κ B via PKA dimerization in airway epithelial cells, suggesting it may induce the gene expressions of various pro-inflammatory cytokines¹⁰. In this study, to clear the role of HOSCN on inflammation in the airway, we analyze its effects on the cytokine production in airway epithelial H292 cell line.

Methods

Cell culture

H292 cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GE Healthcare Life Sciences), 0.05 mg/mL gentamicin sulfate (Wako) at 37°C in 5% CO₂ incubator.

Exposure of cells to HOSCN

H292 cells at 5 x 10⁵ cells/mL of concentration were cultured in RPMI medium at 37°C for 1 day until 95% of the confluences. Cells were incubated in RPMI medium without red phenol (Gibco) plus 50 mM HEPES, 3 mM NaSCN, 5% FBS for 1 hr at 37°C. Then, cells were exposed to HOSCN that produced in culture system containing 16 mU/ml GOX and 10 μ g/ml LPO for 7 hr. Cells were collected for RNA isolation to quantify the mRNA expression of cytokine genes at 7 hr post exposure. The stimulated cells were incubated in RPMI medium containing 10% FBS until 24 hr at 37°C, the cell culture supernatants were collected to measure the cytokine concentration.

Isolation of RNA and quantification of the gene expressions

Total RNA were isolated from cells by Isogen/chloroform extraction and isopropanol precipitation. The concentration of RNA was determined using Nanodrop. cDNA was synthesized with ReverTra Ace[®] qPCR RT Master Mix with gDNA remover (Toyobo). A 8 μ l mixture containing 500 ng total RNA and 2 μ l gDNA remover were incubated at 37°C for 10 min. Then, 2 μ l 5x RT Master Mix II was added in the reaction and incubated at 37°C for 20 min, 50°C for 10 min, 98°C for 5 min. Gene expression was validated by Realtime-PCR (qPCR) using 10 μ l 2X Power SYBR[®] Green PCR Master Mix (Applied Biosystems), 5 μ l cDNA (5-fold dilution) and 10 pmol

specific primers of IL-6 (interleukin-6), IL-8 (interleukin-8), TNF- α (tumor necrosis factor- α), IL-1 β (interleukin-1 β), M-CSF (macrophage-colony stimulating factor), GM-CSF (granulocyte macrophage-colony stimulating factor) and G-CSF (granulocyte-colony stimulating factor) cytokine genes. The cycle conditions of qPCR were 95°C for 20 s, 40 cycles of (95°C for 3 s, 60°C for 30 s), 95°C for 15 s, 60°C for 60 s.

Measurement of the cytokine concentration

Concentrations of Interleukin-6 and Interleukin-8 in cell culture fluids were determined using Human Interleukin-6 and Interleukin-8 immunoassay ELISA kit (Quantikine, R&D systems) following the manufacturer's instructions.

Statistical analysis

Data as presented as mean \pm standard deviation. Statistical analysis of results was performed by Student's t-test. Differences with p values <0.05 were considered significant.

Results

H292 cells were exposed to HOSCN for 7 hours to analyze the effect of HOSCN on the pro-inflammatory cytokine gene expressions. The mRNA expression levels of IL-6 and IL-8 were remarkably increased in H292 cells with HOSCN exposure in comparison to that in H292 cells without HOSCN exposure ($p=0.002$ and 0.0015 , respectively) (Fig. 1a). And expression levels of TNF- α and IL-1 β were slightly enhanced by HOSCN ($p=0.047$ and 0.028 , respectively) (Fig. 1a). Therefore, we measured the amounts of IL-6 and IL-8 cytokines in the culture fluids. Concentration of IL-6 and IL-8 highly increased in H292 cells exposed to HOSCN than that in cells without HOSCN exposure for 7 hr ($p=0.016$ and 0.018 , respectively) and 24 hr stimulation ($p=0.01$ and 0.004 , respectively) (Fig. 1b). It was suitable to the mRNA level of IL-6 and IL-8 genes. These results confirmed that HOSCN induced the pro-inflammatory cytokine expression.

In addition, M-CSF was significantly increased (about 75 folds) in H292 cells exposed to HOSCN than that in cells without HOSCN exposure ($p=0.048$) (Fig. 1a). But, there was no difference of GM-CSF expression between cells with and without HOSCN exposure ($p=0.2$) (Fig. 1a). The expression of G-CSF was not detected.

Discussion

It is thought that HOSCN is a major oxidant generated by MPO in the airway and its excessive or misplaced generation may contribute to lung diseases^{10,11}. In this study, we found that the exposure of epithelial cells to HOSCN increases the mRNA level of IL-6, IL-8, TNF- α and IL-1 β genes, which are driven by NF- κ B, in concordance with previous data that it activates NF- κ B¹⁰. These cytokines may enhance the recruitment and activation of neutrophils, resulting in amplifying the inflammation at early stages of influenza virus infection (Fig. 2).

At inflammatory sites during IFV infection, recruited monocytes differentiate to macrophages. Recent studies show that macrophages are divided into M1 and M2 phenotypes, characterized by pro-inflammatory and anti-inflammatory functions, respectively¹². The polarization of M1/M2 macrophage differentiation depends on the cytokines in the environment where monocytes and macrophages reside. We observed the strong induction of M-CSF gene expression after exposure of airway epithelial cells (H292) to HOSCN. M-CSF is known to skew monocytes toward M2 macrophages via the activation of interferon regulatory factor 4, which is an important transcription factor for M2-phenotype related gene expressions^{13,14}. Therefore, we speculate that HOSCN may impact on the balance of M1/M2 development of macrophages, skewing it toward M2 macrophages (Fig. 2). If our hypothesis is correct, the anti-inflammatory M2 macrophages increased by HOSCN are thought to dampen inflammation during IFV infection. Previous studies showed that it took around 6 days for the differentiation from monocytes to M2 macrophages¹⁵. Therefore, HOSCN may have a role for leading to converge inflammation at late stages of influenza virus infection.

In summary, under the oxidative stress with HOSCN, epithelial cells produced pro-inflammatory cytokines and simultaneously expressed M-CSF cytokine that may skew differentiates macrophage toward M2 phenotype to attenuate inflammation. That may be a defence mechanism of epithelial cells to HOSCN induced-inflammation.

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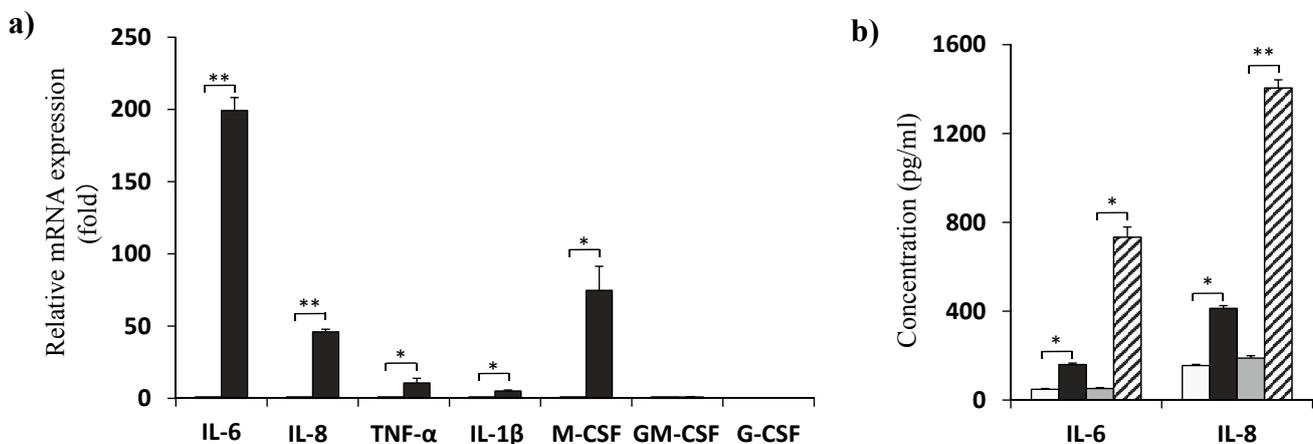


Fig. 1. Induction of cytokines by HOSCN

a) Relative mRNA level of cytokine genes of H292 cells (white bars), and H292 cells that exposed to HOSCN (black bars) after 7 hr stimulation.

b) Production of IL-6 and IL-8 cytokines of H292 cells at 7 hr (white bars), 24 hr (grey bars); and H292 cells that exposed to HOSCN at 7 hr (black bars), 24 hr (stripe bars) post stimulation. Data as presented as MEAN \pm SD of the results of three independent experiments, * $p < 0.05$, ** $p < 0.01$ (Student's t-test).

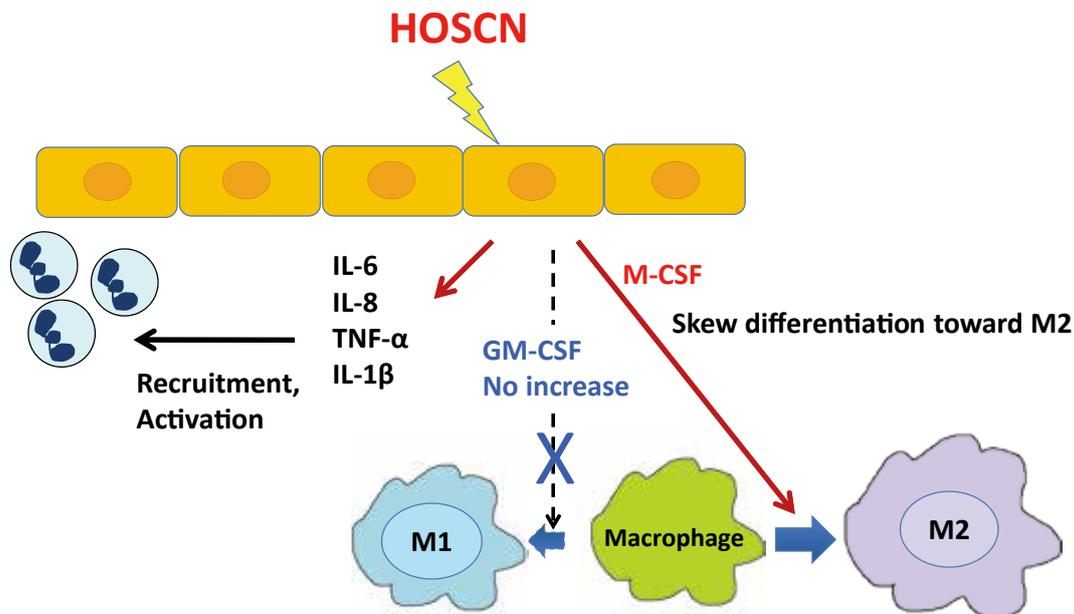


Fig. 2. HOSCN may associate with the differentiation of macrophage and the inflammation of lung epithelial cells

An excess generation of HOSCN induced the production of pro-inflammatory cytokines in the epithelial cells. These cytokines may recruit and activate neutrophils result in damage of cells. An excess generation of HOSCN also induced the production of M-CSF which may skew differentiates macrophage toward M2 phenotype to attenuate inflammation and epithelial damage.

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