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Inhibitory Effect of Chlorogenic Acid on the Expression of the TLR7 Signaling Pathway in the Presence of H1N1 Infection *In vitro*

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Chlorogenic acid, a natural compound widely found in a great variety of natural products, with its wide range of potentially practical values, has been quite frequently researched in many basic scientific experiments for its biological activities, such as antimicrobial properties, and other physicochemical effects. In this study, we investigated the anti-virus role of chlorogenic acid on the toll-like receptor 7 (TLR7) signaling pathway in diverse immune cells infected with H1N1. Through the course of obtaining peripheral blood mononuclear cell(PBMC) from fresh umbilical cord blood, and stimulations by inducing factors, rhGM-CSF and rhIL-4, separately, we established a co-culture system of distinct immune cells with the medium of the H1N1 virus-infected cells(human respiratory epithelial cell). Then chlorogenic acid was added into the system as an drug intervention. The cell supernatant and immune cells were collected for RT-PCR to determine the expression levels of cytokines related to the TLR7 pathway. The results indicated that chlorogenic acid could ameliorate H1N1 virus-infected cell survival and down-regulate the expression levels of the TLR7 pathway cytokines, thus avoiding too much damage from the over-reactive immune system.

Key words: Chlorogenic acid, H1N1, monocytes, dendritic cells, macrophages,TLR7.

Influenza virus, with its frequent incidence of episode and rapid variation of gene, has always been considered as a big headache to the patients and doctors in daily routine, especially in the seasonal flu peak. Nowadays, the main way to hold back the infection with flu is to get a yearly flu vaccine(Connell *et al.,* 2011). The catastrophic viral genetic mutation, along with its species variability, are still limiting the sphere of our countermeasure in the medical field, either by symptomatic treatment or intensification of our body immunity. Oseltamivir and Zanamivir, as[1] the neuraminidase inhibitors, frequently used to treat severely infectious patients in current clinical practice, seem to have no substantial effect in inhibiting the spread and occurrence of the disease(Carrat *et al.,* 2012; Del *et al.,* 2011). However, the antiviral drugs we are widely prescribing and researching primarily focus on the antagonism of the invasion and replication of the virus, rather than virus recognition and activation of the immune system(Baumgarth *et al.,* 1999;Tortorella *et al.,* 2000). When a flu virus invades, innate immune system always function as a powerful weapon to defend against the foreign pathogen through the recognition pattern by which body immunity could signal the first message

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to the succeeding reaction from within, the adaptive immune response. TLR7, an innate signaling receptor, that recognizes single-stranded viral RNA, could be activated by viruses that cause persistent infections through the ignition of the downstream signaling(Walsh *et al.,* 2012). And as we have already known, there are still many similar pattern recognition receptors closely associated with the virus invasion, such as retinoic acid inducible gene-I like RNA helicase(RLH) and melanoma differentiation-associated protein 5(MDA5). As a result, the research and development of a new antiviral drug should not be deviated from the role of recognition in the immune system and the production of cytokines(Thompson *et al.,* 2007; Takeuchi *et al.,* 2007; Takeuchi *et al.,* 2008). In this study, we focused on the signaling expression of TLR7 on the immune cells, and its result whether, once the virus attacks, the immune signals of TLR7-related cytokines would change in terms of the expression levels. Recently, the herb honeysuckle has been extensively used clinically for the treatment of influenza virus infectious disease(Chen *et al.,* 2011; Hudson, 2009). However, Chlorogenic acid, a main ingredient of honeysuckle, and many other antiviral herbal monomer extracts have been studied and explored for trying to elucidate the molecular mechanism how could these herb drugs function to fight against virus, but it is still poorly understood. This study therefore intended to illuminate the antiviral mechanism of Chlorogenic acid through the TLR7 signaling pathway, thus providing a scientific basis for the clinical application of anti-flu herbal drugs.

MATERIALS AND METHODS

H1N1-Infected Model Establishment

16HBE (human respiratory epithelial cells, bought from Xiangfu Biotech Corp., Shanghai, China) were cultured in a 6-well plate, 1ml for each well, with RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a 5% CO_2 incubator at 37°C for all night long. The concentration of 16HBE was prepared to 3×107/ml. Then we infected the cell supernatant with the indicated H1N1 influenza virus A/E/13(provided by the Institute for Tropical Diseases, Southern Medical University, China.) for generating the virus-infected cell model.

Determination of Optimized Drug Concentration(MTT assay)

The concentration of Chlorogenic acid(C16H18O9, CAS Number: 327-97-9, bought from Nanjing Tcm Institute Of Chinese Materia Medica Nanjing) was specified by the MTT experiment. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide(MTT) assay was performed on the 16HBE cell line,100ul for each hole in a 96-well micro-plate, with five identical wells added by the same drug dose for each time point. Another two sets of wells were determined as the control wells. The medium were incubated at 37°C, in a 5% CO_2 incubator. After that course, 5 mg/ml MTT solution(20ml) was added to each well and incubated for 4 hours at 37°C in humidified CO2. MTT conversion to formazan by metabolically viable cells was monitored by a spectrophotometer at 570 nm. The experiment was repeated at least three times.

Inducement and Isolation of the Immune Cells

Mononuclear cells were obtained by Ficoll density gradient centrifugation (Haoyang TBD Bio, Tianjin, China) from umbilical cord blood and cultured in RPMI-1640 supplemented with 10% FBS at 37°C in a 5% humidified CO_2 incubator. Mature DCs were harvested using 100 ng/ml rhGM-CSF and 50 ng/ml rhIL-4 (PeproTech, US) for 7 days. On the 7th day, 50 ng/ml rhTNF-α was added into the medium to induce DC maturation. Macrophages were obtained by inducing mononuclear cells with 100 ng/ml rhGM-CSF for 7 days. In this way, we procured the experimentally requisite cells in the succeeding co-reaction systems.

Co-Culture Reacting System

The virus-infected cell model was co-cultured with immune cells(DCs, Macrophages, monocytes) using Millicell Culture Insert (PIRP30R48, Millipore). The 16HBE cell model was seeded in the outer side of the PET membrane and then DC and macrophage were separately seeded in the inner side of the PET membrane. The special membrane function as a free passage for the cytokines and other elements in the reacting solution. The co-cultured system was put into a 5% CO_2 incubator at 37°C for 24 h.

Quantitative RT-PCR analysis of the TLR7

DCs, macrophages and monocytes were harvested after co-culture with the virus-infected

cell model for 24 h. Total RNA was isolated with Biozol reagent (Biomiga, Inc., China) following the manufacturer's instructions. cDNA synthesis and real-time PCRs were performed using a Quantscript RT kit (KR103, Tiangen Biotech Co., Beijing) and Real Master Mix (SYBR Green, FP202, Tiangen Biotech Co., Beijing) according to the manufacturer's instructions. Primers were synthesized by Generay Biotech Co., Shanghai. Analysis of relative gene expression data was performed through the 2-ΔΔCT method: ΔCt = ΔCt(target gene)-Ct(reference gene) and ΔΔCt = ΔCt(control group) - ΔCt(model group). A gene was considered deregulated if its expression value was higher or lower than the cut-off value set for each gene (mean±SD).

Statistical analysis

Statistical analyses were performed using SPSS 16.0 for windows (SPSS software, IBM). All data are presented as the means ± SD. Variance analysis (ANOVA) and Student's t test were used for multiple analysis, $P<0.05$ was considered statistically significant.

RESULTS

Induced maturation of DC and Macrophages

Extracted PBMC were cultured in a 5% CO_2 incubator at 37° for 6 h. Then, the supernatant was removed and the cells washed twice with PBS to remove non-adherent cells. Each hole was then treated with 2 ml of 1640 medium containing 10% fetal bovine serum and stimulating factors for cultivation at 37° in 5% CO_2. Finally, stimulation of mature DCs and macrophages was successfully accomplished by adding varying stimulators, such as rhGM-CSF, rhIL-4 and rhTNF-α. (Fig. 1).

Concentration measurement of Chlorogenic acid

Through MTT experiment, we found the inhibition rate of the drug for influenza virus was 54.3475%±0.63073% when the concentration was adapted into 400μg/ml. At 1600μg/ml, 65.8475%±1.34982% inhibition rate was observed, and the increasing dose of the drug failed to significantly inhibit the virus futher. Otherwise, When the cells were treated with Chlorogenic acid, the inhibition rate of cell growth was less than 20%, showing that Chlorogenic acid did not significantly inhibit the growth of cells at 400 μg/ml (the inhibition rate was 11.8050±0.68169) (Table 1). This concentration was found as the optimal concentration of Chlorogenic acid for anti-viral effects and used throughout the entire experiments.

Cytokines expression on the TLR7 pathway

As the results of RT-PCR analysis indicated, the H1N1 virus could strengthen the

Table 1. Anti-viral inhibition rates (ER) of Chlorogenic acid

Concentration (mg/ml)	100	200	400	800	1600
Cell cytotoxicity(%)	8.6500± 0.07071	11.2550± 0.73763	11.8050± 0.68169	21.8625± 0.70249	31.9200± 1.45838
virus inhibition (%)	11.9500± 0.60536	31.5225± 0.77487	54.3475± 0.63073	61.5700± 0.89930	65.8475± 1.34982

Table 2. Expression levels of mRNA in DC group ($2^{-\Delta\Delta Ct}$)

gene	16HBE+DC	16HBE+DC+V	16HBE+DC+V+CA
TLR7	1.00±0.00	5.23±0.07*	0.47±0.03$^{\Delta\Delta}$
MyD88	1.00±0.00	3.89±0.06*	0.35±0.04$^{\Delta\Delta}$
IRAK-1	1.00±0.00	4.36±0.17*	0.56±0.04$^{\Delta\Delta}$
TAK-1	1.00±0.00	3.25±0.07*	0.62±0.22$^{\Delta\Delta}$
JNK	1.00±0.00	2.98±0.12*	0.35±0.42$^{\Delta\Delta}$
P38	1.00±0.00	2.72±0.13*	0.41±0.17$^{\Delta\Delta}$

*$P<0.01$ refers to a statistical difference between the virus-infected cells model group(16HBE+DC+H1N1) and the normal group (16HBE+DC);

$^{\Delta\Delta}P<0.01$ refers to a statistical difference between the treatment group(16HBE+DC+H1N1+CA) and the virus-infected cells model group(16HBE+DC+H1N1).

gene expression of the TLR7 signaling pathway in diverse immune cells (Fig. 2). The cytokine expression of (myeolid differentiation factor 88(MyD88), leukocyte interleukin-1 receptor-associated kinase (IRAK-1), transforming growth factor activated kinase 1(TAK1), c-Jun N-terminalprotein kinase (JNK)and p38mitogen-activated protein kinase(p38/MAPK) mRNA expressions) were elevated significantly compared to normal groups (16HBE+DC/macrophage/

Table 3. Expression levels of mRNA in Macrophage group ($2^{-\Delta\Delta Ct}$)

Gene	16HBE+Mϕ	16HBE+Mϕ+V	16HBE+Mϕ+V+CA
TLR7	1.00±0.00	3.05±0.13*	$0.54\pm0.03^{\Delta\Delta}$
MyD88	1.00±0.00	4.62±0.03*	$0.44\pm0.01^{\Delta\Delta}$
IRAK-1	1.00±0.00	4.07±0.04*	$0.61\pm0.02^{\Delta\Delta}$
TAK-1	1.00±0.00	3.44±0.05*	$0.69\pm0.02^{\Delta\Delta}$
JNK	1.00±0.00	2.45±0.06*	$0.43\pm0.03^{\Delta\Delta}$
P38	1.00±0.00	2.21±0.08*	$0.44\pm0.02^{\Delta\Delta}$

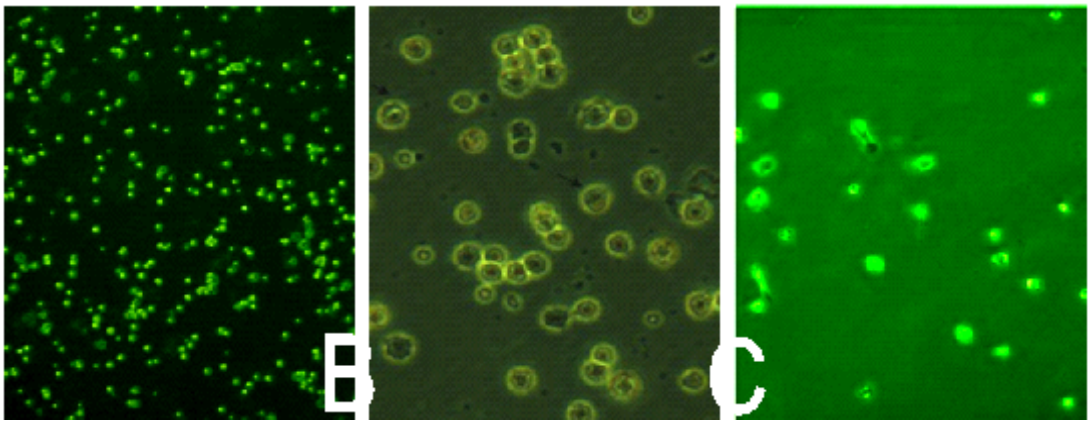
*P<0.01 refers to a statistical difference between the virus-infected cells model group(16HBE+Mϕ+H1N1) and the normal group (16HBE+DC);
$^{\Delta\Delta}$P<0.01 refers to a statistical difference between the treatment group(16HBE+Mϕ+H1N1+CA) and the virus-infected cells model group(16HBE+Mϕ+H1N1).

Table 4. Expression levels of mRNA in Monocyte group ($2^{-\Delta\Delta Ct}$)

Gene	16HBE+MON	16HBE+MON+V	16HBE+MON+V+CA
TLR7	1.00±0.00	2.66±1.82*	$0.76\pm0.25^{\Delta\Delta}$
MyD88	1.00±0.00	2.37±1.51*	$0.72\pm0.29^{\Delta\Delta}$
IRAK-1	1.00±0.00	2.50±1.55*	$0.80\pm0.20^{\Delta\Delta}$
TAK-1	1.00±0.00	2.13±1.16*	$0.83\pm0.17^{\Delta\Delta}$
JNK	1.00±0.00	1.84±0.87*	$0.72\pm0.29^{\Delta\Delta}$
P38	1.00±0.00	1.73±0.76*	$0.74\pm0.27^{\Delta\Delta}$

*P<0.01 refers to a statistical difference between the virus-infected cells model group(16HBE+MON+H1N1) and the normal group (16HBE+DC);
$^{\Delta\Delta}$P<0.01 refers to a statistical difference between the treatment group(16HBE+MON+H1N1+CA) and the virus-infected cells model group(16HBE+MON+H1N1).

monocyte) (P<0.01) when the virus invaded(Table 2;Table 3;Table 4). With the Chlorogenic acid treatment, however, the expression of TLR70IRAK-10MyD880TAK10JNK and p38/MAPK were shown to be significantly reduced (P<0.01)(Table 2;Table 3;Table 4). Therefore, in response to viral infection, the expression of cytokines in the TLR7 signaling pathway and downstream cytokines (TLR70IRAK-10MyD880TAK10JNK and p38/MAPK) increased. The immune recognition for influenza virus invasion was thus clearly activated, boosting downstream IFN and other inflammatory cytokines for self-defense. However, when we used the Chlorogenic acid treatment, the expression of cytokines in TLR7 signaling in different immune cells was reduced(Fig. 3), suggesting that Chlorogenic acid could down-regulate the expression of TLR7 signaling pathway. For this reason, the body defense system could ward off over-exuberance of self-protection and overreaction, which might lead to certain potentially disastrous damages.



A) adherent monocytes cultured for 6 h were harvested by removing the suspended cells; B) matured DCs, induced by 100 ng/ml rhGM-CSF and 50 ng/ml rhIL-4 for 8 days and induced by 50 ng/ml rhTNF-á for 1 day ; C) macrophages, stimulated by 100 ng/ml rhGM-CSF for 7 days.

Fig. 1. Immune cells observed under inverted phase contrast microscope (200×)

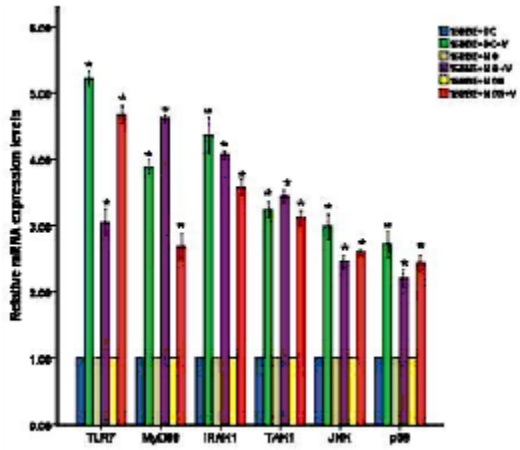


Fig. 2. Expression levels of mRNA in each group without intervention

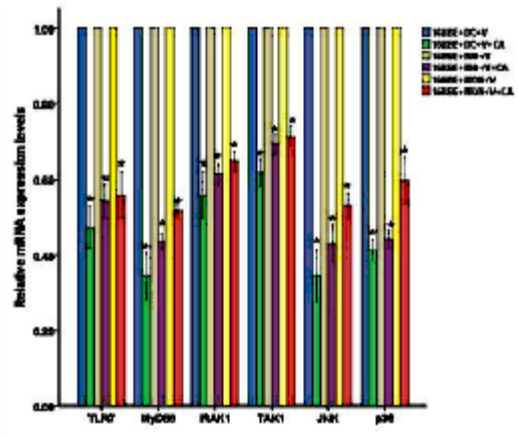


Fig. 3. Expression levels of mRNA in each group with intervention

DISCUSSION

Vaccination against influenza A and B viruses currently is the main effective way to prevent the spread of the devastating disease, although once a body has been already infected with the virus, it seems not that useful to antagonize the disease. Two classes of the drugs are currently licensed in many countries for the treatment of influenza. The M2 ion channel blockers, or amantadanes (amantadine and rimantadine), and the neuraminidase inhibitors(NAIs) (zanamivir and oseltamivir). Since their introduction in 1999, the proportion of influenza viruses resistant to NAIs among circulating influenza viruses has been low, generally less than 1% of isolates tested worldwide (Monto *et al.,* 2006; Sheu *et al.,* 2008; Mungall *et al.,* 2004).

However, readily transmissible drug-resistant viruses develop frequently during amantadane treatment every year(Alexander 2004). Although ribavirin shows in vitro activity against influenza viruses, clinical data are not consistent with in vitro data in many cases (Graci *et al.,* 2006; Riner *et al.,* 2009; Chan-Tack *et al.,* 2009).

Advances in understanding the mechanism implicated in influenza virus replication have revealed a number of potential targets that might be exploited in the development of new agents. But how could immune system recognize the invasion of the influenza virus and respond to defend the normalcy of body system still remains vaguely explored. We have already known that TLRs play an important role in the recognition of components of pathogens and subsequent activation of innate immunity, which then leads to development of adaptive immune responses. And it is well known that both Toll-Like Receptors (TLR) and cytosolic receptors (RIG-1 and MDA-5) are involved in recognition of viral RNA genomes and subsequently trigger IFN-I responses (Medzhitov *et al.,* 2000; Aderem *et al.,* 2000; Stetson *et al.,* 2006; Yoneyama *et al.,* 2004).

Toll like receptors (TLRs) detect a wide variety of microbial products and in DCs they are crucial in linking innate to adaptive immunity (Palm *et al.,* 2009). The mediator is too much cascading inflammatory cytokines. Numerous studies have shown that the main cause of death after IAV infection is due to a"cytokine storm" or excessive secretion of pro-inflammatory cytokines (Teijaro JR *et al.,* 2011). As such, we set a H1N1-infected cell model to observe the innate immune reaction, especially its molecular activation of inflammatory factors, and try to find a way to solve the problem. Indeed, pharmacological intervention in order to limit cytokine responses and thus to reduce the immunopathogenicity of influenza virus has been shown to be beneficial for the host (Teijaro *et al.,* 2011; Ichinohe *et al.,* 2008; Ehrhardt *et al.,* 2010).

In this study, we established an infected-cell model using an influenza virus (H1N1) to infect respiratory epithelial cells, and then immune cells were co-cultured with the infected cells. We found that mRNA expression levels of the TLR7 signaling pathway were increased when encountered with the infection of influenza virus, and that once the systems were treated with antiviral drugs, administration of CA could reduce expression of the cytokines on the TLR7 signaling pathway.

CA could also modulate the inflammatory response and reduce the over-expression of interferon, thus reducing the response of the very first sentinel inducing the cascading inflammation.

Data analysis showed that the expression levels of TLR70IRAK-10MyD880TAK10JNK and p38/MAPK in each group increased significantly when virus attacked ($P < 0.05$). This outcome suggested that these factors played an important role in identifying the invasion of virus and increasing the production of interferon. Otherwise, CA could reduce the expression of these TLR7 signaling-related cytokines, thus we could make good use of its efficacy for treating the severe symptoms at the later phase of infection. In a nutshell, this study have shown that TLR7-mediated immune reaction can provide a potential molecular target for the treatment of flu in humans, and a clue that the use of herbal drug Chlorogenic acid could attenuate the H1N1 virus-induced cell mortality.

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