The Antiadenovirus Activities of Cinnamaldehyde In Vitro

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Abstract

Background: Cinnamomum cassia is a common prescription compound in traditional Chinese medicine.

Methods: In this study, the inhibitory effect of cinnamaldehyde from Cinnamomi cortex on adenovirus (ADV) type 3 (ADV3) was investigated in vitro; virus inhibition ratio was detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method; ultrastructure changes in the ADV3 surface were observed by atomic-force microscope (AFM); apoptosis degree of caspase-3, caspase-8, and caspase-9 protein expression in cells infected with ADV3 were determined by using immunohistochemistry and Western blotting.

Results: Our results showed that the cinnamaldehyde (0.0195–0.315 mg/mL) inhibited the growth of ADV3 in a concentration-dependent manner with the virus inhibition rate of 3–58.6%, and the apoptosis degree of caspase-3, caspase-8, and caspase-9 protein expression was lower in the cinnamaldehyde-treated group than in the virus control group.

Conclusions: The results conclude that anti-ADV3 capabilities of cinnamaldehyde may associate with decreasing the apoptosis level and inhibiting the caspase-3, caspase-8, and caspase-9 protein expression.

Cinnamomum cassia Presl is an important medicinal and edible plant growing mainly in the Guangdong and Guangxi provinces in China. Cinnamomum cassia Presl, also known as C. cassia, belongs to the family Lauraceae. Cortex cinnamomi is the name given to the dry bark of the C. cassia Presl plant; it contains an essential oil whose main chemical composition is cinnamaldehyde. The essential oil also contains some cinnamic acid, cinnamon ether, cinnasol, and its glycosides. Cinnamaldehyde extracted from the C. cassia Presl plant has antibacterial, anti-inflammatory, and anti-tumor effects.

The adenovirus (ADV) is an important etiological agent that can cause human respiratory and alimentary infection. ADV infection in humans can lead to many diseases, such as acute upper and lower respiratory infection, fulminating conjunctivitis, acute hemorrhagic cystitis, rheumatoid arthritis, immunodeficiency disease, cerebritis, cerebral meningitis, and infant gastroenteritis. The reported structure and etiology of ADV are becoming increasingly diverse. Since only a few studies have been performed to examine the antiviral function of C. cassia Presl, the anti-ADV3 function of cinnamaldehyde was investigated in the study.

Materials and Methods

Cell Lines

The HeLa cell line used in this study was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and preserved by the Department of Hygienic Microbiology of Harbin Medical University in China.

Cell Culture

HeLa cells were cultured in 10% cell culture medium (Invitrogen, Carlsbad, CA) containing 10% newborn calf serum (NCS), penicillin (100 units/mL), and streptomycin (100 μg/mL). Conventional methods were used to culture and passage the cells. For the antiviral assay, the medium was supplemented with 2% NCS and the antibiotics.

Inoculation of the Virus

Human ADV type 3 (ADV3) was obtained from the American Type Culture Collection. The ADV3 solution (0.5 mL) was inoculated into the culture flask containing 70% single HeLa cells and allowed to stand for 120 minutes at 37°C. RPMI-1640 (Invitrogen) maintenance medium (1.5 mL) containing 2% NCS was added to the culture flask, and the mixture was incubated at 37°C under 5% CO2. When the HeLa cytopathic effect (CPE) reached more than 75%, the solution containing ADV3 was collected after a process of freezing (−80°C, 10 min) and melting at room temperature (RT) 3 times.

Determination of ADV3 Toxicity

The viral toxicity was assessed using the 50% cell culture infective dose (CCID50). A 10-time serial dilution of the virus-infected fluid was inoculated into a monolayer of HeLa cells, and the CPE was observed at 72 hours. The CCID50 can be calculated with the Reed-Muench method.

Determination of Cinnamaldehyde Toxicity

Different concentrations of cinnamaldehyde (Sigma Chemical, St. Louis, MO) were added to a 96-well cell-culture plates containing a monolayer of HeLa cells. The conventional MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Chemical) method was used to determine cinnamaldehyde toxicity. Optical density values were read using a microplate reader at 2 wavelengths (540nm and 690nm).

The Anti-ADV3 Effect of Cinnamaldehyde Determined by CPE and MTT

A 20 μL/well 100CCID50 ADV3 solution was inoculated into 96-well cell-culture plates, incubated for 2 hours at 37°C, and then the virus fluid was removed. Different concentrations of cinnamaldehyde in an atoxic range were added to the 96-well cell-culture plates (200 μL/well). For each cinnamaldehyde concentration, 8 duplicate wells, a cell control group, and a virus control group were tested simultaneously. When the virus control group CPE reached above 75%, the cell survival rate was detected by MTT method. Experiments were repeated 3 times.
AFM Analysis

The ability of cinnamaldehyde to influence the ultrastructure in ADV3 surface was tested by diluting the latter in 100 CCID_{50} with PBS and then balancing the mixture with TC_{0} cinnamaldehyde. After incubation for 1 hour at RT, the virus was observed with the aid of an AFM (PicoPlus, Molecular Imaging, Tempe, FL). The instrument’s own software was used for image data gathering and processing. All images were collected using a smoothing process only.

Immunohistochemistry Analysis

A streptavidin-biotin-peroxidase complex (SABC) immunohistochemical method was used to observe the effects of cinnamaldehyde on ADV3. Changes in cell morphology were detected using SABC kits (R&D, Shanghai, China) according to the manufacturer’s protocol. After hematoxylin counterstaining, the ADV3-infected cells were observed with the aid of a light microscope. Cells whose nucleus had a buffy granular appearance were apoptotic cells. Cells whose cytoplasm had a buffy granular appearance were the caspase-3+ cells, caspase-8+ cells, and caspase-9+ cells.

Western Blotting Analysis

Sample protein lysate was mixed with 6× loading buffer solution, denatured for 5 minutes at 100°C, and then subjected to 12% sodium dodecylsulfate-polyacrylamide gel electrophoretic separation followed by transffer to the protein to the nitrocellulose filter. The nitrocellulose filter was blocked with 1% bovine serum albumin overnight. It was then reacted with rabbit-anti-β-actin polyclonal antibody (BAB), rabbit-anti-caspase-3 BAB, rabbit-anti-caspase-8 BAB, or rabbit-anti-caspase-9 BAB (Invitrogen) respectively for 2 hours at 37°C, rinsed with Tris-buffered saline with Tween 20 (TBST) 3 times for 10 minutes each, and then incubated with alkaline-phosphatase-labeled goat-anti-rabbit IgG antibody (Invitrogen) for 1 hour at 37°C. The lysine was then rinsed twice for 10 minutes with TBST solution again, and finally stained using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Statistical Analysis

The data were processed with SAS (version 9.13). The drug median toxic concentration (TC_{50}) and the medium inhibitory concentration (IC_{50}) were calculated using the linear-regression method. A dependability analysis of the medicine dosage and its cytological effect (ie, cell survival rate, inhibition ratio) was then carried out to establish whether or not there was a dose-effect relationship. Variance analysis was used for group comparison. The following calculations were made:

HeLa cell survival rate (%) = medicine group absorbance value/cell control group absorbance value × 100%
Inhibition ratio (%) = 100% – survival rate
Virus inhibition ratio = (medicine treatment group absorbance value – virus control group absorbance value) / (cell control group absorbance value – virus control group absorbance value) × 100%
Therapeutic index (TI) = (TC_{50}) / (IC_{50})

Data were expressed as mean ± S.D. Statistical analysis was performed by using 1-way analysis of variance, and the differences between means were tested by using Duncan’s multiple range tests. A P-value of less than 0.05 was considered to be statistically significant.

Results

ADV3 Toxicity Assay

The suitable challenging dose of virus was identified by calculating the CCID_{50} using the Reed-Muench method. The CPE was observed with the aid of an inverted microscope. Compared with the normal cell group, HeLa cells infected with ADV3 became round and then clumped together, showing the typical “bunches of grapes” change. When 50% of the cells exhibited a CPE, they were regarded as CPE+ cells. If the cells did not exhibit any CPE after 72 hours, they were regarded as CPE− cells. The CCID_{50} of ADV3 in HeLa cells was 10^{3.95}. The challenging dose of virus used in the experiments was 100 CCID_{50}/100μL.

Toxic Effect of Cinnamaldehyde on HeLa Cells

The atoxic range of cinnamaldehyde was assessed by measuring the TC_{0} and TC_{50} using the MTT method. The toxic effect of cinnamaldehyde on HeLa cells reduced with decreasing concentration, with a concomitant gradual increase in the cell survival rate. The TC_{0} of cinnamaldehyde was 0.319mg/mL (Figure 1).

The Anti-ADV3 Effect of Cinnamaldehyde Assessed by the CPE and MTT

The MTT method has been proven to be a sensitive and accurate method for screening anti-ADV agents. Healthy HeLa cells were polygon- or diamond-shaped and arranged regularly. When viewed at a high magnification, the epithelium appeared normal, and the endochylema was clear. The virus exerted the following CPE on HeLa cells, as evidenced by the control group; cells developed a vacuole and swell, and became round; they fused together, exhibited the typical “bunches of grapes” change; and finally became necrotic and exfoliate. In the cinnamaldehyde-treated groups, the cells appeared normal, with...
only a few cells exhibiting the CPE characteristics: atrophy-like, string-like, overlapping, and exfoliation (Image 1). The virus inhibition rate positively correlated with the concentration of cinnamaldehyde (0.0195–0.315mg/mL), and its scope was from 3% to 58.6% in a concentration-dependent manner (Figure 2).

Effect of Cinnamaldehyde on ADV3 Observed Using AFM

This tool revealed the ADV3 nucleocapsid had a slightly globular but regular shape, and its surface was smooth. It was approximately 100 nm in diameter and approximately 6.09 nm in height. The cinnamaldehyde-treated nucleocapsid was smaller, with a diameter of approximately 40 nm and had an irregular shape (Image 2), which suggests that cinnamaldehyde...
can directly affect the surface ultrastructure of ADV3.

The Effect of Cinnamaldehyde on ADV3 Revealed by Immunohistochemistry and Western Blotting

The potential mechanism that cinnamaldehyde affected the ADV3 was investigated by assessing the changes in protein expression and cell morphology in HeLa cells infected with ADV3. When the experiment was finished, the percentage of apoptotic cells and the expression of caspase-3, caspase-8, and caspase-9 proteins were determined. Immunohistochemistry revealed that 4 of these parameters were increased in the virus control groups compared to the cinnamaldehyde-treated group (P<0.05; Table 1; Images 3-4). Western blotting further showed the expression of caspase-3, caspase-8, and caspase-9 proteins was negatively correlated with the concentration of cinnamaldehyde, confirming the immunohistochemistry results (P<0.05; Figure 3).

**Table 1. The Effect of Cinnamaldehyde on ADV3-Induced Apoptosis and Expression of Caspase-3, Caspase-8, Caspase-9 Proteins**

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptosis</th>
<th>Caspase-3</th>
<th>Caspase-8</th>
<th>Caspase-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>52.42 ± 5.91**</td>
<td>50.47 ± 5.86**</td>
<td>60.31 ± 6.57*</td>
<td>49.56 ± 5.39**</td>
</tr>
<tr>
<td>Virus control</td>
<td>83.65 ± 9.83</td>
<td>76.26 ± 6.39</td>
<td>77.53 ± 8.28</td>
<td>71.53 ± 5.28</td>
</tr>
</tbody>
</table>

Results are presented as the arithmetic mean (± SE) of 5 random fields of view. Single asterisk (*) and double asterisks (**) indicate P<0.05 and P<0.01, compared with virus control group. The data are representative of 3 separate experiments.

**Discussion**

In infants aged 6 months to 3 years, ADV cannot only cause viral pneumonia, but it can also give rise to long-term lung damage.19 In individuals with an impaired immune response, life-threatening ADV infections are common. Among these are patients with leukemia or acquired immunodeficiency syndrome (AIDS), and recipients of kidney or bone marrow allograft.20-21 Currently, the drug treatment is a main therapy for ADV infections; however there remains a lack of specific drugs.
for the adenoviral diseases that do not have strong side effects. Therefore, the search for highly-efficient anti-ADV drugs with fewer side effects has become a challenge to life sciences. Cinnamaldehyde is a major component from the volatile oil of cinnamon in traditional Chinese medicine. Over the past 30 years, many studies have shown that cinnamaldehyde had many pharmacological effects and low toxicity.22-25 However, there are few studies on the antiviral effect of cinnamaldehyde22 and no precise basis for the theory and experiment. Therefore, a study was performed to illustrate the anti-ADV effect of cinnamaldehyde. In the present study, the inhibition rate of virus, ultrastructure changes of virus surface, the extent of apoptosis, and expression of caspase-8, caspase-9, and caspase-3 proteins were detected to determine the anti-ADV effect and potential mechanism of cinnamaldehyde.

First, the results from the MTT method showed cinnamaldehyde (0.0195–0.315 mg/mL) inhibited the growth of ADV3 with the virus inhibition rate of 3–58.6%, which is concentration-dependent. The results from the CPE method demonstrated that most of the HeLa cells maintained a normal cellular shape with a normal epithelium, and only a small proportion of the cells presented pathological changes such as shrinking, clustering, overlapping, or exfoliation in the cinnamaldehyde groups. Thus, the 2 methods used in the study revealed that cinnamaldehyde obviously has an anti-ADV3 effect.

In order to observe the nucleocapsid change of cinnamaldehyde-treated ADV3 at physiological conditions, the external morphology of virus was detected by AFM. As expected, ADV3 treated with cinnamaldehyde had a small virus particle with a diameter of about 40nm, irregular shape, and uneven envelope. These findings confirm that the cinnamaldehyde directly kills and damages ADV3, further showing that cinnamaldehyde can work against ADV3.

Apoptosis or programmed cell death is a highly regulated process involving activation of a series of molecular events leading to cell death. Some studies have indicated that ADV has a cytotoxic effect on human tumor cells, inducing apoptosis.28 In order to clarify the potential anti-ADV3 mechanisms of cinnamaldehyde, the degree of apoptosis and the expression of caspase-3, caspase-8, and caspase-9 proteins were evaluated by using immunohistochemistry and Western blotting. Our results showed that the apoptosis degree in cinnamaldehyde-treated groups was obviously lower than that in virus control groups, suggesting the anti-ADV3 effect of cinnamaldehyde possibly correlated with the apoptotic mechanism. In addition, the decrease in expression of caspase-3 in cinnamaldehyde-treated groups further demonstrated that the anti-ADV3 effect of cinnamaldehyde may inhibit the caspase-mediated apoptosis pathway because many studies have reported that induction of caspase-3 is the only way of activating the apoptosis protease cascade reaction.29-31

Previous studies have reported caspase-8 and caspase-9 are essential proteases in 2 caspase-mediated apoptosis processes involving the extrinsic death receptor and intrinsic mitochondrial pathways.32 In our study, the results of immunohistochemistry and Western blotting showed the expression of caspase-8 and caspase-9 significantly decreased in the cinnamaldehyde-treated group, implying that the anti ADV3 mechanism of cinnamaldehyde may inhibit the caspase-mediated intrinsic and extrinsic apoptotic pathways in a concentration-dependent manner. The Acknowledgements: We thank Ms. Weijun Lu and Ms. Yuehui Zhao for their assistance with laboratory techniques. This study was supported by National Natural Science Foundation of China (grant No. 30771909), Ministry of Education of China (grant No. 20070226007), and a grant from the key project of natural science foundation of Heilongjiang Province of China (grant No. ZJY 0701). Corresponding author: Zhang-yi Qu, PhD.