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Original Article

Characterization of Proinflammatory Cytokine Profiles Produced in Different Serotypes of Adenoviruses-infected Human Lung Alveolar A549 Cells

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Approximately 5%-10% of children with acute airway infections can be attributed to adenovirus infection. Adenovirus (AdV) infection usually causes fever and further severe acute respiratory and conjunctival infection, which may lead to death in vulnerable subjects such children younger than 6 years old. In clinics, infectious AdVs are divided into 6 species and 51 serotypes. Among the identified infectious AdVs, species B (serotypes AdV3 and AdV7) and species C (serotypes AdV1, AdV2 and AdV5) are highly associated with respiratory tract infections and may persist in children without causing symptoms for years. Intriguingly, infection of AdV3 and AdV7 usually causes severe syndromes compared to infections by other serotypes AdV. The immune responses induced by the different serotypes AdV are rarely investigated. In the present study, we collected AdV samples isolated from patients with respiratory infection in middle Taiwan during 2004-2005 and measured the different proinflammatory cytokines produced in the AdV-infected lung alveolar A549 cells. Production of cytokines was quantitated using sandwich ELISA. Our results revealed that AdV3 induced the highest level of interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) compared to the other AdV1, AdV2, AdV5 and AdV6. These findings provide evidence that AdV3, compared with other AdVs, robustly induces the production of various inflammatory cytokine, which may further contribute to severe airway inflammation found clinically.

Key Words: Adenovirus, serotypes, proinflammatory cytokines, airway infection

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Introduction

Acute viral lower respiratory tract infection is a leading cause of hospitalization for infants and young children in developed countries and is a major cause of death in developing countries[1-3]. Adenovirus (AdV) is a major cause of acute

respiratory tract infection (RTI) in children, and the infection usually causes fever and might further induce severe acute respiratory and conjunctival infections, which may further lead to death particularly in children younger than 6 years old[4].

Detection of AdV in viral culture can be performed by immunofluorescence and enzyme-linked immunosorbent assay. Typing and subtyping is performed by immunofluorescence, neutralization tests, polymerase chain reaction (PCR), and restriction fragment length polymorphism[5,6]. In Taiwan, AdV is a major pathogen causing viral pneumonitis and is responsible for 5-10% of acute RTIs in children younger than 5 years old[7]. However, only a few descriptions of the molecular epidemiology have been published[8-10].

Among the identified infectious AdV, Subgenus B1, including AdV serotypes 3 and 7 (AdV3 and 7), subgenus C [serotypes 1, 2, 5 and 6, (AdV1, 2, 5 and 6)], and subgenus E [serotype 4, AdV4)] are common causes of RTIs[11]. AdV3 and 7 are responsible for most epidemics of lower RTI in children, although they are also identified in sporadic cases[12-14]. Infection by subgenus B1 (AdV3 and AdV7) usually causes more severe symptoms than infection by the other serotype subgenus in children[15]. However, the pathogenic mechanisms leading to the severe illness by subgenus B-AdV are incompletely understood.

Because the inflammatory cytokine storm induced by virus may cause more tissue injury than the cell death caused by virus replication[16,17], the present study aimed to profile the proinflammatory cytokines induced by different serotype AdV and provide etiological evidences to interpret the correlation between severity of infection and subgenus of AdV for younger children in Taiwan.

Materials and Methods

Isolation and characterization of AdV

SHuman lung alveolar cell line A549 was purchased from Biosource Collection and Research Center (Hsinchu, Taiwan) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and 100 μg/mL penicillin/streptomycin (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Fifty μL of specimen obtained from respiratory infection patients was inoculated onto a monolayer of A549 cell culture grown over culture plate after the medium was removed. The inoculated culture was kept in a shaker for 30 min, and then DMEM supplemented with 1% v/v fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) was added. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2, and monitored daily by phase-contrast microscope (Nikon, Tokyo, Japan) for the presence of cytopathic effects. The isolation of AdV was confirmed by immunofluorescence analysis using antibodies against hexon protein of human AdV (MAB805, Chemicon International, Harrow, UK) and anti-mouse IgG conjugated with FITC (Cell Signaling Technology, Beverly, MA, USA). Immunofluorescent image was acquired by a fluorescence microscope and photographed (Axioplan, Zeiss, Germany).

For purity examination, all the isolated AdV samples were analyzed by PCR and RT-PCR to detect specific DNA sequence deduced human adenoviral hexon region and human enterovirus genome, respectively. Only the isolated virus samples which were positive for human AdV by both PCR analysis and culture tests and negative for human enterovirus genome by RT-PCR analysis were collected. Serotype identification for the collected AdV samples were performed by Control of Disease Center (CDC; Department of Health, Excusive Yuan, R.O.C). The AdV samples whose seroptypes had been identified by CDC were used for further investigation.

Virus infection and cytokine quantitation

AdV serotypes 1, 2, 3, 5, and 6 identified by The Center for Disease Control of Taiwan were used to infect A549 cells. The virus infection was performed as described previously. Briefly, A549 cells at densities of $2x10^5$ cells/mL in 6-cm culture plate were infected with AdV from a stock culture (20 p.f.u. cell). After the infection with AdV for 8, 24 and 48 hours, cultured medium was collected

for cytokine quantitation. The concentrations of IL-1β, IL-6, IL-8, and TNF-α were determined by sandwich ELISA (R&D systems, Minneapolis, MN, USA) according to manufacturer's instructions. Three independent infections were measured for statistical analysis.

Statistical analysis

Data were expressed as means ± SEMs for the three independent experiments. Significance was analyzed by using 1-way ANOVA followed by Dunnett for multiple comparisons with the control or the impaired 2-tailed Student *t* test. The differences were considered significant if *p* values were less than 0.05.

Results

Infection of AdV

To confirm the infection of AdV, A549 cells infected with different serotypes of AdV were analyzed by immunofluorescence assay. As shown in Fig. 1, adenovial hexon proteins presented on infected A549 cells were detected by the specific monoclonal antibodies. These data indicated that the infection of AdV was successful and pathogenic. In addition, the average infection rate of each AdV serotype ranged from 72 - 77%. There was no significant difference between different AdV serotypes. Therefore, this infection protocol was used for analyzing proinflammatory cytokines produced by AdV-infected A549 cells.

A549 cells infected with AdV3 produced the highest level of IL-1β

After being infected with a serial serotypes of AdV for different incubation times (8, 24 and 48 h), the cultured media were collected for IL-1β quantitation by sandwich ELISA method. As shown in Fig. 2, IL-1β production induced by the infections with all the AdV serotypes for 8, 24, or 48 h was significantly increased up to 43.6-fold compared to the control (no infection). Interestingly, after 24 h-incubation, IL-1β production induced by the infection with AdV3 was significantly increased compared to infection with the other AdV ($P<0.05$). In addition, IL-1β production was insignificantly changed among the

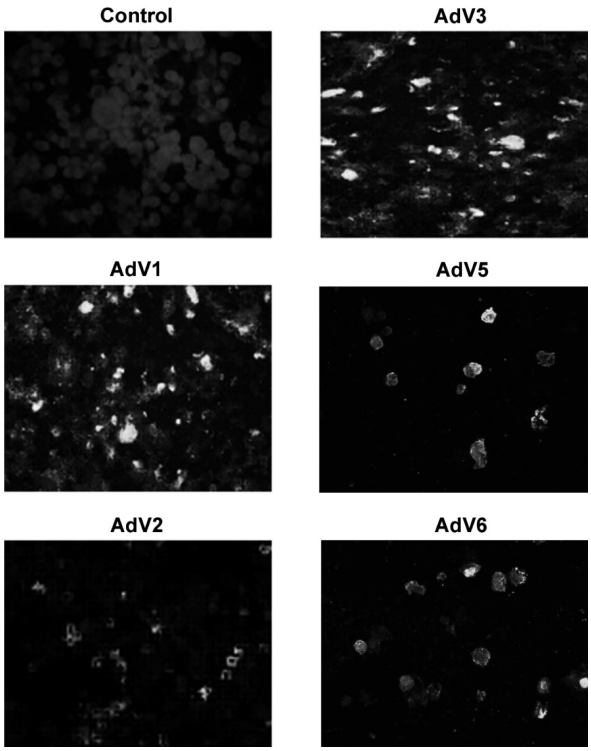

Figure 1. Identification of serotypes. A549 cells were infected with isolated AdVs as described in Materials and methods. Specific antigen presented on the infected cells was determined by using immunofluorescence staining. Identified serotypes were indicated (AdV1, AdV2, AdV3, AdV5 and AdV6).

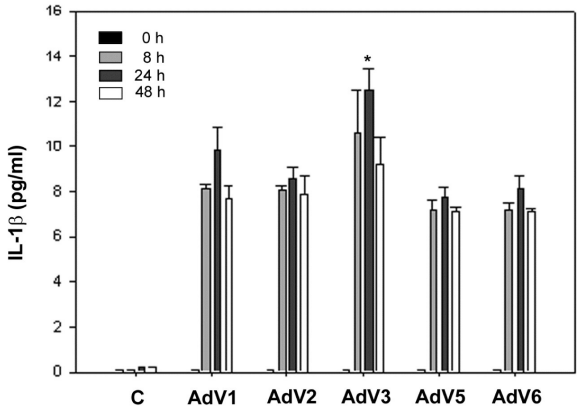

Figure 2. IL-1β production by AdV-infected A549 cells. Cells were infected with individual serotype AdV and incubated for 8, 24 and 48 h. After the incubation, cultured medium was collected for IL-1β quantitation using sandwich-ELISA assay. Three independent experiments were performed for statistical analysis. *, P <0.05 as compared to the AdV1 with the same incubation time.

infection with AdV1, 2, 5 and 6 for 24 h (P=0.359), as well as among the infection with AdV1, 2, 3, 5 and 6 for 8 h or 48 h. These results revealed that 24 h-infection with AdV3 induced the highest level of IL-1β production compared to the other tested AdV.

Increase of IL-6 produced by A549 cells infected with different serotypes of AdV

Since IL-1β production was significantly increased by AdV infection, production of other important proinflammatory cytokines was further analyzed. As shown in Fig. 3, IL-6 production by the infections with all the AdV serotypes for 8, 24, or 48 h was increased up to 4.2-fold compared to the control (no infection). Notably, after 24 h- and 48 h-incubation, IL-6 production induced by the infection with both AdV1 and AdV3 was significantly increased compared to infections by the other AdV (P<0.05). In addition, IL-6 production was insignificantly changed by infections with AdV2, 5 and 6 for 24 h (P=0.102), as well as by infections with AdV1, 2, 3, 5 and 6 for 8 h. These results reveal that AdV1 and AdV3 induce the higher levels of IL-1β production than the other tested AdV.

A549 cells infected with AdV3 produced the highest level of IL-8

IL-8 plays a pivotal role in airway inflammation attributed to viral infections[18]. Thus, whether IL-8 production is elevated by AdV infections was also analyzed. As shown in Fig. 4, IL-8 production by the infections with all the AdV serotypes for 8, 24, or 48 h was significantly increased up to 19.6-fold compared to the control (no infection). After 8 h- and 24 h-incubation, IL-8 production induced by the infection with AdV3 was significantly elevated compared to the infections with the other AdV (P<0.05). In addition, IL-8 production was negligibly changed among the infections by AdV1, 2, 5 and 6 for 8, 24, or 48 h (P=0.425, 0.884, and 0.716, respectively). These results reveal that 8 h- and 24 h-infection with AdV3 induced the highest level of IL-8 production compared to the other tested AdV.

Significant increase of TNF-α produced by A549 cells infected with AdV3

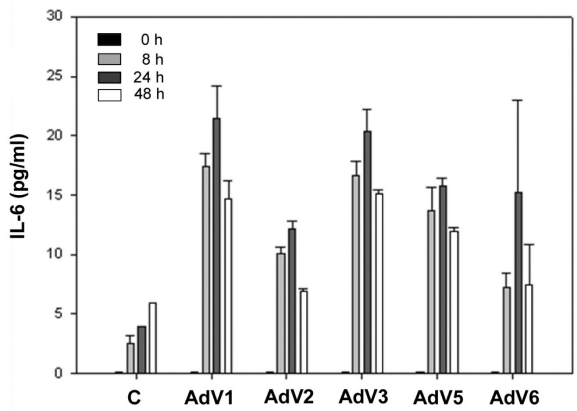

Figure 3. IL-6 production by AdV-infected A549 cells. Cells were infected with individual serotype AdV and incubated for 8, 24 and 48 h. After the incubation, cultured medium was collected for IL-6 quantitation using sandwich-ELISA assay. Three independent infections were performed for statistical analysis.

TNF-α has been regarded as a central proinflammatory cytokine leading to lung injury[19]. Accordingly, whether TNF-α production is induced by AdV infections was investigated. Our results revealed that TNF-α production was significantly enhanced by the infections with AdV1, AdV3 and AdV5 for 24 or 48 h compared

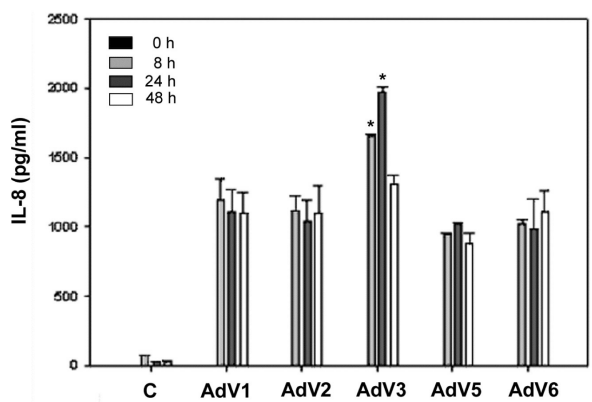

Figure 4. IL-8 production by AdV-infected A549 cells. Cells were infected with individual serotype AdV and incubated for 8, 24 and 48 h. After the incubation, cultured medium was collected for IL-8 quantitation using sandwich-ELISA assay. Three independent infections were performed for statistical analysis. *, *P* <0.05 as compared to the AdV1 with the same incubation time.

to the control (no infection) (Fig. 5). Intriguingly, infection with AdV3 for 8, 24, or 48 h induced the highest production of TNF-α compared to the other infections of AdV, respectively ($P<0.05$) (Fig. 5). In addition, TNF-α production induced by AdV3 infection was proportional to the incubation time (Fig. 5). Notably, TNF-α production by infection with AdV1 and AdV5 for 8 h, as well as infection with AdV2 for 8, 24 and 48 h, was insignificantly altered compared to the control (no infection) ($P>0.15$). These findings showed that AdV3 infection induced the highest level of TNF-α production compared to the other tested AdV, while AdV2 infection only negligibly induced TNF-α production.

Discussion

AdV was the second commonest respiratory virus reported in hospitalized children between 1997 and 1999 in Taiwan[20,21]. It has also been reported as the major cause for acute respiratory infections and severe pneumonia on children younger than 2 years old in children hospitals in Bucharest, Romania[22]. However, serotype distribution for the hospitalized children has not been comprehensively reported. The higher overall incidence of respiratory infection with AdV in these patients in autumn and winter is consistent with the data from the Centers for Disease Control in Taiwan. In the present study, we investigated the profiles of proinflammatory cytokines induced by AdV infections, and the results revealed that the profile induced by individual AdV serotype is specific. These findings suggest that infection with each AdV serotype triggers a different immune response, which may lead to unique syndrome as observed in clinical cases.

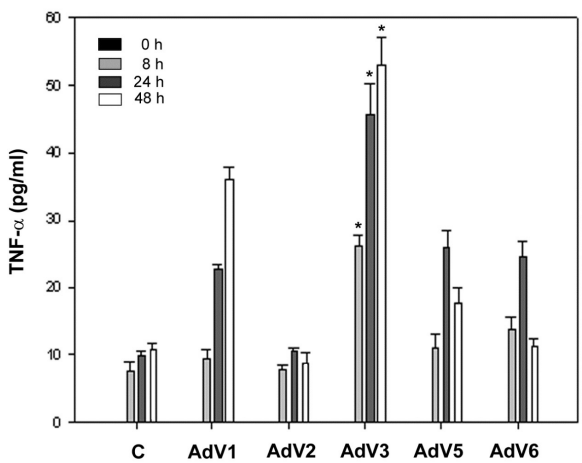

Figure 5. TNF-α production by AdV-infected A549 cells. Cells were infected with individual serotype AdV and incubated for 8, 24 and 48 h. After the incubation, cultured medium was collected for TNF-α quantitation using sandwich-ELISA assay. Three independent infections were performed for statistical analysis. *, $P<0.05$ as comparing to the AdV1 with the same incubation time.

From middle 1999 to early 2000, AdV3 infection was the main cause hospitalizations of children compared to the other common AdV infections[15]. AdV infection has been reported to induce the expression of several proinflammatory cytokines including TNF-α, IL-1β, and IL-6, accompanying the infiltration of the lung by alveolar macrophages and neutrophils[23,24]. In addition, infection of recombinant adenoviral vectors is reported to enhance the expression of TNF-α, IL-1, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), IL-6, and granulocyte/macrophage colony-stimulating factor (GM-CSF)[25,26]. Our findings revealed that 24 h-infection of AdV1, 2, 3, 5, and 6 significantly elevated production of IL-1β, IL-6, IL-8 and TNF-α compared to our non-treatment controls. In addition, 24 h-infection of AdV3 induced the highest production of IL-1β, IL-6, IL-8 and TNF-α, suggesting that AdV3 infection can contribute to the acute respiratory inflammation, a finding consistent those of clinical observation.

In conclusion, we isolated and identified five AdVs from patients with acute respiratory infection, and demonstrated that the each AdV infection triggered a significant production and a specific profile of proinflammatory cytokines by lung alveolar cell. These findings provide evidences that AdV3 is a potent risk factor among the AdVs commonly causing acute respiratory infection and airway inflammation and AdV3 may attribute its robust capability of inducing proinflammatory

cytokine production.

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Original Article

不同血清型腺病毒感染肺癌細胞株A549所分泌之前發炎細胞激素研究

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5%-10%的兒童急性呼吸道感染皆由腺病毒感染所引起，並進而造成嚴重的急性呼吸道感染、結膜感染甚至死亡。人類腺病毒目前已知有6個亞屬包含51種血清型。血清型第1、2、3、5、6型為常見造成呼吸道感染的腺病毒，其中又以血清型第3型與第7型為最容易導致嚴重病症。腺病毒呼吸道感染及進一步誘發的呼吸道發炎與損傷機制至今尚未完全明瞭，但一般認為前發炎細胞激素的誘發扮演相當重要的角色。因此，本研究擬由2004-2005年間感染腺病毒的病人檢體中，分離與鑑定不同血清型的腺病毒，並進一步探討其對於誘發肺癌細胞株A549所分泌的前發炎細胞激素之類型與濃度進行分析探討。我們利用分離所得之血清型第1、2、3、5、6型腺病毒進行對A549細胞的感染，培養8、24、48小時後收集培養基進行前發炎細胞激素IL-1β、IL-6、IL-8及TNF-α的定量分析。結果顯示，腺病毒感染後確實顯著提升前發炎細胞激素的分泌，這些與先前的臨床檢驗相符。此外，相較於本實驗的其他腺病毒，血清型第3型的腺病毒所誘發的前發炎激素最高，推測可能與血清型第3型腺病毒在臨床上最容易導致呼吸道重症有關。綜合本研究的結果，發現不同血清型腺病毒會誘發不同程度的前發炎細胞激素之分泌，而且這些差異與其在臨床上所引起之症狀嚴重情形相關。另外，基於本研究結果，我們建議監控腺病毒感染患者的IL-1β、IL-8及TNF-α的濃度，可以在完成病毒血清型鑑定完成之前，或許可以幫助醫師評估病人的病情發展與監測藥物療效。

關鍵詞：腺病毒、血清型、前發炎細胞激素、呼吸道感染

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