Hepatic damage caused by coxsackievirus B3 is dependent on age-related tissue tropisms associated with the coxsackievirus-adenovirus receptor

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This study brings a novel angle on coxsackievirus B (CVB) pathogenesis, as most of the previous studies have focused on heart or pancreas disease. Here, it is shown that infection by the human isolate CVB3 results in hepatocyte necrosis and apoptosis. The role of the viral receptor CAR is also studied, and amelioration of the symptoms after treatment with anti-CAR antibody is demonstrated, thus hinting at the potential importance of targeting CAR for therapeutic development.

Keywords
coxsackievirus B; coxsackievirus-adenovirus receptor; hepatitis; receptors.

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Abstract
Coxsackievirus B (CVB) and enterovirus 71 (EV71) are important causes of severe enteroviral diseases in neonates or young children in Taiwan. CVB can cause fulminant hepatitis, myocarditis or meningoencephalitis. This study was designed to explore the role of coxsackievirus-adenovirus receptor (CAR) in the pathogenesis of CVB3-infected hepatocytes via in vitro and mice studies. CVB3 (CVB3/2630) was isolated from liver tissue of a neonate with fulminant hepatitis. Cell lines A549, HeLa, HEp2 and Huh-7 were maintained in Dulbecco's modified Eagle’s medium. Mice progeny 1 or 7 days old were used in the experiments. Viremia was noted in 7-day-old ICR mice 2 h after intraperitoneal injection. The highest viral titers were detected in blood, liver and spleen. Histopathological studies of the liver demonstrated polymorphonuclear cell infiltration, massive hepatic cell necrosis and apoptosis. CAR was expressed more in liver than in other tissues. Expression of CAR decreased with mouse age. Anti-CAR monoclonal antibody prevented infection of Huh-7 cells from CVB3. Furthermore, anti-CAR monoclonal antibody pretreatment can reduce mortality and decrease the level of liver enzymes in CVB3-infected mice. These findings indicate that CAR plays an important role in the initiation of CVB infections and is closely associated with hepatotropism and age-specific susceptibility.

Introduction
The coxsackieviruses, polioviruses, echoviruses and other enteroviruses belong to the genus Enterovirus in the family Picornaviridae. Previously, coxsackieviruses were classified as subgroups A and B according to their pathogenicity in newborn mice (Hyypiä et al., 1997). Coxsackievirus B (CVB) 1-6 has been classified in the human enterovirus B (HEV-B) species (Fauquet et al., 2005). CVB infection may induce complications of the central nervous system (CNS), liver, heart, pancreas, and occasionally muscle (Crowell & Landau, 1997; Verboon-Maciolek et al., 2002). Some serotypes of echoviruses and coxsackieviruses can also cause severe hepatitis in neonates (Kaplan et al., 1983; Wong et al., 1989; Wang et al., 1998; Abzug, 2001). Prior to the introduction of universal vaccination in 1984, hepatitis B accounted for 65% of childhood fulminant hepatitis in Taiwan (Chang et al., 1987; Chen et al., 1987, 2004; Hsu et al., 1988). CVB is at present one of the most important causes of fulminant hepatitis among infants in this country (Wang et al., 1998). Maternal–infant transmission is thought to be the major route of infection.

Several outbreaks of CVB infections were reported in Taiwan from 1994 to 2005. The most common serotypes were CVB1, CVB3 and CVB4. In these outbreaks, fatal cases were usually associated with fulminant hepatitis,
characterized pathologically by extensive hemorrhagic hepatic necrosis and viremologically by isolation of CVB from liver tissue. Laboratory findings showed coagulopathy associated with thrombocytopenia, prolonged clotting times, and increased fibrin degradation products (Wang et al., 1998; Abzug, 2001). Although most affected neonates have a mild illness, nonspecific fever or rash, a small proportion of infected newborns may develop severe illness in the first 2 weeks of life (Verboon-Maciolek et al., 2002).

Attachment to specific host cell surface receptors is required for initiation of enterovirus infections. Based on studies of absorption of CVB1 and CVB3 in tissue homogenates, the presence and relative abundance of enterovirus-specific receptors in different tissue types has been proposed as a mechanism that accounts for differences in host range, viral tropism and age-specific virulence (Kunin, 1962). Coxsackievirus-adenovirus receptor (CAR) plays a major role in CVB infection and may be involved in CVB host cell interactions. CAR is a member of the immunoglobulin (Ig) superfamily with one variable and one constant Ig domain, a single-pass transmembrane domain, and a cytoplasmic domain (Tomko et al., 1997). CAR mRNA has been detected in a variety of murine and human tissues (Bergelson et al., 1997; Fechner et al., 1999). The mouse CAR (mCAR) has been shown to be a receptor for CVB by transfecting mCAR into nonpermissive CHO cells (Tomko et al., 1997). It has been postulated that the lack of CAR expression in placenta may help prevent vertical transmission of virus to the fetus (Koi et al., 2001).

The aims of the current study were to (1) compare the growth and infectivity of CVB3/2630 strain in various cell lines, including HEp2, A549, HeLa and Huh-7; (2) perform CVB infection in a mouse model to determine the histological findings; (3) characterize expression of CVB infection and its association with age; and (4) study the role of anti-CAR antibody in blocking CVB infection.

Materials and methods

Viruses and cell culture

Cell lines A549, HeLa, HEp2 and Huh-7 (derived from a human hepatoma) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone) plus penicillin (200 U mL⁻¹), and streptomycin (100 µg mL⁻¹) at 37 °C in a 5% CO₂ incubator. CVB3/2630, isolated from a patient with fulminant hepatitis, was kindly provided by the virology laboratory of National Cheng Kung University Hospital.

One-step growth kinetics and cytotoxicity of CVB3

A549, HeLa, HEp2 and Huh-7 cell monolayers in 24-well plates (2 × 10⁵ cells) were infected with CVB3/2630 at a multiplicity of infection (MOI) of 0.1 for 1 h at 37 °C. The cells were washed in phosphate-buffered saline (PBS) and then cultivated in DMEM containing 2% FBS. Virus titers were determined at different intervals by plaque assay using A549 cell monolayers in 24-well plates. For comparison of the cytotoxicities of CVB strains, Huh-7 cells (5 × 10⁵) were seeded in 96-well plates for 18 h at 37 °C before infection with CVB3. Cell viability was monitored at different intervals by a colorimetric assay (Cell Counting Kit-8; Dojindo Molecular Technologies, Gaithersburg, MD).

Plaque assay

A549 cells were seeded in 24-well plates containing 10% FBS DMEM medium, incubated overnight. The supernatants were removed sequentially from each well and immediately replaced with 100-µL samples, serially diluted with 2% FBS DMEM medium. Plates were incubated for 1 h at 37 °C and drained every 10 min. The overlay medium (1 mL per well of DMEM containing 2% FBS and 0.5% methylcellulose) was then added after washing. Plates were incubated at 37 °C. After 2 days, the medium was removed and replaced with a crystal violet solution for 1 h. The plates were washed with water and the plaque number was counted.

TUNEL assay

Apoptotic cells were examined according to a terminal transferase-mediated dUTP nick-end labeling (TUNEL) kit (ApoAlert DNA fragmentation assay kit; BD Clontech, Palo Alto, CA). Tissue sections were dewaxed and dehydrated for 15 min. After deparaffinization, sections were digested with proteinase K (20 mg mL⁻¹, Sigma Chemical Co. Ltd, Gillingham, Dorset, UK) for 5 min at room temperature. After washing with PBS, slides were immersed in TdT equilibration buffer for 10 min. Specimens were then reacted with the TdT reaction mixture (TdT solution plus digoxigenin-11-dUTP and dATP) for 60 min at 37 °C. After washing with PBS, the slides were treated with 2× standard saline citrate (SSC) for 15 min. Apoptosis was assessed by fluorescence microscopy (Olympus).

Blocking of CVB3 infection by anti-CAR monoclonal antibody

Huh-7 cells (1.5 × 10⁵) were seeded in 24-well plates and incubated for 24 h at 37 °C. Cells were pre-incubated with 100 µL anti-CAR monoclonal antibody (Upstate, Lake Placid, NY) at 1:100, 1:200 or 1:400 dilutions at 37 °C for 1 h. Cells were then challenged with 100 µL of 10-fold serial dilutions of the stock CVB3 preparation (6 × 10⁶ PFU µL⁻¹). The overlay medium was added to the wells and incubated for 48 h. The cells were stained with a crystal violet-methanol solution and washed with water.

Mice

Specific-pathogen-free ICR mice 6 to 8 weeks old were purchased from the Laboratory Animal Center, National Cheng Kung University. Mice progeny 1 or 7 days old were used in current experiments. This study was carried out in strict accordance with the recommendations in the guide for the care and use of laboratory animals of the National...
Science Council, Republic of China. The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Cheng Kung University.

Virus inoculation of animals
ICR mice 7 days old were inoculated by intraperitoneal route with 3 × 10^5 plaque-forming units (PFU) of CVB3 or 2% FBS DMEM medium. Mice were killed by pentobarbital sodium anesthesia (5 mg per mouse). Blood was collected in tubes containing EDTA prior to perfusing the organs with sterile normal saline. The liver was immediately removed and weighed. It was frozen in 1 mL of 2% FBS DMEM medium and homogenized with a glass homogenizer. Then the homogenates were frozen and thawed three times and centrifuged at 4000 g for 20 min. The supernatants were collected and serially diluted for viral titer assays. Further, 1- or 8-week-old ICR mice were pretreated intraperitoneally with anti-CAR monoclonal antibody (2 or 5 μg per mouse) and then the mice were infected with CVB3 or added medium.

Liver enzymes levels and histopathological studies
Whole blood obtained from ICR mice was centrifuged at 4000 g for 20 min. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) in the supernatants were measured with an automatic analyzer (Hitachi 747, Japan). Following sacrifice, the mice were perfused with 5 mL 3.7% paraformaldehyde followed by 5 mL of sterile normal saline solution. Tissues were removed and immersed in 3.7% formaldehyde/PBS for 48 h. Paraffin blocks were sectioned and stained by hematoxylin and eosin for histological examination. For the histopathological study, the mice were killed at 0, 6, 24 and 48 h postinfection.

RNA extraction and RT-PCR
ICR mice 7 days old were sacrificed and perfused with sterile normal saline. The heart, lung, liver, spleen and brain were removed and homogenized. Total tissue RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Tissue RNA (5 μg) was mixed with oligo-dT and then subjected to thermal cycling for 5 min at 65 °C, cooled to 25 °C for tissue RNA for denaturing and annealed with primers. Samples were added to a reverse transcriptase (RT) reaction mixture containing 32 U RNase inhibitor, 0.5 mM dNTPs, buffer (Promega, Madison, WI), and 10 U AMV reverse RT (Promega) and reverse transcribed at 42 °C for 90 min and then at 90 °C for 5 min to inactivate RT. The cDNA was further used for PCR amplification in the reaction solution of 0.2 mM dNTP, 0.2 mM sense and antisense primers, 1× reaction buffer (KST), and 1 unit of Biothermal DNA polymerase (KST). The reaction was performed in a PCR thermal cycler (Applied Biosystems 2720, Singapore) for 12 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 40 s at 58 °C, 60 s at 72 °C and, finally, 10 min at 72 °C. PCR products were separated by 1.2% agarose gel, stained with ethidium bromide, and viewed under UV light. The primers for detection were as follows: GADPH cDNA (304-bp) sense: 5′-CAGGACACTTCAACACACCAG-3′ antisense: 5′-CAGTCTTTCGAGTAGGCAGTG-3′, CAR cDNA (549-bp) sense: 5′-GGCCGCCCCTACTGTGCTTCG-3′ antisense: 5′-CTGCCAGCCATGGCGTAGGC-3′.

Statistical analysis
Data were expressed as mean ± SD. Statistical significance was determined by paired or non-paired nonparametric tests, using SPSS software (version 11.5, Chicago, IL). P < 0.05 was considered to be significant.

Results
Replication of CVB3 in Huh-7, A549, HEp2 and HeLa cells
Clinical isolate CVB3/2630 was used to determine the kinetics of one-step growth curves (MOI of 0.1) in Huh-7, A549, HEp2 and HeLa cell lines (Fig. 1). There were differences in the one-step growth curves of these isolates at earlier time points, but they were similar at 48 h in all four cell lines as determined by growth rates and production of infectious virus. The A549 cells were infected less efficiently and evidently more cycles were needed to produce the same level of the virus. It is known that this cell line is heterogeneous which may be one reason for this finding.

Distribution of CVB3 in mouse organs
To investigate the tissue tropisms of CVB3, three 7-day-old ICR mice were injected intraperitoneally with 3 × 10^5 PFU. Blood samples and the organs were collected at 0, 2, 6, 24 and 48 h postinfection (pi). Viral titers were determined by plaque assay (Fig. 2a). CVB3 was initially detected in the blood and liver at 2 h pi; in lung, liver and spleen at 6 h pi; and in all tested organs, including the brain and heart, at
The highest viral titers were detected in blood and liver followed by spleen. CVB3 was initially detected in the blood and liver at 2 h post infection. The highest viral titers were detected in blood and liver followed by spleen.

CVB3 was detected from blood through 0–48 h of infection. To study the change of liver function after CVB3 infection in vivo, 7-day-old ICR mice were inoculated with DMEM containing 2% FBS (mock control) or with 3 × 10^5 PFU CVB3 per mouse by the intraperitoneal route. Serum levels of AST, ALT, and LDH were elevated 6 h after infection and were significantly elevated 48 h postinfection (Fig. 2b).

Histopathological and apoptotic studies of CVB3-infected mice

To examine the histopathological features of CVB3 infection, 7-day-old ICR mice were inoculated intraperitoneally with DMEM (mock control) or CVB3 (3 × 10^5 PFU per mouse). The tissue were examined at the 0, 6, 24 and 48 h. Histological examinations revealed polymorphonuclear cell infiltration and death of hepatic cells 6 h pi. Massive hepatic cell necrosis with more extensive polymorphonuclear cells infiltration was noted at 48 h pi (Fig. 3a). The level of hepatic cell death increased along with time after infection. To determine the apoptotic change associated with hepatic cell death following CVB3 infection, the liver sections were examined by TUNEL assay. Apoptotic hepatic cells could be detected by TUNEL staining and increased with the time of infection from 6 to 48 h (Fig. 3b). These findings indicate that CVB3 caused the death of hepatic cells by both necrosis and apoptosis.
Expression of CAR in tissues of CVB3-infected mice

To investigate the effect of age on the expression CAR, organs obtained from 1-, 7-day and 8-week-old ICR mice were homogenized and subjected to RT-PCR assay. mCAR was found to be expressed predominantly in brain, heart, lung and liver, and to a lesser extent in the spleen of 1-day-old ICR mice (Fig. 4a). The expression of mCAR decreased with increasing mice age, especially in liver and spleen of mice older than 8 weeks. The age-dependent CAR expression in liver was shown by immunohistochemistry (Fig. 4b).

Blocking of CVB3 infection by anti-CAR monoclonal antibody

To further elucidate the role of CAR in the pathogenesis of CVB3 hepatic damage, the ability of anti-CAR monoclonal antibody to block CVB3 infection was determined in Huh-7 cell lines. Anti-CAR monoclonal antibody was able to inhibit CVB3 replication in a dose-dependent manner in vitro (Fig. 5). This experiment provides further evidence of the key role of the CAR in the initiation of CVB3 infection in the hepatoma cell line. Further, using anti-CAR monoclonal antibody (2 or 5 µg per mouse) 1-day-old ICR mice were pretreated and then infected with CVB3. Anti-CAR monoclonal antibody treatment significantly improved the survival rate of mice (Fig. 6a). The change of the level of liver enzymes was also tested in 7-day-old ICR mice pretreated with anti-CAR antibody and then infected by CVB3. The serum levels of AST, ALT and LDH in these mice were decreased (Fig. 6b).

Discussion

CVB is currently one of the most important causes of fulminant neonatal hepatitis in Taiwan. It was found that CVB3/2630, isolated from a newborn with fulminant hepatitis, replicates at about the same rate in A549, HEp2, HeLa and Huh-7 cell lines. The highest viral titers in CVB3-infected ICR mice were in blood and liver. Blood levels of AST, ALT and LDH in CVB-infected mice peaked at 48 postinfection. This was associated with histopathological findings of polymorphonuclear cell infiltration and massive hepatic necrosis and apoptosis. Expression of mCAR receptors was most prominent in brain, heart, lung and liver in 1-day-old mice and decreased with increasing age. Finally, anti-CAR monoclonal antibody blocked CVB3 infection of Huh-7 cells in a dose-dependent manner. Anti-CAR monoclonal antibody treatment also reduced the mortality and decreased the serum level of liver enzymes in ICR mice infected with CVB3. Early studies (Kunin, 1962) also showed that CVB1 and CVB3 were absorbed in a temperature- and age-dependent manner by a heat-labile substance present in homogenates of mouse brain and liver.

Neonatal CVB disease may range from asymptomatic infection to overwhelming systemic illness or even death.
Common clinical complications associated with severe neonatal CVB infections include meningoencephalitis, pneumonia, myocarditis and hepatitis. In Taiwan, severe CVB complications in neonates are more commonly associated with hepatic than neurologic or cardiac disease (Wang et al., 1998). Transmission of enteroviruses from mother to infant is relatively common (30–50%) (Brown & Karunas, 1971). It is thought to occur through contact with maternal blood and secretions perinatally. There is accumulating evidence that horizontal transmission may occur within neonate nurseries (Rantakallio et al., 1970). In the current study, high titers of CVB in blood of mice were found after infection and during the first 48 h thereafter. This supports the notion that the infection is spread by the hematogenous route. The elevation of liver enzymes was similar to those found in human neonates with fulminant CVB hepatitis (Wang et al., 1998). The high virus titers in the liver are also in accord with our findings from isolating the virus directly from the liver of a fatal neonate with CVB fulminant hepatitis (Wang et al., 1998).

CVB infection has been shown to induce apoptosis in mouse pancreatic acinar cells and myocardiocytes (Huber et al., 1999; Henke et al., 2000). CVB3 also induces degenerative morphological changes in infected HeLa cells accompanied by caspase 3-activation (Carthy et al., 1998). Joo and colleagues also found that CVB4 induces apoptotic phenotypes within 24 h postinfection in cultured rat neuronal cells and in permissive Vero cells (Joo et al., 2002; Ahn et al., 2003). These findings were confirmed by demonstrating induction of apoptosis in infected liver cells as early...
as 6 h postinfection using the TUNEL assay. Interaction between the CVB3 capsid protein VP2 and the proapoptotic protein Siva, which is involved in the CD27/CD70-transduced apoptotic pathway, may also play a role in the pathogenesis of CVB3 hepatic apoptosis (Henke et al., 2000).

Human and mice are most susceptible to invasive CVB infections during the early neonatal period. Immunohistochemical studies in rats and mice reveal a broad neural and epithelial expression of CAR during early development that decreases with age (Tomko et al., 2000). The expression of mCAR was found by RT-PCR and immunohistochemistry to decrease with increasing age in mice, in agreement with previous studies (Tomko et al., 1997; Bergelson et al., 1998). mCAR was present predominantly in brain, followed by heart, lung and liver of 1-day-old ICR mice. Thus the expression of CAR appears to be highly regulated with respect to both cell type and developmental age. CAR is required for CVB infection (Bergelson et al., 1998). It has been proposed that regulation of CAR by mediators of inflammation may play a role in the susceptibility to infection (Ito et al., 2000; Vincent et al., 2004).

Harvala et al. (2005) reported that CVB3 highly replicated in the liver in adult BALB/c mice. This finding showed that the CVB3 capsid is essential for liver tropism, and suggested that receptor interactions play a key role in the susceptibility of these tissues to infection. Antibody against mCAR blocked CVB3 infections in the mouse liver cell line, also strongly supporting the hypothesis that interaction between the capsid protein of CVB3 and CAR is needed for the infection of hepatic cells (Harvala et al., 2005). The current finding that anti-CAR monoclonal antibody blocked infection of the mouse liver cells by CVB3 and improved the survival rate in mice, supports the concept that the relative abundance of this receptor is a major determinant of tissue tropism.

All CVB viruses require CAR to initiate a productive infection, whereas affinity for the decay accelerating factor (DAF) co-receptor appears to be more variable (Shieh & Bergelson, 2002). Single amino acid changes allow non-binding CVB3 isolates to interact with DAF and thus broaden their tropism. For example, mutation of VP3-234E to Q permitted CVB3-Nancy to bind DAF and infect rhabdomyosarcoma (RD) cells (Pan et al., 2011). DAF plays a chaperone-like role in polarized gut epithelial cells during entry, directing the virion within cell–cell tight junctions where the major CAR entry receptor resides (Coyne & Bergelson, 2006). DAF-binding variants within a virus population may have a selective growth advantage in the intestinal lumen, where DAF is readily accessible but CAR is not. On the other hand, it has been reported previously that CVBs that use human DAF as a receptor do not bind murine DAF (Spiller et al., 2000).

The use of anti-CAR monoclonal antibody or soluble CAR (sCAR) in vivo may provide a suitable approach to evaluate CAR-based therapeutic strategies to combat CVB3 infection (Fischer et al., 2009). Efficient CVB3 neutralization using CAR and DAF analogues in vitro, fused to the Fc region of IgG. sCAR proteins inhibit CVB infection of susceptible
target cells in vitro and in vivo (Lim et al., 2006). Antibody to the Fc region promotes the solubilization of the receptor. Thus, the incorporation of the Fc-fused receptor analogue blocks virus infection.

In conclusion, CVB3-infected hepatic cells were damaged by both necrosis and apoptosis. CAR is necessary for CVB3 attachment and entry into cells, and accounts for organ and age-specific susceptibility to infection. It is theoretically possible that antibodies against CAR might protect against disseminated neonatal CVB disease in the early course of the infection.

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