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Histone deacetylase inhibitors suppress coxsackievirus B3 growth *in vitro* and myocarditis induced in mice

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Summary. – Clinical importance of myocarditis, predominantly caused by coxsackievirus B3 (CVB3), is recently rising. However, a detailed mechanism of pathogenesis of CVB3 myocarditis still needs to be clarified. Recently, it has been reported that histone modifications including acetylation are involved in coxsackievirus replication. To examine whether the CVB3 replication requires histone acetylation, histone deacetylase (HDAC) inhibitors were employed. We found that the HDAC2 activity increased in virus-infected cells at 12 hrs p.i. and that HDAC inhibitors suppressed the virus replication *in vitro*. This suggests that the HDAC2 activity may be required for the virus replication. Eventually, a HDAC inhibitor trichostatin A protected against CVB3-induced myocardial injury *in vivo*. Our results suggest that HDAC may be a novel therapeutic target for treating viral myocarditis.

Keywords: coxsackievirus B3; histone acetyltransferase; histone deacetylase; HDAC inhibitors, trichostatin A; apicidin; valproic acid; shRNA; myocarditis; mouse

Introduction

Some types of virus-induced myocarditis occur in neonates and young children (Estandiarei and McManus, 2008) and among these, coxsackievirus B3 (CVB3) is clinically important. CVB3 comprises a group of non-enveloped, positive single-stranded human pathogenic enteroviruses in the family *Picornaviridae* (King *et al*., 2011). Although many reports have revealed the mechanism of CVB3-induced pathogenesis (Henke *et al.*, 2008), the exact mechanism still requires some clarification. Understanding the mechanism of viral myocarditis will be critical for developing a clinically relevant therapy.

Previously reported mechanisms of CVB3-induced pathogenesis include the following: inhibition of Src-like protein phosphorylation reduced virus replication; phosphorylation of JNK1/2, p38 and ERK1/2 MAPK increased in virus-infected HeLa cells; inhibition of glycogen synthase kinase 3β suppressed coxsackievirus-induced cytopathic effects; and Cyr61 activation is required for CVB3-mediated cell death (Estandiarei and McManus, 2008; Kim *et al.*, 2004). Moreover, CVB3-induced cardiomyocyte injury is linked somehow to the PKA, PKB and PKC pathways, cell growth cycle, and extracellular matrix structure (Estandiarei and McManus, 2008).

We hypothesized that disturbing some or all of the aforementioned factors could be a strategy for the treatment of CVB3-induced myocarditis. Thus, identifying the signaling pathways involved with CVB3-induced myocarditis may provide clues to new drug targets. Recently, epigenetic modification has been implicated in many human diseases, including cancer, aging, and several types of virus infections (Issa, 2002). Therefore, to investigate the epigenetic regulation of virus growth, we examined the effects of histone acetyltransferases and histone deacetylase (HDAC) inhibitors on CVB3 growth in HeLa cells and on the myocarditis induced in mice by CVB3.

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Abbreviations: CVB3 = coxsackievirus B3; HDAC = histone deacetylases; p.i. = post infection; shRNA = small hairpin RNA; TSA = trichostatin A

Materials and Methods

Virus, cells and animals. HeLa cell line was maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2. The infectious virus used in this study was derived from a cDNA copy of the Woodruff variant of CVB3, acquired from Dr. Jeon (Lim *et al.*, 2005). The mouse experiment was reviewed and approved by the Animal care and use committee of the Catholic University of Korea (Korea). Four-week-old BALB/c male mice were purchased from Orient (Korea). CVB3 was injected intraperitoneally into each mouse at 1×10^6 PFU/100 μl. Trichostatin A (TSA; Sigma Aldrich, USA) was administered to each mouse every day at 0.5 mg/kg by intraperitoneal injection.

Histology. The hearts from mice infected with CVB3 and the control mice infected with cell culture supernatant were excised after the animals were killed. The apical parts of the hearts were fixed in 4% formalin, embedded in paraffin, and stained by H&E.

Virus titration. The heart tissues were homogenized in DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml). After centrifugation, the viral titers in the supernatants were determined by plaque-forming assay, using standard procedures (Shim *et al.,* 2011).

Detection of VP1 protein by Western blot analysis Virus-infected HeLa cells were lysed in lysis buffer. The samples were transferred to a PVDF membrane (Bio-Rad, USA). The blot was incubated overnight at 4°C with a primary monoclonal anti-VP1 antibody (NCL-Entero; Novocastra Laboratories Ltd, UK) to detect viral protein expression. The membranes were washed three times and the immune complexes were detected by reactions with enhanced chemiluminescence reagents (GE Healthcare, USA), as described previously (Park *et al.*, 2009).

HDAC2 activity assay. HDAC2 activity was measured with a Colorimetric HDAC2 Activity Assay kit, according to the manufacturer´s instructions (BioVision, USA). Briefly, cells were infected with CVB3 at a MOI of 10. The cell lysate was subjected to a standard immunoprecipitation procedure. Precipitation was measured with the HDAC2 Activity Assay kit.

shRNA targeting HDAC2 mRNA. HeLa cells were transfected with an shRNA (20 mmol/l) targeting human HDAC2 mRNA (Genolution, Korea) using Lipofectamine 2000 Reagent (Invitrogen, USA), as recommended by the manufacturer. The target shRNA sequence was 5′-GGTCAATAAGACCAGATAACATGTC-3′.

Results

HDAC inhibitors suppress CVB3 replication in HeLa cells

The ability of HDAC inhibitors to inhibit virus replication was examined. CVB3-infected HeLa cells (MOI = 10) were treated with TSA, apicidin, or valproic acid. HDAC inhibitors prevented the expression of viral capsid protein (VP1) in a dose-dependent manner, indicating that the inhibitors were specific (Fig. 1). Also, the viral titer in the cell culture supernatant of each inhibitor-treated sample was lower depending on the dose of TSA (data not shown), indicating that both intracellular replication and extracellular spread of CVB3 were inhibited.

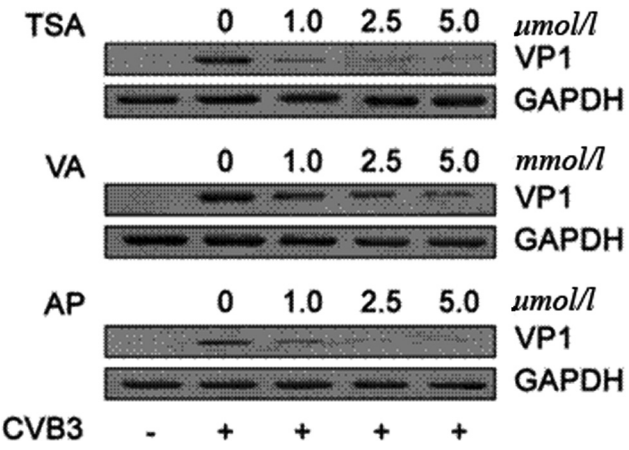

Fig. 1

HDAC inhibitors suppress CVB3 replication in HeLa cells

VP1 detected in CVB3 infected cells (+) and non-infected control (-) by Western blot analysis at 24 hrs p.i.

CVB3 infection raises HDAC2 activity in HeLa cells

TSA and valproic acid inhibit both class I and II HDACs. Apicidin is a specific inhibitor of class I HDACs, with the exception of HDAC8 (Park *et al.*, 2009), suggesting that HDAC class I might play a role in CVB3 replication. Other studies have reported that HDAC2, a class I HDAC, mainly affects transcriptional regulation, cell-cycle progression, and other developmental events (Wilting *et al.*, 2010). Based on these observations, we inferred that HDAC2 might be involved in activity changes in CVB3-infected HeLa cells. We investigated the activity of HDAC2 in CVB3-infected HeLa cells and found that virus infection enhanced the activity of HDAC2 from an early point during infection (4 hrs p.i.). This increase in activity culminated 12 hrs after infection, and this increased activity was maintained for an extended period thereafter (Fig. 2). Thus, CVB3-induced cell cytotoxicity may affect HDAC2 activity optimally up to 12 hrs after infection and suboptimally thereafter.

shRNA targeting HDAC2 mRNA suppresses CVB3 replication in HeLa cells

Next, we investigated the role of HDAC2 in CVB3 replication. Interestingly, the specific knockdown of

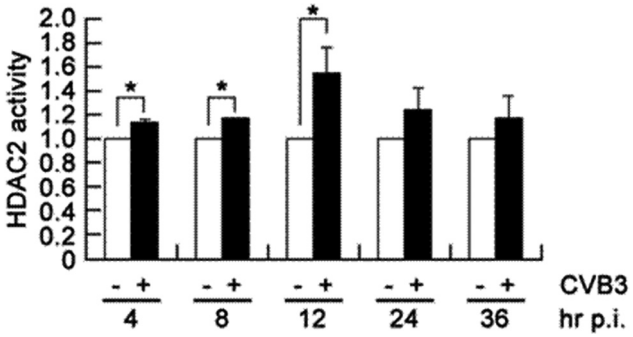

Fig. 2

CVB3 infection raises HDAC2 activity in HeLa cells

HDAC2 activity in CVB3 infected (+) or uninfected control (-) HeLa cells.

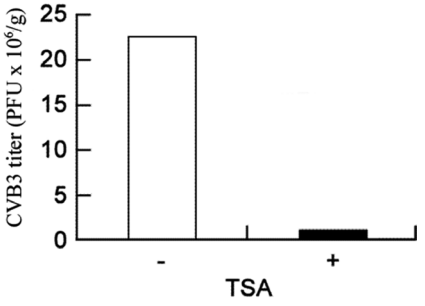

Fig. 4

TSA reduces CVB3 titers in the heart of CVB3-infected mice

Virus titers of CVB3 infected HeLa cells after TSA treatment (+) or in controls (-) after 72 hrs p.i.

HDAC2 expression by shRNA caused a dose-dependent block in VP1 expression (Fig. 3). These results indicate that HDAC2 activation is necessary for CVB3 replication *in vitro*.

TSA reduces CVB3 titers in the heart and protects mice from myocarditis

To determine whether treatment with an HDAC inhibitor can protect against myocarditis, we injected male BALB/c mice first with TSA and then 24 hrs later injected their peritoneal cavities with CVB3. TSA was then administered every day at a concentration of 0.5 mg per kg of body weight. The TSA-treated mice showed approximately 10-fold lower virus production in the heart compared with the untreated mice 3 days after infection (Fig. 4).

TSA also protected against virus-induced myocarditis in the mouse 7 days after infection compared with CVB3-infected mice lacking TSA treatment (Fig. 5). When the heart tissues were stained with H&E to detect myocardial inflammation, the blue-purple regions indicated areas of basophil infiltration and the bright-pink areas showed the eosinophilic cytoplasm of the myocardial cells (Fig. 5). The H&E-stained tissue sections from the CVB3-infected mouse hearts showed much more blue-purple regions than the TSA-treated mouse hearts, indicating that TSA protects against CVB3-induced heart damage. These data indicate that TSA is a potential therapeutic agent for viral myocarditis.

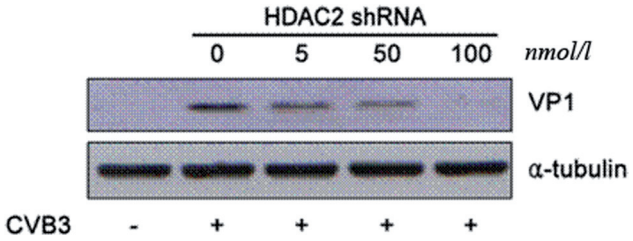

Fig. 3

shRNA targeting HDAC2 mRNA suppresses CVB3 replication in HeLa cells

VP1 detected in CVB3 infected cells (+) and in non-infected control (-) by Western blot analysis.

Discussion

We demonstrated that HDAC inhibitors, especially TSA, suppress CVB3 growth and protect against CVB3-induced myocarditis *in vitro* and *in vivo*. Although the exact mechanism of HDAC inhibitors is unknown, they modify the activities of histone and non-histone proteins that are affected by acetylation and cause a general increase in transcription (Spange *et al.*, 2009). Therefore, we speculate that HDAC inhibitors might increase the transcriptional activity of some unknown cellular factor that is a potential inhibitor of CVB3 replication. Contrary to our results, some HDAC inhibitors can escape from virus latency and reactivate viruses, including human immunodeficiency virus (Archin *et al.*, 2009). However, an alternative explanation is that during the subacute phase after CVB3 infection, CVB3 stimulates the migration of natural killer cells and macrophages to the site of injury, followed by the induction of pro-inflammatory cytokines and the subsequent infiltration of activated T cells (Estandiarei and McManus, 2008).

In addition to the direct injury to the heart caused by CVB3, the immune responses directed against the virus are important mechanisms of CVB3 pathogenesis. That is, CVB3 infection triggers immune cell infiltration of myocytic tissue, inducing myocyte inflammation, and ultimately injuring myocytes (Lindner *et al.*, 2012). The

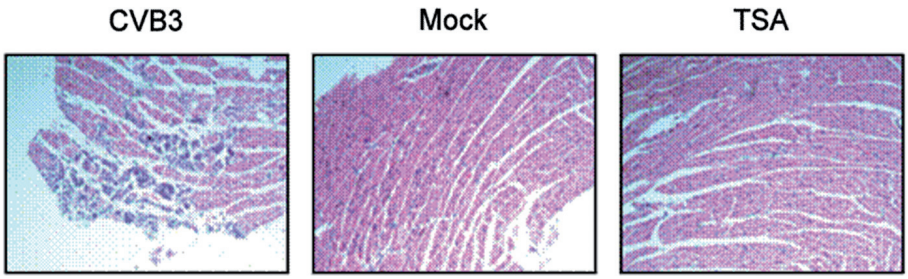

Fig. 5

TSA protects mice from CVB3-induced myorcarditis

H&E staining at day 7 p.i. Magnification 200x.

activation of CVB3-specific T cells and/or the induction of adhesion molecules in myocytes are prerequisites for the infiltration of immune cells into the heart (Whitten, 2002). Other studies have also shown that HDAC inhibitors regulate immune cell activation by regulating several cellular factors (such as STAT1, STAT3, and NF-κB) and also by suppressing the expression of key adhesion molecules (such as VCAM-1) thus leading to fewer activated monocytes binding to the inflamed endothelium (Lindner *et al.*, 2012; Deb *et al.*, 2012). Therefore, HDAC inhibitors may suppress the activation of CVB3-specific T cells and the induction of myocyte adhesion molecules. This may be one mechanism underlying the action of HDAC inhibitors, explaining how they suppress CVB3-induced myocarditis. However, intensive research is required to understand the roles of HDAC inhibitors in protecting against CVB3-induced myocarditis.

HDAC inhibitors are being applied in the clinic for treating cancer, neurodegenerative diseases, and inflammatory diseases. Potential applications of HDAC inhibitors may easily be expanded to include other diseases, such as CVB3-induced myocarditis. Thus, we believe that HDAC inhibitors have great potential in the clinical context as a therapeutic agent for CVB3-induced myocarditis.

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