THE ROLE OF ERK1/2 ACTIVATION IN THE INFECTION OF HELA CELLS WITH HUMAN COXSACKIEVIRUS B3

S.M. KIM[1], E.J. KIM[1], S.I. PARK[1], J.H. NAM[2*]

[1]Division of Cardiovascular Research, Department of Biomedical Sciences, National Institute of Health, 5 Nokbun-dong, Eunpyung-gu, Seoul, 122-701 Korea; [2]Division of Biotechnology, The Catholic University of Korea, Bucheon, Gyeonggi-do, 420-743 Korea

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Summary. – Human coxsackievirus B3 (CVB3) is known to trigger in host cells a biphasic activation of extracellular signal-regulated kinase (ERK1/2); i.e., early transient and late sustained activation. In this study, we explored (i) the role of ERK1/2 activation in virus entry into cells and virus replication and (ii) cellular genes influenced by this activation in CVB3-infected HeLa cells. Pretreatment of the cells with an ERK1/2 inhibitor, PD98059 showed that early transient ERK1/2 activation is not be related to virus entry, but late sustained ERK1/2 activation plays a role in virus replication. To identify which cellular genes are influenced by the ERK1/2 activation after virus infection, a cDNA microarray analysis was performed. In HeLa cells pretreated with PD98059 and then infected with the virus, the number of influenced cellular genes was higher compared to that in infected cells not pretreated with the inhibitor (15 vs 77 at 10 mins post infection (p.i.) and 347 vs 91 at 9 hrs p.i. Thus the virus infection affected several host genes through ERK1/2 activation.

Key words: Human coxsackievirus B3; cDNA microarray; ERK1/2

Introduction

CVB3 is an important human pathogen that induces acute and chronic viral myocarditis in children and young people (Kawai, 1999). About 5–50% of myocarditis and its end-stage dilated cardiomyopathy are attributable to CVB3 infection (Maisch *et al*., 2002). CVB3 infection induces biphasic ERK 1/2 activation (phosphorylation) in HeLa cells, in which early transient ERK1/2 activation appears at 10 mins p.i., while late sustained activation at 9 hrs p.i. These activations are important for CVB3 replication and contribute to virus-mediated changes in host cells (Luo *et al*., 2002). Recently, it has been established that in several different types of viral infections the ERK1/2 activation regulates many viral and cellular functions including viral gene expression, virus replication, and virus-induced apoptosis (Johnson *et al*., 2001; Sontag *et al*., 1993; Jacque *et al*., 1998; Pleschka *et al*., 2001). These findings suggested that the ERK1/2 activation influences a number of cellular genes that are related to viral growth or host defense mechanism. This is also supported by *in vivo* data showing that the ERK1/2 activation in mouse heart infected with CVB3 may contribute to differential host susceptibility to viral myocarditis (Opavsky *et al*., 2002). In addition, the ERK1/2 activation by CVB3 infection precedes caspase-3 activation (Cunningham *et al*., 2003). However, the role of each ERK1/2 activation in biphasic activation and particular cellular genes that are influenced by the ERK1/2 activation in CVB3-infected cells are so far unknown.

The purpose of this study was to determine (i) whether the biphasic ERK1/2 activation in CVB3 infection of HeLa cells plays a role in early events, such as viral entry and in late events, such as viral growth, and (ii) which cellular genes

*Corresponding author. E-mail: jhnam@catholic. ac.kr; fax: +822-3880924.

Abbreviations: CVB3 = Human coxsackievirus B3; DCM = dilated cardiomyopathy; DMEM = Dulbecco's Modified Eagles Medium; ERK1/2 = extracellular signal-regulated kinase 1/2; IFA = immunofluorescence assay; MOI = multiplicity of infection; p.i. = post infection

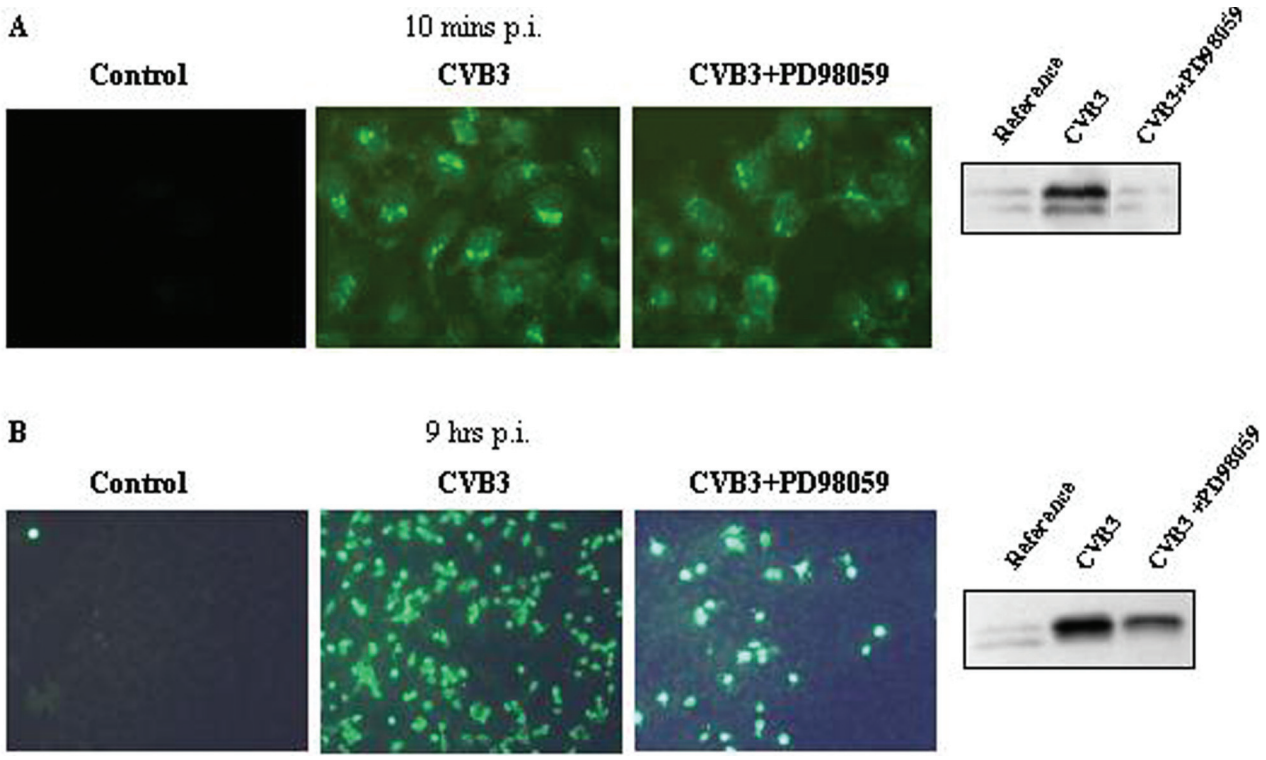

Fig. 1

Effect of PD98059 on ERK1/2 activation, virus entry and virus replication in CVB3-infected HeLa cells at 10 mins (A) and 9 hrs (B) p.i.

Left panels: virions in the cells stained by IFA, magnification 400x (A) and 100x (B). Right panels: ERK1/2 activation assayed by Western blot analysis.

are influenced by the ERK1/2 activation. For this purpose, we tested the effects of PD98059, an ERK1/2 inhibitor targeting MEK1/2 upstream of ERK1/2 on early events in CVB3 infection. Also, we used the cDNA microarray technique to identify cellular genes affected by the ERK1/2 activation. Some data obtained by cDNA microarray technique were checked by RT-PCR.

Materials and Methods

Virus and cells. A Woodruff variant of CVB3 H3 strain (Knowlton *et al*., 1996) was propagated in HeLa cells in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% of heat-inactivated fetal bovine serum (growth medium) at 37°C. Virus infectivity was determined by plaque assay (Henke *et al*., 1998). Virus stock was stored at -80°C.

Virus infection and inhibitor pretreatment. For the inhibitor pretreatment, HeLa cell cultures in 70–80% confluency were incubated with 50 μmol/l PD98059 (A.G. Scientific, USA) in serum-free DMEM for 1 hr. The cells were infected with the virus at a multiplicity of infection (MOI) of 10 PFU/cell for 1 hr, washed three times with PBS and cultured in the growth medium. All steps were carried out at 37°C.

Immunofluorescence assay (IFA) of the CVB3 VP1 antigen was performed according to Nam *et al.* (1999). Briefly, infected HeLa cells on glass coverslips were fixed at 10 mins or 9 hrs p.i. with 4% paraformaldehyde in PBS and were first incubated with a monoclonal antibody to Coxsackievirus B5 VP1 (Clone 5-D8/1, Novocastra Laboratories Ltd, UK) and then with an anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology, USA), each time for 1 hr at 37°C. The cultures were washed, mounted and examined by fluorescence microscopy (Carl Zeiss Jena, Germany).

Western blot assay of phosphorylated ERK1/2 was performed according to Luo *et al.* (2002). Samples of cell lysates containing equal amounts of proteins were electrophoresed on a 12% polyacrylamide gel in the presence of SDS and blotted onto a nitrocellulose membrane. The blot was blocked with 5% skim milk in a Tris-buffered saline (137 mmol/l NaCl and 10 mmol/l Tris pH 7.4) for 1 hr and then incubated with a primary antibody to phosphorylated ERK1/2. A horseradish peroxidase-conjugated anti-mouse immunoglobulin (both from Santa Cruz Biotechnology, USA) served as secondary antibody. The immunoreactive bands were visualized by chemiluminescence (ECL, Amersham Pharmacia, Sweden).

cDNA microarray technique has been described earlier (Nam *et al*., 2002, 2003; Lee *et al*., 2003). The microarray contained more than 7,400 distinct sequences, half of which with unknown functions. Briefly, total RNA was prepared from cells infected with

CVB3 and from cells pretreated with PD98059 and infected with CVB3. Fluorescein-labeled cDNA was prepared by reverse transcription of total RNA in the presence of aminoacyl-dUTP, followed by coupling with the Cy3 dye (for non-infected cells) or Cy5 dye (for infected cells) (Amersham Pharmacia). The Twin Chip Human-8K Digital Genomics (Korea) cDNA microarray was hybridized with a mixture of fluorescein-labeled cDNAs from non-infected and infected cells at 42°C for 16 hrs. After washing, the DNA chips were scanned using the ScanArray Life (Perkin-Elmer Life Sciences, USA). Scanned images were analyzed with the GenePix software (Axon Instruments, USA) to obtain gene expression ratios (infection versus control). Logged gene expression ratios were normalized by LOWESS regression (Yang *et al*., 2002).

cDNA microarray data analysis. The fluorescence intensity of each spot was calculated by local median background subtraction. A robust scatter-plot smoother LOWESS function was used for an intensity-dependent normalization for gene expression. Scatter plot analysis was done using the Microsoft Excel 2000 software. Genes were considered differentially expressed when their logarithmic gene expression ratios were higher than +1 or lower than –1 (i.e. only at least 2-fold differences were considered) and q-values were lower than 0.1.

Results

Effect of PD98059 on ERK1/2 activation in HeLa cells infected with CVB3

Virus-infected cells either pretreated with the inhibitor or non-pretreated and non-infected cells were subjected to IFA (viral antigen), Western blot analysis (ERK1/2 phosphorylation) and cDNA microarray analysis (cellular genes expression) at 10 mins and 9 hrs p.i. (Fig. 1). At 10 mins p.i., ERK1/2 was not phosphorylated after the PD98059 pretreatment (Fig. 1A) in accord with earlier results of Luo *et al*. (2002). Under the same conditions, IFA revealed that the virus was able to enter the cells. This finding indicates that the ERK1/2 activation is not related to the virus entry into cells. At 9 hrs p.i. the ERK1/2 phosphorylation was lower in the inhibitor-pretreated cells compared to the non-pretreated cells (Fig. 1B). The fact that the reduction was partial but not complete could be explained by the absence of the inhibitor during the 9 hr period p.i. and possible partial recovery of the ERK1/2 activity. Also, the number of cells containing viral antigen was lower compared to the cells not pretreated with the inhibitor. This result demonstrates that late ERK1/2 activation is required for viral replication and confirms earlier reports (Luo *et al*., 2002; Opavsky *et al*., 2002).

Effect of PD98059 on cellular gene expression in HeLa cells infected with CVB3

Virus-infected cells either pretreated or non-pretreated with the inhibitor and non-infected cells either pretreated or non-pretreated with the inhibitor were subjected to cDNA microarray analysis at 10 mins and 9 hrs p.i. (Table 1 and Fig. 2). At 10 mins p.i., a total of 77 genes were affected by the virus infection in the absence of PD98059. Whereas most

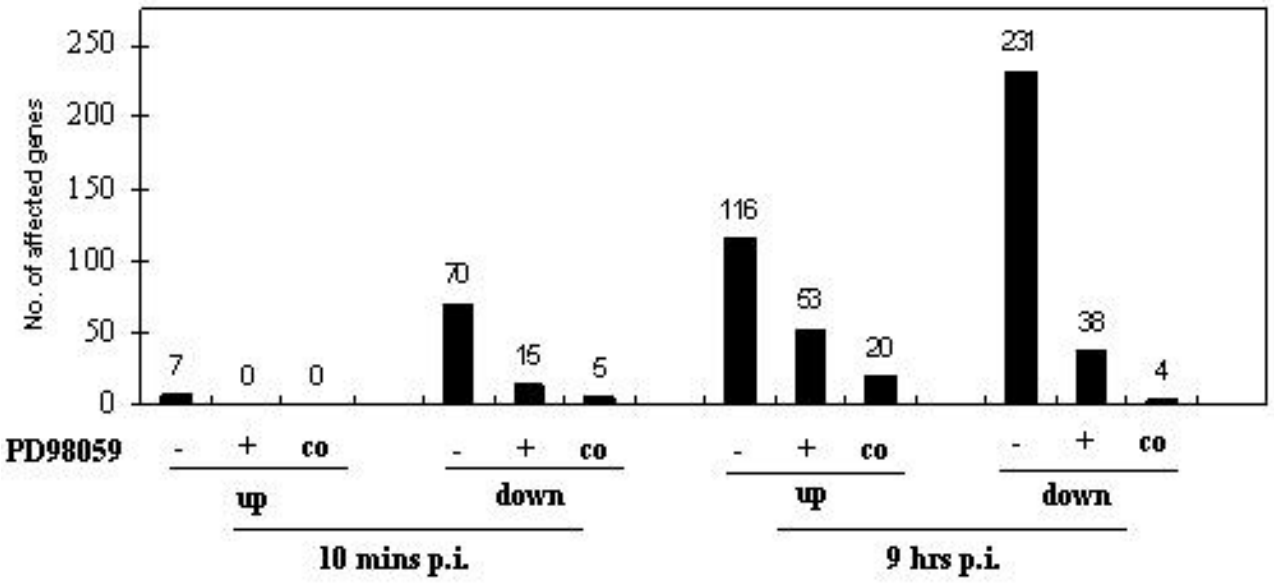

Fig. 2

Number of cellular genes affected by ERK1/2 activation in CVB3-infected HeLa cells

cDNA microarray analysis. +/– = the presence/absence of PD98059, co = the number of genes influenced in both the presence and absence of PD98059, up/down = up-regulated/down-regulated genes.

Table 1. Expression of cellular genes affected by ERK1/2 activation in HeLa cells infected with CVB3

GenBank Acc. No.	Gene	PD98059			
		Absent	Present	Absent	Present
		10 mins		9 hrs	
	Cell growth and maintenance				
X72755	CXCL9 (Chemokine ligand 9)			1.04[a]	
N50741	TREM2 (Triggering receptor expressed on myeloid cells 2)			1.16	
AF067724	NME5 (Nonmetastatic cells 5, protein expressed in, nucleoside-diphosphate kinase)			1.14	
AF011905	RAD 1 (RAD1 homolog, S. pombe)			1.41	
X51405	CPE (carboxypeptidase E)	1.71			
D88894	BACH (brain acyl-CoA hydrolase)			2.25	1.84
Y12084	CYR61 (cysteine-rich, angiogenic inducer, 61)			1.63	2.24
U19927	WAS [Wiskott–Aldrich syndrome (eczema-thrombocytopenia)]			1.19	
M19154	TGFB2 (transforming growth factor, beta 2)			1.19	
M63193	ECGF1 [endothelial cell growth factor 1 (platelet-derived)]			1.14	
X78947	CTGF (connective tissue growth factor)			1.08	1.73
	Transport/channel/cytoskeleton				
U61500	TMEM 1 (transmembrane protein 1)	1.82			
U17077	BENE (BENE protein)			2.18	2.43
M24173	HBZ (hemoglobin, zeta)			1.7	
AW051032	HTR1D [5-hydroxytryptamine (serotonin) receptor 1D]			1.53	1.08
AF026030	TIMM4 [translocase of inner mitochondrial membrane 44 homolog (yeast)]			1.4	
AB014606	KIF1C (kinesin family member 1C)			1.25	
N24732	GABPA (GA binding protein transcription factor, alpha subunit 60kDa)			1.18	
X12901	VIL1 (villin 1)			1.11	
	Signal transduction				
AA932219	PILRA (paired immunoglobin-loke type 2 receptor alpha)	1.34			
Z34974	PKP1 (plakophilin 1, ectodermal dysplasia/skin fragility syndrome)			1.91	
NM_003357	SCGB1A1 [secretoglobin, family 1A, member 1 (uteroglobin)]			1.73	1.31
S73591	TXNIP (thioredoxin interacting protein)			1.57	1.33
D14874	ADM (Adrenomedullin)			1.53	1.1
AI598150	JUN [v-jun sarcoma virus 17 oncogene homolog (avian)]			1.37	1.01
S62539	IRS1(insulin receptor substrate 1)			1.25	
	Immune response				
W03765	CSF2RB (Colony stimulating factor 2 receptor, beta, low-affinity)	1.18			
NM_006919	SERPINB3 [serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3]			1.73	1.69
X13967	LIF [leukemia inhibitory factor (cholinergic differentiation factor)]			1.24	
U73529	CXCR6 [chemokine (C-X-C motif) receptor 6]			1.14	
	Transcription				
AA948679	KIAA1750 (KIAA1750 protein)			1.55	
AI275084	TRAP2B [transcription factor AP-2 beta (activating enhancer binding protein 2 beta)]			1.2	
AF006514	CHD2 (chromodomain helicase DNA binding protein 2)			1.16	1.02
M78435	MLL2 (myeloid/lymphoid or mixed-lineage leukemia 2)			1.35	1.02
AF028008	TIEG2 (TGFB inducible early growth response 2)			1.3	1.01
	Cell cycle				
AF011905	RAD1 [RAD1 homolog (S. pombe)]			1.41	
J03358	FER [fer (fps/fes related) tyrosine kinase (phosphoprotein NCP94)]			1.15	
	Cell death				
AI185199	IER3 (immediate early response 3)			1.43	1.55
AF067724	NME5 (nonmetastatic cells 5, protein expressed in nucleoside-diphosphate kinase)			1.14	
AA099277	TUCAN (tumor up-regulated CARD-containing antagonist of caspase nine)			1.11	
	Miscellaneous				
AI792251	PRAC (small nuclear protein PRAC)			1.66	
U65637	CHST1 [carbohydrate (keratan sulfate Gal-6) sulfotransferase 1]			1.21	1.05

[a]Values for the logarithmic gene expression ratios obtained by cDNA microaray.

of these genes were down-regulated, only 7 genes were up-regulated, namely CPE, TMEM 1, PILRA, CSF2RB, CACNG3, FLJ20356, and KIAA0773. In contrast, with PD98059 no genes were up-regulated. This finding indicates that the up-regulated genes were under the control of the ERK1/2 activation.

At 9 hr p.i., most infected cells were morphologically changed and displayed rounding, a typical sign of cell death (data not shown). This observation suggests that many cellular genes could be affected by the virus infection. In fact, 347 such a genes were detected. Among them, 116 were up-regulated and 231 were down-regulated. However, the pretreatment with PD98059 reduced the number of both up- and down-regulated genes to 91. Interestingly, 24 of 347 genes in virus-infected cells were affected regardless of the pretreatment with PD98059.

As the CVB3 protein 2A cleaves eIF4G, an important factor in host cap-dependent translational machinery, total protein synthesis in virus-infected cells is reduced (Carthy *et al*., 1998). We predicted that, beyond a certain time point p.i. the gene expression in CVB3-infected cells would be reduced. Indeed, at 18 hrs p.i., most cellular genes were dramatically down-regulated or were indetectable. Morphologically, all cells showed a typical cell death pattern: rounding, detaching from the surface and floating in the medium. Only two genes were still up-regulated, CYR61 and v-myc. (data not shown). We were interested in the up-regulated genes at 9 hr pi because we thought that these genes might be closely related to the virus growth or to the host defense system.

Discussion

When a virus infects the host cell, it may cause changes in the intracellular environment that are appropriate for virus replication through virus-host signal transduction. HeLa cells infected with CVB3 show the biphasic, early and late ERK1/2 activation. An inhibition of the biphasic ERK1/2 activation prevents CVB3 replication in infected cells (Luo *et al*., 2002; Johnson *et al*., 2001). This demonstrates that the ERK1/2 activation is important for optimalization of the host environment for virus replication. We suspected that the early ERK1/2 activation may be related to early events of virus reproduction cycle including the entry mechanism, because viral endocytosis requires in general supply of energy (Mellman, 1996). However, although the early ERK1/2 activation was blocked by the inhibitor, the virus was able to enter host cells regardless of the inhibitor pretreatment. This finding demonstrates that the inhibition of the early ERK1/2 activation did not affect, at least, virus entry. However, the inhibition of the late ERK1/2 activation impaired virus replication. Recently, Cunningham *et al*. (2003) have reported that the early ERK1/2 activation is not due to CVB3 infection but to the cell culture medium, while only the late ERK1/2 activation is related to the virus infection. Our data suggest that the early ERK1/2 activation has no role in CVB3 infection.

To identify cellular genes that are under the control of the ERK1/2 activation, we tested the effects of CVB3 infection and PD98059 on the expression of cellular genes in HeLa cells using the cDNA microarray analysis.

From the sensitivity to the inhibitor we deduced that the expression of 91 cellular genes at 9 hrs p.i. was influenced by the ERK1/2 activation. On the other hand, 24 genes were up- or down-regulated regardless of the ERK1/2 activation. Among them, CYR61 is interesting because it was up-regulated at 9 hrs pi regardless of the presence of the inhibitor and the up-regulation was maintained up to 18 hrs p.i., although most genes were down-regulated or non-detectable. CYR61, an immediate early gene was up-regulated; its protein product promotes cell adhesion and migration and is normally secreted into the extracellular matrix (O'Brien *et al*., 1990; Kireeva *et al*., 1996). Recently, it has been reported that CYR61 has a role in neuronal cell death through JNK activation (Kim *et al*., 2001). According to our cDNA microarray data, JUN, which is downstream of JNK, was up-regulated. Therefore, it is possible that CVB3 infection may induce CYR61 through JNK activation and the produced CYR61 as growth factors may re-induce by feedback JNK in other cells. The induced JNK may have many biological functions in infected cells, including apoptosis (Harper and LoGrasso, 2001). This cascade is independent of the ERK1/2 activation. However, further detailed studies are necessary to confirm the role of JNK in CVB3 infection. To date, we already proved that CVB3 infection induces CYR61 activation via JNK (Kim *et al.,* 2004.).

CTGF, similarly to CYR61 is also encoded by immediate early genes. Both CTGF and CYR61 are included in the CCN family and have a similar biological role (Brigstock, 2003). In particular, both CYR61 and CTGF are co-induced by several factors including TGF-ß (Chen *et al*., 2001). Interestingly, both CYR61 and CTGF are up-regulated in CVB3-infected HeLa cells at 9 hrs p.i. and TGF-ß, which stimulates both genes is up-regulated too. Thus, it is suggested that TGF-ß may have a role in CVB3-induced signal transduction.

Summing up the results of this study, the early transient ERK1/2 activation is apparently not related to virus entry into cells, but the late sustained ERK1/2 activation plays a role in virus replication. Many cellular genes are affected by the late ERK1/2 activation. However, some cellular genes influenced by CVB3 infection are not related to the ERK1/2 activation.

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