Enhancement of enteric adenovirus cultivation in a novel Ras-overexpressing cell line

Jiyeon Si, Misoon Kim, Mi Young Lim and GwangPyo Ko

Department of Environmental Health, School of Public Health, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-742, South Korea

Enteric human adenoviruses (HAdVs; serotypes 40 and 41) have been identified as an emerging cause of drinking water contamination. Due to their fastidious characteristics, HAdVs are difficult to cultivate and therefore not detected easily by standard mammalian cell cultivation methods. Here we found that human embryonic kidney 293 cells, transformed transiently with Ras, enhanced HAdV replication by more than threefold. We also constructed a stable cell line overexpressing the Ras protein, 293-Ras, in which the replication of three HAdV strains of serotypes 40 and 41 was increased markedly. However, only HAdV replication was enhanced; infection of 293 and 293-Ras cells with human rhinovirus-6 showed no significant differences in replication rate. Infected 293-Ras cells exhibited an increased level and phosphorylation of extracellular regulated kinase (ERK). In addition, the Ras-mediated increase in HAdV replication was impaired by the mitogen-activated protein kinase/ERK kinase (MEK1) inhibitor U0126, suggesting direct involvement of the MEK1/ERK pathway in enhanced HAdV replication. Based on these results, we suggest that the 293-Ras cell line be used for the efficient detection and cultivation of HAdV strains in both clinical and environmental specimens.

INTRODUCTION

Human adenoviruses (HAdVs) comprise 57 serotypes that are subdivided into seven groups (A–G) (Tebruegge & Curtis, 2012). These viruses cause a wide range of infections in humans, including gastroenteritis, upper respiratory tract infections, pneumonia, pharyngitis and conjunctivitis, depending on the serotype (Gray et al., 2005). Furthermore, AdVs are a source of contamination of drinking water. The US Environmental Protection Agency is currently adding AdVs to the list of drinking water contaminant candidates (Contaminant Candidate List 3; http://water.epa.gov/scitech/dws/ccl/ccl3.cfm) due to their prevalence and transmission in drinking water. Enteric HAdVs (serotypes 40 and 41) belonging to group F are aetiological agents of infantile gastroenteritis, second only in terms of incidence to rotaviruses (Sherwood et al., 2007). They are resistant to tap water, sea water and UV radiation when compared with other enteric viruses (Enriquez et al., 1995; Thurston-Enriquez et al., 2003). However, the cultivation of this virus in cells yields low titres; indeed, cultivation of HAdV-41 results in a 20-fold lower yield than cultivation of AdV-2 (Siqueira-Silva et al., 2009). Given that the standard method of detection of virus in environmental samples requires cultivation in mammalian cells, the fastidious nature of HAdVs may lead to an underestimation of the importance of these viruses (Jaykus, 1997).

Ras is a membrane-bound oncoprotein involved in a myriad of signal transduction pathways that regulate the communication involved in cell growth, proliferation, differentiation and death (Aoki et al., 2008). Ras activation occurs through guanine nucleotide exchange factors, which exchange GDP for GTP, relaying downstream signals by phosphorylation (Goodsell, 1999). The primary downstream targets of Ras are mitogen-activated protein kinase (MAPK) signalling pathways that play key roles in conveying extracellular signals to induce appropriate responses (Zhang & Liu, 2002). The best-studied MAPKs include extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase and phosphatidylinositol 3-kinase (PI3K). Of the kinase pathways, Ras/ERK is known to support vesiculovirus, reovirus and influenza virus replication in certain cancer cell lines (Norman et al., 2004; Noser et al., 2007; Pleschka et al., 2001). In the present study, we constructed both transient and permanent cell lines that overexpressed Ras, and evaluated their effect on the replication of HAdV strains.

RESULTS

HAdV-41 replication by transient transfection with a Ras expression vector

To evaluate the effect of Ras signalling on HAdV replication efficiency, we transfected transiently 293 cells with a H-RasV12-expressing vector and infected them with HAdV-41. HAdV replication was estimated at the viral mRNA and DNA levels by real-time PCR. As shown in Fig. 1, HAdV-41
replication rates were around three- and sevenfold higher at the mRNA and DNA levels, respectively. These results indicate that viral gene expression and replication were facilitated in 293 cells by activation of Ras signalling.

Construction of a Ras-overexpressing cell line
To obtain transformed cell lines that expressed constitutively the activated Ras protein, newly transfected cells were selected under G418 conditions. After several passages, the chromosomal integration of the Ras gene and its expression in transformed 293 cells (293-Ras) were verified by PCR and reverse transcription (RT)-PCR. A 636 bp amplicon was detected by PCR in the chromosomal DNA of the cell lines (Fig. 2a) and the same amplicon was amplified by RT-PCR from the RNA samples (Fig. 2b). As the parental 293 cells exhibit intrinsic expression of the Ras gene, part of the Ras-containing vector was amplified to differentiate the intended Ras overexpression. Western blotting using an anti-Ras antibody revealed that Ras protein level was greater in the 293-Ras cell line than in the parental 293 cells (Fig. 2c).

Cultivation and replication of HAdVs in 293-Ras cells
To assess the replication efficiency of HAdV in the stable cell lines, 293 and 293-Ras cells were infected with 3.2 × 10^5 TCID_{50} ml^{-1} of HAdV-41. The infected cells were enumerated after 24 h by flow cytometry (Fig. 3). HAdV infection of 293 cells resulted in an infection rate of 8.20 %, whereas 293-Ras cells displayed a 22.05 % infection rate. These results confirm that 293-Ras cells were more susceptible to viral infection and therefore more suitable for cultivation. We also examined the cells infected with 2.7 × 10^5 TCID_{50} ml^{-1} of HAdV-41 by transmission electron microscopy (TEM) (Fig. 4). At 2 days post-infection (p.i.), viral particles began entering 293-Ras cells by phagocytosis and groups of viruses were observed in the cytosol. At 3 days p.i., progeny viral particles were found within the nucleus of 293-Ras cells; however, it was difficult to observe AdVs in 293 cells until 3 days p.i. Furthermore, the replication in 293-Ras cells of three HAdV strains was investigated by real-time PCR (Fig. 5): HAdV-40 and HAdV-41 from clinical stool samples, and the laboratory-adapted HAdV-41 strain. DNA levels of the three HAdV strains were increased significantly by 209 % (P=0.077), 15 851 % (P=0.028) and 231 % (P=0.001) in 293-Ras cells compared with 293 cells. To determine if the effect of Ras is specific for HAdVs, human rhinovirus (HRV)-6 was inoculated into 293-Ras cells. As shown in Fig. 5, there was no significant difference in HRV-6 replication between 293 and 293-Ras cells (P<0.05).

Ras-dependent enhancement of HAdV replication
To dissect the signalling cascade involved in the Ras-mediated enhancement of HAdV replication, 293-Ras cells that had been infected with HAdV-41 were treated with the MAPK inhibitors U0126 (MEK inhibitor), JNK inhibitor II (JNK inhibitor), LY29400 (PI3K inhibitor) and SB203058 (p38 inhibitor). At 3 days p.i., viral DNA was quantified by real-time PCR. As shown in Fig. 6a, Ras-mediated enhancement of viral replication was abolished only by treatment with U0126, which inhibits the MEK/ERK pathway. The other inhibitors did not ameliorate significantly the enhancement of viral replication. The level of ERK phosphorylation in 293-Ras cells in comparison with that in 293 cells was assessed. Both 293 and 293-Ras cells were infected with 3 × 10^4 genomic copies of HAdV-41 for 3 h and subjected to Western blotting. The 293-Ras cells showed stronger ERK phosphorylation in 293-Ras cells in comparison with that in 293 cells. At 6 h post-infection, both the 293 and 293-Ras cells were infected with 3 × 10^4 genomic copies of HAdV-41 for 3 h and subjected to Western blotting. The 293-Ras cells showed stronger ERK phosphorylation, and AdV infection further intensified activation in both the 293 and 293-Ras cells (Fig. 6b). To verify the effects of the Ras/MEK/ERK signalling pathway on AdV replication, viral replication rates in 293 and 293-Ras cells were compared after treatment with the MEK inhibitor (Fig. 6c). The inhibitor significantly reduced the levels of the virus by ~600-fold in 293 cells (P=0.046) and ~1500-fold in 293-Ras cells (P=0.043). These results indicate that the Ras/MEK/ERK pathway is involved directly in Ras-mediated enhancement of AdV replication in 293-Ras cells.

DISCUSSION
Here, we developed a new cell line overexpressing the Ras oncogene, which exhibited enhanced HAdV growth. AdV,
specifically group F, is known to grow very slowly and have low titres than other AdVs. Our work with the cytomegalovirus (CMV) transactivator protein IE1 improved AdV cultivation by promoting the expression of the essential viral genes, E1A and hexon (Kim et al., 2010). Transient transfection of 293 cells with CMV IE1 showed two and four times higher levels of viral DNA and RNA, respectively, compared with the 293 control cells. However, the same transient transfection with the Ras gene yielded a greater degree of AdV replication than CMV IE1 (Fig. 1). Additionally, TEM images revealed more rapid replication of AdV in 293-Ras cells than in 293-CMV cells. When infected with the same amount of HAdV-41, observation of virus in 293-CMV cells required 3 days, whereas the presence of virus was confirmed on the second day after infection in 293-Ras cells (Fig. 4). Adenovirus replication is divided into two phases (Russell, 2000). The first phase facilitates the penetration of virus into cells and entry of the viral genome into the nucleus. Once transcription is initiated in the nucleus, the late phase begins with the assembly and maturation of infectious virus particles in the nucleus. Accordingly, the infection that spreads within the nucleus of 293-Ras cells at 3 days p.i. is suspected to comprise progeny virus. In 293 cells, it was difficult to examine the virus even at 3 days p.i., possibly due to a lower rate of virus replication compared with 293-Ras cells.

Further study is warranted to confirm if the absence of AdV in 293 cells is due to the delayed entry of the virus. The enhanced HAdV replication in 293-Ras cells was validated by flow cytometry, real-time TaqMan PCR of stool specimens and cultivation assays (TCID₅₀). According to the flow cytometry results, 293-Ras cells yielded approximately three times more HAdV-41 virus than did 293 cells (Fig. 3). Cells were infected with HAdV-40 and HAdV-41 isolated from diarrhoeal patients, and the virus was quantified by real-time TaqMan PCR (Fig. 5). As an internal control, the expression of glyceraldehyde 3-phosphate dehydrogenase was analysed by SYBR Green quantitative PCR. The result showed no significant difference between the two cell lines both before and after the virus infection. Although the HAdV-40 titre was not significantly greater in 293-Ras cells than 293 cells, replication of the virus was twice as successful in 293-Ras cells ($P=0.077$). Both clinically isolated and laboratory-adapted HAdV-41 strains demonstrated significantly greater viral DNA loads in 293-Ras cells ($P=0.028$ and $P=0.001$, respectively). TCID₅₀ assays also confirmed the enhanced cultivation of laboratory-adapted HAdV-41 in 293-Ras cells at passage 30. Viral titres were more than five times higher in 293-Ras cells compared with 293 cells. However, HRV-6 replication was not significantly different...
between 293 and 293-Ras cells ($P<0.05$). Therefore, the effect of Ras is likely specific to AdV.

Many DNA and RNA viruses regulate replication by manipulating MAPK signalling pathways in the infected host (Bruder & Kovesdi, 1997; Jacque et al., 1998; Luo et al., 2002). Among the downstream targets of Ras, we investigated the involvement of MAPKs, including ERK, JNK, p38 and PI3K. The Ras/MEK/ERK signalling pathway has been shown to be involved in the propagation of influenza virus and coxsackievirus B3, and inhibition of this pathway has been found to negatively affect viral protein synthesis and release of progeny virus (Luo et al., 2002; Pleschka et al., 2001). In addition, reovirus was found to require p38 kinase for virus replication (Norman et al., 2004). Only inhibition of the MEK/ERK pathway by U0126 abolished viral replication in 293-Ras cells (Fig. 6a). Furthermore, a stronger ERK phosphorylation was induced in 293-Ras cells (Fig. 6b). Comparison of the inhibitory effect of U0126 on HAdV-41 replication also indicated that twice the degree of viral inhibition occurred in 293-Ras cells. These data suggest that Ras/MEK/ERK is involved directly in HAdV replication in 293-Ras cells.

The exact mechanism underlying the promotion of virus replication by the Ras signalling pathway warrants further study. Ras was recently shown to reduce signal transduction and activation of transcription proteins STAT1 and STAT2 through the MEK pathway (Christian et al., 2009; Klampfer et al., 2003). Indeed, a previous study improved HAdV-40 yield in cells that expressed defective STAT1 signalling (Sherwood et al., 2007). Inhibition of STAT1 may lead to a reduced IFN-mediated antiviral response, facilitating infection of cells. The Ras/Raf/MEK pathway was also found to prevent the IFN response during vesicular stomatitis virus propagation (Battcock et al., 2006; Noser et al., 2007). Consequently, it is conceivable that Ras hinders the host defence system through links with the IFN and STAT downstream pathways. In addition, the Ras oncogene plays both pro- and anti-apoptotic roles (Mi et al., 2001). Early apoptosis does not provide adequate time for virus propagation. Alternatively, successful exploitation of this cellular response will facilitate more efficient virus propagation (Hay & Kannourakis, 2002). A great advantage of apoptosis associated with virus replication is that the virus can be disseminated without activation of the host immune system (Teodoro & Branton, 1997). The Ras/MEK/ERK pathway can be either pro- or anti-apoptotic (Cox & Der, 2003). Ras suppressed c-Myc-induced apoptosis through the PI3K/Akt pathway, but promoted apoptosis through the Raf/ERK pathway in serum-starved cells (Kauffmann-Zeh et al., 1997). Another study tested

**Fig. 3.** Flow cytometry assessment of HAdV-41 expression in 293 and 293-Ras cells. An FITC-conjugated anti-AdV antibody was used to determine the percentage of positive cells, shown as the proportion of region M1. The cells were either infected with $6.6 \times 10^4$ viral genome copies ml$^{-1}$ (infected), incubated with culture medium without virus (mock-infected) or cultured in the absence of virus (control).
the process of cell transformation by activating c-Myc and Ras, and revealed that blocking of apoptosis by MEK/ERK is a prerequisite for cell transformation (Tsuneoka & Mekada, 2000). Thus, the balance between Ras signalling pathways may facilitate viral dissemination in 293-Ras cells.

In summary, we constructed a cell line that exhibits enhanced growth of fastidious HAdVs. The newly constructed cells were shown to be highly competent in terms of supporting the growth of various HAdV strains, particularly those from clinical samples, and may prove useful for cultivation of viruses from environmental samples. Rapid detection of HAdVs will be useful in public health control with regard to ensuring efficient monitoring and prevention of waterborne diseases caused by the presence of this virus in drinking water.

**METHODS**

**Virus culture and stock.** HAdV-41 was obtained from the American Type Culture Collection (ATCC VR-930) and propagated in human embryonic kidney 293 cells that were grown in Eagles’ minimal essential medium (Gibco) with 10% FBS (Gibco). The infected cell lysates were purified by chloroform extraction and centrifuged at 4000 g for 15 min at 4 °C. The supernatant was recovered and ultrafiltered using Amicon Ultra-15 (Millipore) to concentrate the virus. These virus stocks were stored at −80 °C prior to use. The quantity of viral stock was estimated by serial dilution and a PCR amplification of viral DNA, and shown to be 3.3 × 10⁶ viral genome equivalents.

Fig. 4. TEM images: (a) 293 cell line at 3 days p.i. with HAdV-41 (bar, 2 μm), (b) 293-Ras cell line at 2 days p.i. with HAdV-41 (bar, 1 μm), (c) 293-Ras cell line at 2 days p.i. with HAdV-41 (bar, 2 μm), and (d) 293-Ras cell line at 3 days p.i. with HAdV-41 (bar, 2 μm). Arrowheads indicate HAdVs.
Viral replication and transient transfection with the Ras gene. The H-RasV12-expressing retroviral vector pBABE was kindly provided by Kensuke Hirasawa (Memorial University of Newfoundland). The Ras-expressing vector was transfected transiently into 293 cells using Lipofectamine (Invitrogen) according to the manufacturer’s instructions, with pcDNA3 as a control. After a 24 h incubation, each well was inoculated with 3.2 × 10^2 TCID_{50} ml^{-1} of HAdV-41. At 3 days p.i., viral DNA was extracted using a QIAamp Viral DNA Mini Kit (Qiagen). Total RNA extraction was carried out using an Easy-spin Total RNA extraction kit (Intron). To prevent genomic DNA contamination, the mRNA extract was treated with RQ1 RNase-Free DNase (Promega) for 30 min at 37 °C. Real-time TaqMan RT-PCR was performed using an ABI 7300 real-time PCR system (Applied Biosystems) as described previously (Ko et al., 2005). Quantification of viral DNA and mRNA was performed using a standard curve generated from 10-fold serial dilutions of viral capsid gene of known copy number. The primer and probe set targeting the hexon region was used in this study. Amplification of an internal gene of known copy number. The primer and probe set targeting the standard curve generated from 10-fold serial dilutions of viral capsid gene.

Fig. 5. Viral replication efficiency of 293-Ras cells. Three enteric HAdV strains and an HRV strain were used to infect 293 and 293-Ras cells. Viral DNA was amplified using real-time PCR at 5 days p.i. for the viruses isolated from clinical samples and at 3 days p.i. for the laboratory-adapted HAdVs. The copy number of control DNA was set as 100 %. Laboratory-adapted HAdV-41 titres were determined by the TCID_{50} assay at 4 days p.i. Error bars, SE of three and two independent experiments for real-time PCR and TCID_{50} assay, respectively. *P<0.05 based on Student’s *t*-test.

Construction of a Ras-expressing cell line. The 293 cells were harvested at 2.5 × 10^5–2.5 × 10^7 cells ml^{-1}. Electroporation was performed with 600 μl of the cell suspension and 20 μg of pBABE in a prechilled electroporation cuvette (Bio-Rad) at 260 V, 960 μF using a Gene Pulser II unit (Bio-Rad). After a 5 min post-pulse incubation on ice, the cell suspension was grown in T25 flasks under the same culture conditions as above. Transformed cells were selected under G418 (Gibco) as described previously (Kim et al., 2010). Introduction and expression of the H-Ras gene were confirmed by PCR and RT-PCR. Genomic DNA was extracted using a G-spin Cell/Tissue kit (Intron). Primer sequences were: mod T7-F, 5’-ATT AAT ACG ACT CAC TAT AGG GAG-3’ (forward); Ras-R, 5’-CAC ACT TGC AGC TCA TGC AGC-3’ (reverse). The primers were designed to amplify part of the Ras-expressing vector due to the intrinsic Ras oncogene expressed in the parental 293 cells. PCR was performed with an initial denaturation for 2 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C 30 s and extension at 72 °C for 30 s. The real-time PCR assay was performed using the fixed sample volume in this study. TGA TGA CCC TTT-3’. The amplifications were carried out with an initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C 30 s and extension at 72 °C for 30 s. The real-time PCR assay was performed using the fixed sample volume in this study.
verify ERK levels, the cells were inoculated with HAdV-41 secondary anti-mouse–HRP antibody (1 : 5000 dilution; Abcam). To cytometry (Seoul National University Hospital Clinical Trial Center).

Subsequently, 50 μl was added to acetone/methanol (1 : 1) and washed with 1 % FBS. They were then fixed in 1 % paraformaldehyde and washed with PBS. They were then added to a 1 : 1000 dilution of polyclonal anti-ERK and anti-p-ERK antibodies (Santa Cruz), and a 1 : 2500 dilution of secondary anti-rabbit and a 1 : 5000 dilution of secondary anti-mouse-HRP antibodies. Quantification of the band intensities was carried out using ImageJ 1.47t software.

**Western blotting.** The Ras protein level in the new cells was confirmed by Western blotting as described previously (Kim et al., 2010). Antibodies used were anti-Ras mAb (1 : 2000 dilution; Millipore), anti-β-actin antibody (1 : 10000 dilution; Abcam) and secondary anti-mouse–HRP antibody (1 : 5000 dilution; Abcam). To verify ERK levels, the cells were inoculated with HAdV-41 (1.6 × 10⁵ TCID₅₀ ml⁻¹) for 3 h and probed in a 1 : 1000 dilution of polyclonal anti-ERK and anti-p-ERK antibodies (Santa Cruz), and a 1 : 2500 dilution of secondary anti-rabbit and a 1 : 5000 dilution of secondary anti-mouse-HRP antibodies. Quantification of the band intensities was carried out using ImageJ 1.47t software.

**Enteric HAdV replication in cells overexpressing Ras protein.** The 293 and 293-Ras cells were infected for 5 days with 3.2 × 10⁵ TCID₅₀ ml⁻¹ of laboratory-adapted HAdV-41 or HAdV-40/41 from clinical stool samples. Viral DNA was quantified as described above. The level of HAdV-41 protein in the new cells was estimated by flow cytometry (BD Biosciences). Cells were prepared and infected with 3.2 × 10⁵ TCID₅₀ ml⁻¹. At 1 day p.i., the cells were fixed in 1 % paraformaldehyde and washed with PBS. They were then added to acetone/methanol (1 : 1) and washed with 1 % PBS. Subsequently, 50 μl of FITC-conjugated anti-Adv antibody (0.1 mg ml⁻¹; Abcam) was added to the cells, followed by incubation at 4 °C for 1 h. The cells were then washed in PBS before being assessed by flow cytometry (Seoul National University Hospital Clinical Trial Center).

In addition, viral particles were examined under a transmission electron microscope (JEOL) at the Seoul National University Hospital Clinical Trial Center. Infected cells were harvested and fixed with 1.5 % glutaraldehyde (Sigma).

**Effect of the Ras signalling pathway on enteric HAdV replication.** The 293 and 293-Ras cells were seeded and infected as described above. At 3 h p.i., inhibitors (40 mM) were added to each well followed by incubation at 37 °C in 5 % CO₂ for 3 days. The inhibitors used were U0126, JNK inhibitor II, LY29400 and SB203058 (Calbiochem). Infected 293 cells were treated only with U0126. As the inhibitors were prepared by dissolving in DMSO, equal volumes of DMSO were used as controls. At 3 days p.i., adenoviral DNA was prepared and quantified by real-time PCR as described above.

**HRV replication in the new cell line.** HRV-6 was purchased from the American Type Culture Collection (ATCC VR-486). The 293 and 293-Ras cells were seeded overnight and infected with 5.2 × 10⁴ TCID₅₀ ml⁻¹. At 5 days p.i., viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen) and quantified by real-time RT-PCR. We used previously designed primers and probes, excluding the reverse primer, which was designed to target a specific rhinovirus serotype (Gambarino et al., 2009). The reverse primer sequence used was 5′-GGT CCG GAA TTG CTC ATT ACG-3′. The conditions for the real-time RT-PCR included a RT step for 10 min at 45 °C and PCR cycling with an initial
denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and annealing/elongation at 60 °C for 45 s.

**Statistical analysis.** The levels of gene expression and copy numbers were analysed statistically using a paired Student’s t-test with SPSS software, version 19 (Armonk).

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