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E1A expression might be controlled by miR-214 in cells with low adenovirus productivity

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ABSTRACT

In this study, we explored the differences in the human adenovirus type 5 (Ad5) production efficiencies of various cell types. The rate of virus production was higher in several cell lines, such as HeLa cells, than in Saos-2 cells. The expression level of the coxsackie and adenovirus receptor (CAR) protein, an adenovirus receptor, was very similar among these cell lines. Although no significant difference in the expression of early region 1A (E1A) mRNA was detected, the amount of E1A protein in the Saos-2 cells was markedly lower than that in HeLa cells. Proteasome inhibitor treatment did not rescue the quantity of E1A in the Saos-2 cells, suggesting that their decreased E1A protein expression is not due to protein decay. To examine the different expression of E1A protein, we employed a bioinformatics approach to identify miRNA that target the 3′-untranslated region (3′-UTR) of E1A mRNA and identified miR-214 as a highly promising candidate. In Saos-2 cells, which have abundant levels of endogenous miR-214, the expression of luciferase was dramatically repressed, when the reporter gene was fused with the 3′-UTR of E1A mRNA including an miR-214 binding site. On the other hand, the activity from the same reporter was unchanged in HeLa cells, which display low-level miR-214 expression. Finally, we confirmed that the knockdown of the miR-214 upregulated the productive efficiency of the virus. These findings indicate that cellular miR-214 is capable of inhibiting adenovirus replication by regulating the translation of E1A protein.

1. Introduction

Adenoviruses are non-enveloped DNA viruses, with a linear, double-stranded DNA molecule of about 36 kilobase pairs in length. The virion is an icosahedral particle measuring 70–100 nm in diameter, and the adenovirus particle is primarily composed of three major capsid proteins, hexons, pentons, and fibers (Hierholzer, 1992; Shenk, 2001). Currently 52 different serotypes of adenovirus have been found in human hosts. The infection of human cells with adenoviruses leads to the expression of several classes of early proteins; the induction of cellular and viral DNA synthesis; the production of late viral proteins; the formation of progeny virions; and finally, cell lysis. It has been shown that the efficiency of virus infection differs between cell lines. Several receptors including coxsackie and adenovirus receptor (CAR) (Bergelson et al., 1997; Roelvink et al., 1998) are suggested to be the cellular factors responsible for the initial contact between adenoviruses and their target cells.

E1A is the first expressed gene product after adenovirus infection, and the transcription of E1A mRNA is activated by host cell transcription factors (Higashino et al., 1993; Bruder and Hearing, 1989). The E1A gene encodes two major proteins of 289 and 243 amino acids in length which arise from differential splicing of the same mRNA. In adenovirus infected human cells, E1A is essential for productive viral infection (Jones and Shenk, 1979). E1A contributes to the activation of almost all other adenovirus genes and multiple host cellular genes at the transcription level by interacting with transcription factors and co-factors such as pRB and p300 (Frisch and Mymryk, 2002).

MicroRNA (miRNA) are highly evolutionally conserved small 20–25 nucleotide non-coding RNA that bind to the 3′-untranslated region (UTR) of mRNA and elicit translational repression, mRNA cleavage, or degradation. So far, more than 1000 miRNA have been identified in various organisms, and they are known to regulate basic cellular functions including proliferation, differentiation, and

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cell death (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Krutzfeldt et al., 2006; Croce, 2009). It has been suggested that mammalian cells inhibit virus infection by targeting viral transcripts with cellular miRNA (Ghosh et al., 2009; Grassmann and Jeang, 2008; Skalsky and Cullen, 2010).

In the present study, we found that differences in the E1A protein level are responsible for the divergent adenovirus replication efficiencies of various cells. The expression of E1A is controlled at the translational level by miR-214, which is abundantly expressed in the cells that show low adenovirus productivity. Therefore, we concluded that cellular miRNA play a role in anti-adenovirus replication by regulating virus gene expression.

2. Materials and methods

2.1. Cells and virus proliferation assay

The oral squamous cell carcinoma cell line HSC3, the non-small lung cancer cell line H1299, the cervical cancer cell line HeLa, and the osteosarcoma cell line Saos-2 were used in this study. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10–% fetal bovine serum and antibiotics at 37 °C in a 5–% CO_2 atmosphere under humidified conditions. Then, cells were infected with adenovirus type 5 (Ad5) variant dl309 (Jones and Shenk, 1979), which is phenotypically wild-type Ad5, in 12 well dishes at an MOI of 10 ifu/cells. All cells were collected at 48 h after infection, and virus lysate was prepared using three cycle of freezing and thawing. The infectious titers of Ad5 dl309 were determined using the Adeno-X™ Rapid Titer Kit (Clontech) and HEK293 cells. Infectious units were calculated according to the manufacturer's protocol.

2.2. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated using the RNeasy Mini kit (QIAGEN) and reverse transcribed using ReverTra Ace (TOYOBO) with random primers. cDNA generated from 1 μg of total RNA was used for the real-time RT-PCR together with SYBR Green Realtime PCR Master Mix Plus (TOYOBO) using specific primers for E1A and β-actin. Quantification was performed using the comparative $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), and the resultant C_T values were normalized to the expression of β-actin.

The expression of miRNA was also analyzed using TaqMan MicroRNA assays (Applied Biosystems). Total miRNA was isolated using the mirVana miRNA Isolation Kit (Applied Biosystems) according to the manufacturer's protocol. TaqMan qRT-PCR was performed according to the manufacturer's protocol using miR-214 and small nuclear RNA U6 specific primers, and the data evaluation was performed using the ΔΔCt method.

2.3. Western blot analysis

The cells were lysed with 1% NP-40 lysis buffer (150 mM NaCl; 50 mM Tris–HCl, pH 7.5; 1% Nonidet P-40) in the presence of protease inhibitors. Twenty μg of each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted onto a PVDF membrane (Millipore). After blocking the membrane with a solution of TBS and 0.1% Tween 20 containing 5% nonfat dried milk at 4 °C overnight, the membranes were treated with primary antibodies against adenovirus E1A (M73, generous gift from T. Shenk) and CAR (RmcB, Millipore), before being incubated with a horseradish peroxidase-conjugated anti-mouse secondly antibody (Jackson Immuno Research). β-Actin was detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit β-actin (I-19) (Santa Cruz Biotechnology). An enhanced chemiluminescence detection system (Perkin Elmer) was used to visualize protein.

2.4. Proteasome inhibitor assay

The cells were infected with Ad5 dl309 in 6-well dishes at an MOI of 50 ifu/cell. The proteasome inhibitors lactacystin (Calbiochem) and MG-132 (Calbiochem) were dissolved in DMSO, and the dl309-infected cells were treated with 20 μM of each proteasome inhibitor for up to 12 h. The control cells were treated with the vehicle alone.

2.5. Surveying candidate miRNA for adenovirus E1A transcript

MicroInspector (Rusinov et al., 2005), an algorithm for predicting miRNA and their binding sites in queried RNA sequences was used to search for miRNA that would be able to bind to the human 3′-UTR of E1A mRNA [Ad5 from nt 1546 to nt 1617, AC_000008]. The hybridization temperature and free energy cut-off value were set at 37 °C and −20 kcal/mol, respectively. During our research of the miRNA database, Homo sapiens was selected in the species category.

2.6. Reporter plasmid reconstruction for miRNA efficiency

To generate a multicloning site (*XbaI/NheI/XhoI/BglII/EcoRI/SpeI*) in pCMVGL (Higashino et al., 2005), a luciferase reporter including the CMV promoter, the oligonucleotides 5′-CTAGAGCTAGCCTCGAGAGATCTGAATTCA-3′ and 5′-CTAGTGAATTCAGATCTCTCGAGGCTAGCT-3′ were annealed to obtain a 30-bp fragment, and the resultant dsDNA was inserted into the *XbaI* site of pCMVGL. In order to produce the pCMVGL-hAd5 E1A 3′UTR, the 71-bp fragment containing the 3′UTR of the human Ad5 *E1A* gene was amplified by PCR using the 293 cell genome with a 5′ primer containing an *XbaI* restriction site (5′-CTAGAGCTAGCCTCGAGAGATCTGAATTCA-3′) and a 3′ primer containing an *EcoRI* restriction site (5′-CTAGTGAATTCAGATCTCTCGAGGCTAGCT-3′), and the fragment was cloned into the *XbaI/EcoRI* site of the pCMVGL reporter plasmid. To produce pCMVGL-hAd5 E1A 3′UTR mut, the sequence 5′-GTTTGCTG-3′ complementary for the seed sequence of miR-214 were changed to 5′-GTAATAAT-3′ by using KOD-Plus-Mutagenesis Kit (TOYOBO). The pRLTK plasmid (Promega) was used as an internal control.

2.7. Reporter assays

Passive lysis buffer (Promega) was used to prepare cell lysates for the reporter expression assays. Luciferase activity was measured in a Lumat LB9507 (BERTHOLD) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The relative reporter activity was obtained by normalizing it to the level of Renilla luciferase activity.

2.8. Knockdown of miRNA

Saos-2 and HeLa cells were transfected with miRZip Lentivector-based Anti-MicroRNA for miR-214 (System Biosciences) and miRZip Lentivector-based Scramble Hairpin Control Anti-MicroRNA (System Biosciences) using Hily Max transfection reagent (Dojindo). After 24 h, the cells were infected with dl309 in MOI 10. At 24 h post infection, cells were harvested to estimate virus titer.

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