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Research paper

Targeting species D adenoviruses replication to counteract the epidemic keratoconjunctivitis

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ABSTRACT

Human adenoviruses are non-enveloped DNA viruses causing various infections; their pathogenicity varies dependent on virus species and type. Although acute infections can sometimes take severe courses, they are rarely fatal in immune-competent individuals. Adenoviral conjunctivitis and epidemic keratoconjunctivitis are hyperacute and highly contagious infections of the eye caused by human adenovirus types within species D. Currently there is no causal treatment available to counteract these diseases effectively. The E2B region of the adenovirus genome encodes for the viral DNA polymerase, which is required for adenoviral DNA replication. Here we propose novel model systems to test this viral key factor, DNA polymerase, as a putative target for the development of efficient antiviral therapy based on RNA interference. Using our model cell lines we found that different small interfering RNAs mediate significant suppression (up to 90%) of expression levels of viral DNA polymerase upon transfection. Moreover, permanent expression of short hairpin RNA based on the most effective small interfering RNA led to a highly significant, more than tenfold reduction in replication for different human group D adenoviruses involved in ocular infections.

1. Introduction

A wide variety of human diseases is caused by adenoviruses. Human adenoviruses (HAdV) are non-enveloped DNA viruses, which are divided into seven species (from A to G) based on their morphological, hemagglutinating and oncogenic properties as well as their genome size and DNA sequence [1–3].

Adenoviruses are known as common cause of human ocular, respiratory and gastrointestinal infections, which can occur throughout the whole lifespan [4]. In the healthy human population, adenoviral infections are in most cases self-limiting, and there is no relation to sex, ethnic origin, social status, or nutritional state [5]. However, potentially life-threatening adenovirus infections occur more frequently in immunocompromised individuals [6,7]. Available data suggests that detectable adenovirus in serum has been associated with fatal adenovirus-related infections in up to 70% of allogeneic stem cell transplant recipients [7].

Human adenovirus type D36 (HAdV-D36) is associated with increased adiposity, and important metabolic alterations in children and adults [8]. There are several reasons why HAdV-D36 infection may result in obesity development. It is known that infection accelerates the differentiation of preadipocytes to adipocytes [9] and stem cells to adipocytes [10]. Also HAdV-D36 infection induces lipid accumulation by increasing glucose uptake in adipocytes and upregulation of adipogenesis-related genes [11]. Several reports indicate HAdV-D37 involvement in metabolic disorders such as obesity and nonalcoholic fatty liver disease [9,12]. HAdV-D37 also causes inflammatory eye diseases [13].

Eye infections caused by human adenoviruses include adenoviral conjunctivitis (AC), an inflammation of the eye mucous membrane – conjunctiva, as well as epidemic keratoconjunctivitis (EKC), a hyperacute inflammation of the conjunctiva and the cornea

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mostly caused by human adenoviruses of species D, in particular, types D8, D19, and D37 [14]. AC and EKC are extremely common and highly contagious ocular infections that can cause significant morbidity, accompanied by high social and quality-of-life costs, and lead to high economic losses in industrial countries. Adenoviral eye infections reportedly account for up to 80% of cases of temporary disability among ophthalmological patients and up to 10–15% of all cases of significant impairment of visual acuity in Russia [15]. More than 300,000 EKC cases are diagnosed in Russia per year [16]. In contrast, 316 EKC cases were reported across Germany in the first eight months of 2010 [5,17].

Currently there is no causal therapy, which is effective to treat AC and EKC. Treatment of these diseases is mainly focused on alleviating symptoms and preventing complications, while the patient immune system resolves the infection [18,19]. Thus, there is an urgent need for new etiology-directed therapies and drugs with selective activity against these viruses.

To address this problem, the adenoviral DNA polymerase seems to be a promising target. The highly conserved *E2B* region of the adenovirus genome encodes for the viral DNA polymerase (*pol*), which is required for efficient replication of adenoviral DNA [20,21] and therefore is a prerequisite for the virus to enter the late phase of its replication cycle. We hypothesized that prevention of *pol* mRNA translation might interrupt the adenoviral lytic replication cycle, due to an effective inhibition of viral genome replication and viral protein synthesis.

RNA interference (RNAi) is a powerful technique for investigating gene functions [22,23] and one of the most promising approaches for gene therapy. Several reports have demonstrated that small interfering (si) RNAs can downregulate the expression of different target genes, and also attenuate viral infection and replication [23–28].

Lentiviral vectors have shown great promise in gene function studies and gene therapy applications, including the stable and efficient delivery of small hairpin (sh) RNAs, precursors of siRNAs [29,30]. Hairpin structures have successfully been used for the inhibition of gene expression during the development of viral infections [31].

Here we asked whether RNAi might be used for targeted inhibition of adenoviral DNA polymerase gene expression, both by the means of synthetic siRNAs and shRNA expressed from highly efficient lentiviral vectors.

2. Materials and methods

2.1. Cell culture

HEK293 [32], A549 (DSMZ ACC107; Brauschweig, Germany) and H1299 [33] cells were cultivated in Dulbecco's Modified Eagles Medium (DMEM) (Life technologies, UK), containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamin. Cells were grown at 37 °C in 95% air/5% CO2 incubator. A549 E2B, H1299 E2B, and H1299-shRNA-E2B cell lines were maintained in the same growth media as their parental lines.

2.2. Lentiviral vectors

All lentiviral vectors were constructed using standard methods of gene engineering [34]. Viral particles were produced by calcium phosphate co-transfection of HEK293 cells. Infectious viral particles were collected 24–72 h later at 12 h intervals. Titers of 5×10^5 to 5×10^6 transducing units (TU)/ml were used for further research. Titration was performed on uninfected A549 and H1299 cells. Detailed protocols on lentiviral vector production and titration are available at www.LentiGO-Vectors.de [34,35].

2.3. Indicator cell lines

Lentiviral vectors encoding either the DNA polymerase of human adenovirus group D type 36 (*pol-D36*) alone or *pol-D36* fused to the FLAG marker sequence (DYKDDDDK epitope) cDNA were used for transduction of A549 and H1299 cells respectively. Selection was carried out using 3 μg/ml puromycin (Sigma-Aldrich, USA) for transduced A549 and H1299 cells respectively. Control A549 and H1299 cells that did not express puromycin-resistance gene died within 1 week. After 10 days of selection A549 E2B and H1299 E2B cells were analyzed by flow cytometry. To obtain homogenous populations A549 E2B and H1299 E2B cells were cloned by limiting dilution. Cell clones were analyzed by flow cytometry. Initially, both A549 and H1299 cells were transduced with lentiviral vectors expressing *pol-D36*, fused to the FLAG marker sequence. Expression levels of HAdV-D36 DNA polymerase protein fused to the FLAG epitope, determined by Western blot, were significantly higher in H1299 cells than in A549 cells (see Supplementary Fig. 1). Therefore we conducted further experiments on H1299 E2B indicator cell line.

Lentiviral vectors expressing anti-E2B-shRNA were used to transduce H1299 cells. To obtain homogenous populations, H1299 anti-E2B-shRNA cells were cloned by limiting dilution. Cell clones were analyzed by flow cytometry.

2.4. siRNAs

We designed 3 siRNAs complementarytothe *pol* mRNA sequences of HAdV-D8, -D19, -D36 and -D37. siRNAs were obtained from Syntol (Russia). 21-mer oligonucleotides carrying two nucleotide (nt) UU overhangs at their 3′ ends were synthesized: siRNA E2B-1 (sense strand 5′-CGGUGACUUCAAUUAUGAAUU-3′, antisense strand 5′-UUCAUAAUUGAAGUCACCGUG-3′); siRNA E2B-2 (sense strand 5′-CGGUUGAAUGUAAGGCUUAUU-3′, antisense strand 5′- UAAGCCUUACAUUCAACCGUG-3′), and siRNA E2B-3 (sense strand 5′-CGGUUGAAUGUAAGGCUUAUU-3′, antisense strand 5′- UAAGCCUUACAUUCAACCGUG-3′). As negative control, non-targeting scrambled siRNA Scr (sense strand 5′-CAAGUCUCGUAUGUAGUGGUU-3′, antisense strand 5′-CCACUACAUACGAGACUUGUU-3′) matching the type of design of the respective targeting siRNAs was used. siRNAs were designed using Whitehead Institute siRNA Selection Program [36].

2.5. Transfection of siRNAs

3×10^4 model or parental cells per well were plated on a 24-well plate in 0.5 ml medium and transfected with siRNA at a concentration 200 nM using Lipofectamine® 2000 Transfection Reagent (Life technologies, USA).

2.6. shRNA

Based on the sequence of the siRNA with the highest level of gene inhibition, anti-E2B-shRNA (sense strand 5′-p-AACGCACCCGTAGAAATCAAGTACTTCTGCAATACTTGATTTCTACGGGTGTTTTTC-3′, antisense strand 5′-p-TCGAGAAAAACACCCGTAGAAATCAAGTATTGCAGGAAGTACTTGATTTCTACGGGTGCGTT-3′) encoding lentiviral vector (anti-E2B-shRNA LeGO-G) and a corresponding control vector encoding a non-targeting Scr-shRNA (sense strand 5'-p-GATCCGCAAGTCTCGTATGTAGTGGCTTCCRGTCACCACTACATACGAGACTTGTTTTTG-3′, antisense strand 5'-p-AATTCAAAAACAAGTCTCGTATGTAGTGGTGACAGGAAGCCACTACATACGAGACTTGCG-3′) were designed. Lentiviral vectors were cloned using standard