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CD46 is a Protein Receptor for Human Adenovirus Type 64

by

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Honors Thesis

Submitted to:

Biochemistry and Molecular Biology Department
University of Richmond
Richmond, VA

May 3, 2024

Advisor: Dr. Eugene Wu

This thesis has been accepted as part of the honors requirements
in the Program in Biochemistry and Molecular Biology.

Eyleen Wu — 2024 April 30

(advisor signature) *(date)*

Joanne Rattelbaum — 4/30/2024

(reader signature) *(date)*

Abstract

Adenoviruses are important gene delivery vectors and causative agents for a variety of human diseases such as the common cold and gastrointestinal infections. Human adenovirus type 64 (Ad64; formerly 19c) and type 37 are associated with epidemic keratoconjunctivitis. Based upon its high homology and similar disease tropism to Ad37, we hypothesized that Ad64 would have the same protein receptor, CD46 (membrane cofactor protein), as Ad37. We show that a recombinant Ad64 containing an enhanced Green Fluorescent Protein transgene (Ad64.eGFP) enters Chinese hamster ovary cells expressing human CD46 (CHO-CD46) on the surface. Entry into human cervical carcinoma (HeLa) cells is increased by the presence of calcium, but that increase can be blocked by an anti-CD46 antibody. Ad64.eGFP gene delivery into human conjunctival epithelial (HCjE) cells can also be blocked by soluble CD46, supporting our hypothesis that Ad64 uses CD46 as a protein receptor on the surface of target cells. CD46 expression on CHO-CD46, HeLa, and HCjE cells was confirmed by flow cytometry and/or western blotting. The identification of CD46 as a receptor for Ad64 makes Ad64 an attractive candidate for gene delivery into eye cells as well as other cells that express CD46.

Introduction

Adenoviruses are non-enveloped viruses containing double-stranded DNA in an icosahedral capsule measuring around 90-100 nm in diameter. Most commonly, adenoviruses are associated with respiratory infections, gastroenteritis, genitourinary infections, and epithelial keratoconjunctivitis. Epithelial keratoconjunctivitis (EKC), known colloquially as pink eye, refers to the inflammation of corneal and conjunctival epithelial cells of the eye. Causes of EKC can be non-infectious, such as allergens, toxins, and immunological conditions, as well as infectious, such as viruses and bacteria. In the infectious category, approximately 75% of all cases are caused by adenovirus, for which there is little to no direct treatment (3). Severe cases of EKC are reported to cause chronic inflammation of the region, scarring, and limbal stem cell deficiencies, all of which pose serious risks to patients around the world (3).

The family of *Adenoviridae* is divided further into seven species ranging from the letter A to the letter G. It is known that adenoviruses from both species B and D cause EKC. Previous research on adenovirus type 37 (Ad37) and adenovirus type 56 (Ad56) has identified CD46, or membrane cofactor protein, and sialic acid as the two main receptors for EKC causing

adenoviruses. CD46 is a surface protein found on a variety of cells in two main extracellular isoforms: BC and C (Figure 1B). Aside from acting as a receptor for other pathogens, its native function involves various pathways in the complement system of the immune response. In virus overlay protein binding assay (VOPBA) experiments, researchers have shown Ad37 to bind via the fiber knob to CD46 (8, 9). On the other hand, sialic acid is an oligosaccharide chain that extends from cell membrane proteins and lipids, creating negative pockets on the cellular surface that attract water, pathogens, and other molecules of interest. The use of neuraminidase, an enzyme that cleaves sialic acids, has been shown to decrease Ad37 infection of A549 lung epithelial cells by 60%, implicating sialic acid as another receptor for EKC causing viruses (2).

Adenovirus type 64 (Ad64), another species D adenovirus, experienced a reclassification from Ad19c in 2012 due to genetic sequencing studies (11). Like Ad37, Ad64 causes EKC and shares the same shaft and fiber knob as Ad37, prompting our interest in investigating Ad64 mechanisms of infection. Previous studies on adenovirus and EKC have used various non-ocular cell types to model infection and blocking experiments in-vitro. While some have used Chang C conjunctival cells, research has indicated their contamination with HeLa cervical cancer cells, rendering them as another non-ocular model (5). This paper aims to add to our model for EKC infection using immortalized human epithelial conjunctival (HCjE) cells and to elucidate the relationship between CD46, sialic acid, and Ad64.

Methods

Cell Culture

HCjE cells were grown in Serum-Free Media (SFM; ThermoFisher Scientific). Media was replaced every other day. For detachment, cells were fully detached with 0.05% Trypsin-EDTA (ThermoFisher Scientific) at 37 ºC for 12 minutes. An equivalent amount of Dulbecco's Modified Eagle Medium (DMEM) was added to the flask to neutralize the trypsin, and the total amount was spun down at 300 g for 5 minutes. Cells were resuspended in SFM and appropriately distributed.

Synthesis of cDNA and Negative RT control (for RT-PCR)

RNA was extracted from approximately 10^6 HCjE cells using 1 mL of Trizol reagent according to the manufacturer's protocols. The resulting washed RNA extract was resuspended

in 20 uL of RNAse-free water. The first strand of cDNA was synthesized according to the following steps: 1) Mix 4 µL SuperScript VILO MasterMix (Invitrogen), 4 µL RNA, and 12 µL RNAse-free water. 2) Incubate at 25 ºC for 10 minutes. 3) Incubate at 42 ºC for 60 minutes. 4) Terminate the reaction at 85 ºC for 5 minutes.

The negative RT control was prepared by mixing 4 µL SuperScript VILO MasterMix (Invitrogen) and 12 µL RNAse-free water. The mixture was incubated at 65 ºC for 10 minutes to heat denature the RT, then 4 µL of RNA was added. The samples then proceeded through the steps 2-4 mentioned above.

Reverse transcriptase polymerase chain reaction

All primers were prepared to a working concentration of 10 µM. For PCR, each sample had a final forward primer concentration of 0.5 µM, final reverse primer concentration of 0.5 µM, final SuperScript VILO MasterMix (Invitrogen) concentration of 1X, and an unknown amount of template DNA that was <250 ng. The constitutive gene chosen as a control was PPIB. Primer sequences are as follows: PPIB_fw – TGTGGTGTTTGGCAAAGT, PPIB_rev – TGGAATGTGAGGGGAGTG, CD46_exon6_fw – TGACAGTAACAGTACTTGGGA, CD46_exon1213_rev – ATCAGTTAGGTATGTGCCTTTC, CD46_exon1214_rev – ACCATCTGCTTTCCCTTTC. PPIB fw and rev pair amplified constitutively expressed PPIB. CD46_exon_6_fw amplified the start of CD46 for all isoforms. The presence or omission of exon 13 dictates tail type. CD46_exon_1213_rev binds tail 1 isoform cDNA. CD46_exon_1214 rev binds tail 2 isoform cDNA. Exon 8 dictates isoform, where omission of the exon corresponds to the C isoform. Thermocycler settings are as follows: 1) 98º 30 sec, 2) 98º 10 sec, 3) 55º 30 sec, 4) 72º 30 sec, repeat steps 2-4 30 times. The resulting samples were run through a 1.5% agarose gel using TAE buffer at 120 V for 70 minutes.

Western Blot

Whole HCjE cell lysate was obtained by lysing a confluent 225 cm^2 flask of cells with Mammalian Protein Extraction Reagent (M-PER; ThermoFisher Scientific). Gel loading samples were prepared using a 3:1 or 5:1 sample:4xSDS buffer ratio and ran through a 7.5% polyacrylamide gel at 150V for 70 minutes. The gel was transferred to polyvinyl difluoride (PVDF) membrane in a tank at 4 ºC at 150 V for 90 minutes. The resulting blot was blocked with

UltraCruz Blocking Reagent (Santa Cruz Biotechnology) at room temperature for 1 hour. Blocking reagent was freshly replaced and the blot was incubated with CD46 Antibody (C-10): sc-166159 conjugated to Alexa Fluor 488 (Santa Cruz Biotechnology) at a 1:1000 antibody:blocking reagent ratio overnight at 4 ºC. Blot was washed with TBS-T and imaged using ChemiDoc MP (Bio-Rad).

Fluorescent Antibody Flow Cytometry

Confluent T75 flasks of A549, HeLa, and HCjE cells were fully detached using TrypLE Express (ThermoFisher Scientific) at 37 ºC. The cells were spun down at 300 g for 5 minutes and washed once with PBS. Samples were resuspended in PBS, control samples were aliquoted, and experimental samples were incubated with CD46 Antibody (C-10): sc-166159 conjugated to Alexa Fluor 488 (Santa Cruz Biotechnology) at a 1:1000 antibody:solution ratio for approximately 4 hours on ice. Cells were spun down and resuspended in a small amount of PBS to for flow cytometry.

Infection-Blocking Experiment

HCjE cells were incubated overnight in a 24-well plate containing 50,000 cells per well. Viral infection was induced at a final viral concentration of 1.43×10^6 particles/mL overnight at 37 ºC. For CD46 blocking, soluble CD46 (GenScript Biotech) was added to virus laden SFM at a final concentration of 0.012 µg/mL and incubated at room temperature for 5 minutes before being added to wells. For sialic acid cleavage, neuraminidase (Sigma-Aldrich) treatment of cells prior to viral infection involved a final concentration of 31 milliunits incubated for 1 hour at 37 ºC. Cells the following day were detached using TrypLE Express (ThermoFisher Scientific) at 37 ºC and analyzed for transgenic eGFP expression using a Becton-Dickinson Accuri C6 flow cytometer.

Fiber Knob Blocking Experiments

Genes encoding recombinant fiber knobs were synthesized and inserted into pET28a-TEV-NHis by Genscript (Piscataway, NJ). BL21(DE3)pLysS cells (Invitrogen, Carlsbad, CA) were transformed with fiber knob plasmids, grown in LB, and induced with 0.5 mM IPTG to overexpress fiber knob protein. Cells were lysed with B-PER lysis reagent (ThermoFisher), and

fiber knobs with N-terminal 6-His tags were purified using batch purification over Ni-NTA Agarose (Qiagen). Fiber knobs were expressed and purified by H. Zhu in the E. Wu lab. HCjE cells were grown at 50,000 cells per well in a 24-well plate overnight. All experimental wells were treated with neuraminidase at a final concentration of 31 milliunits incubated for 1 hour at 37 ºC. Final concentration for Ad64 was titrated appropriately in previous experiments to obtain infection rates of around 20-30% in untreated cells. Ad19p and Ad37 fiber knob final concentrations were 30 µg/mL and 100 µg/mL, which were added into each well before adding virus. The plates were incubated overnight at 37 ºC, were detached using TrypLE Express (ThermoFisher Scientific) at 37 ºC, and were passed through flow cytometry the next day.

Results

HCjE cells express all four major isoforms of CD46:

The expression of the adenovirus receptor, CD46, in immortalized human conjunctival epithelial cells has not been characterized. HCjE cells were subjected to RNA extraction and reverse transcriptase-polymerase chain reaction to determine which isoforms of CD46 are produced by alternative splicing. The forward primer was designed to bind a sequence in exon 6 (present in all common isoforms) while the reverse primers were designed to bind the 3′-end of exon 12 and the 5′-end of exon 13 (tail 1) or the 3′-end of exon 12 and the 5′-end of exon 14 (tail 2). The RT-PCR results indicate that HCjE cells transcribe mRNA encoding for BC-1, BC-2, C-1, and C-2 isoforms of CD46 (Figure 1A). RT-PCR results performed in duplicate show no bands in the heat denatured RT condition. Both replicates showed strong presence of constitutively expressed PPIB gene at the expected ~300 bp, BC-1 and BC-2 isoforms at ~320 bp, and C-1 and C-2 isoforms at ~275 bp. BC isoform bands were present at a higher intensity compared to C isoform bands, but neither were as intense as PPIB (Figure 1A). To determine if CD46 isoforms are expressed in HCjE cells, HCjE lysate was subjected to Western blotting and found to contains both BC and C isoforms of CD46. The C isoform band appears as a streak between 50-60 kD and the BC isoform appears as a streak between 65-75 kD. The smeary appearance on the blot can be attributed to the heavy and variable glycosylation of the extracellular portion of CD46 as well as the hydrophobic transmembrane section (Figure 1C).

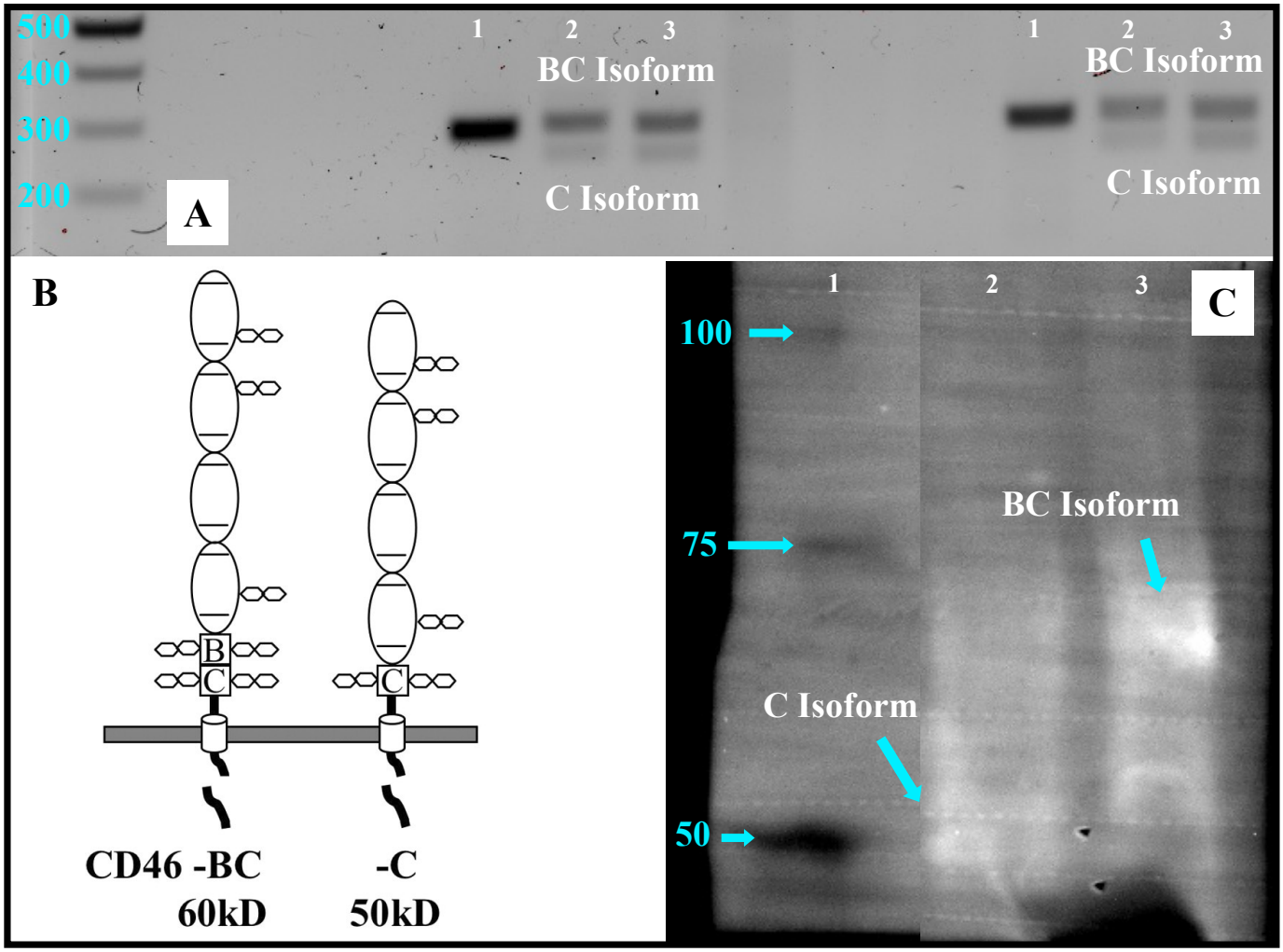

Figure 1. (**A**) RT-PCR duplicate of CD46 mRNA from human conjunctival epithelial cells. Negative control (blank lanes) is heat-denatured RT, experimental control (lane 1) was constitutively expressed gene PPIB at 300 bp, cytoplasmic tail 1 (lane 2), cytoplasmic tail (lane 3). (**B**) Diagram of BC and C isoforms of CD46 extending from the cell surface (9). Long, football-shaped ovals indicate SCR1-4 domains from top to bottom. Horizontal hexagons represent glycosylation. (**C**) Western blot of CD46 in human conjunctival cell lysates. Whole cell lysate using M-PER Reagent. Alexa-Fluor 488 primary antibody incubated in UltraCruz Blocking Reagent. Ladder (lane 1), 3:1 lysate:4x SDS (lane 2), 5:1 lysate:4x SDS (lane 3).

To confirm that expressed CD46 protein makes it to the cell surface as expected, flow cytometry using an anti-CD46 fluorescent antibody was employed to show that CD46 is present on intact A549, HeLa, and HCjE cells (Figure 2). All three control cell lines showed a sharp peak of fluorescence at ~11^3 mean fluorescence intensity (MFI) in the FITC-A channel (green). All three

experimental cell lines showed a sharp increase in fluorescence to ~10^5 MFI. There is a ~40-fold increase in MFI when tagging these cells with a CD46 antibody compared to the control samples.

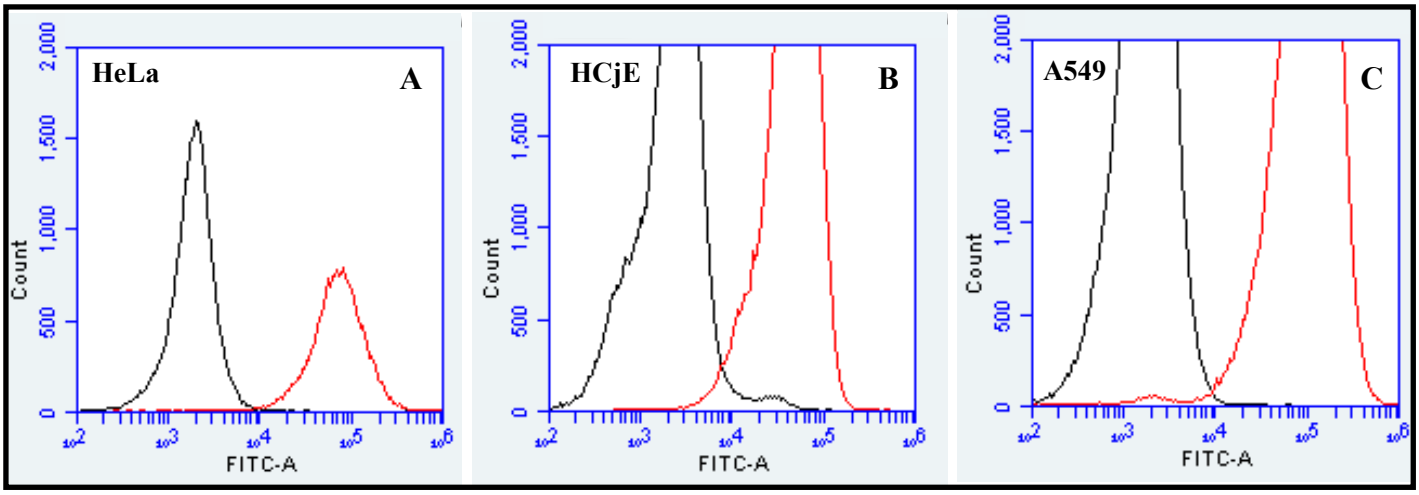

Figure 2. Flow cytometry analysis of CD46 expression. Black histogram represents control. Red histogram represents antibody-laden (Alexa-Fluor 488) cells.

Ad64 infection of HCjE cells can be blocked in various ways:

Expression of CD46 confers susceptibility to Ad64 infection. CD46 knockout Chinese hamster ovary (CHO) cells showed no Ad64 infection at less than 1%. The expression of BC isoform CHO cells significantly increased Ad64 infection to approximately 9.5% as well as C isoform expressing CHO cells to approximately 5% (Figure 3; $p = 0.0173$). I next sought to test Ad64 infection of HCjE cells. I found that HCjE cells were susceptible to infection by Ad64 and that soluble CD46, neuraminidase, and a combination treatment of both significantly decreased Ad64 infection of HCjE cells (Figure 4). Control HCjE cells fluoresced at 5,000 MFI while cells incubated with Ad64 exhibited a 5-fold increase in fluorescence to 25,000 MFI ($p < 0.0001$). Soluble CD46 decreased fluorescence to ~8,000 MFI, neuraminidase decreased to ~14,000 MFI, and the combination treatment decreased to ~10,000 MFI. Ad19p and Ad37 fiber knobs significantly inhibit Ad64 viral infection at both 30 µg/mL and 100 µg/mL ($p < 0.0001$). Ad64 infected HCjE cells fluoresced at 1.0E6 MFI, both Ad19p fiber knob conditions decreased fluorescence to ~7.0E5 MFI, and Ad37 fiber knob conditions decreased fluorescence to ~8.0E5 MFI and ~6.0E5 MFI when increasing fiber knob concentration, respectively (Figure 5).

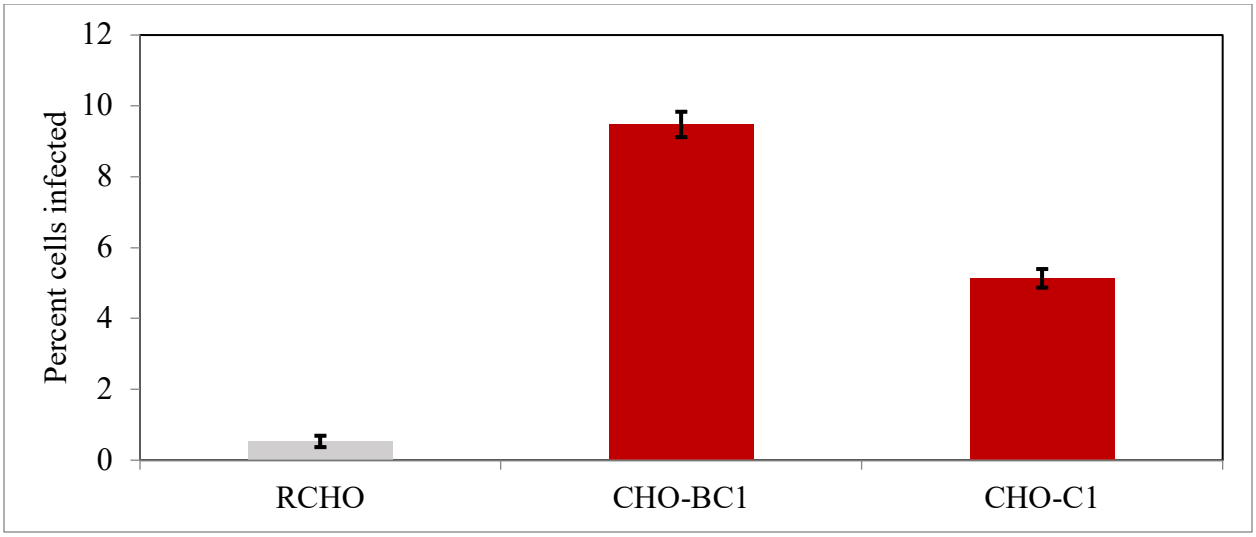

Figure 3. Ad64 mediated gene delivery into Chinese hamster ovary cells transgenically expressing CD46. Treatments were standard viral infection of RCHO (no CD46 expressed), CHO-BC1 (BC1 isoform expressed), CHO-C1 (C1 isoform expressed). Averages represent triplicate and error bars represent standard deviation. Red colored bars denote a significant difference ($p = 0.0173$)

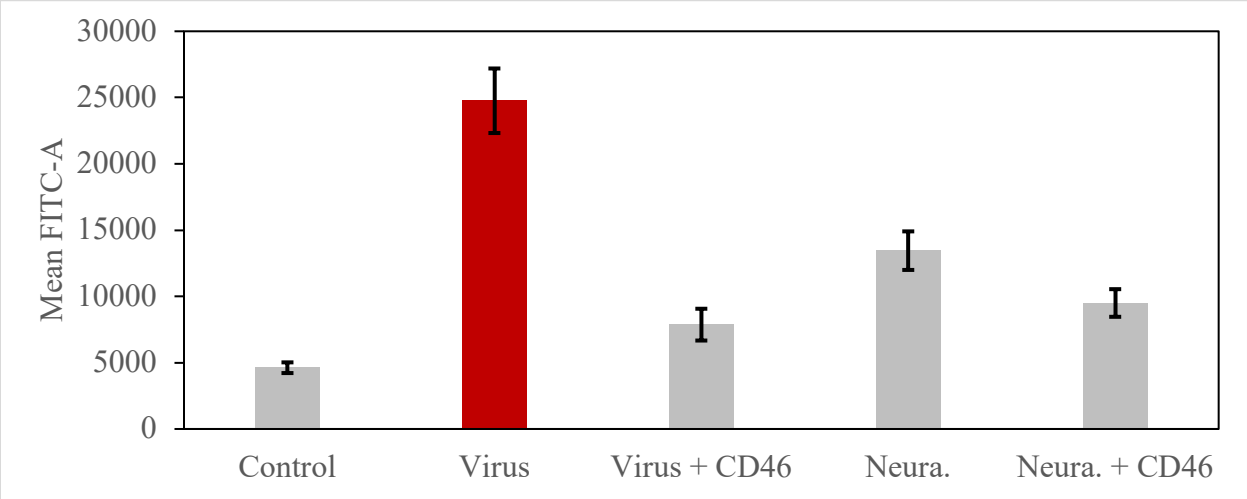

Figure 4. Ad64 mediated gene delivery into human conjunctival epithelial cells and CD46/sialic acid blocking experiments. Infections were done in the following treatments: only virus (+Virus), virus incubated 5 minutes with soluble CD46 before infection (Virus + CD46), cells incubated with neuraminidase for 1 hour prior to infection (Neura.), and the latter two combined (Neura. + CD46). Averages represent quintuplicate and error bars represent standard error. Red colored bar denotes a significant difference ($p < 0.0001$).

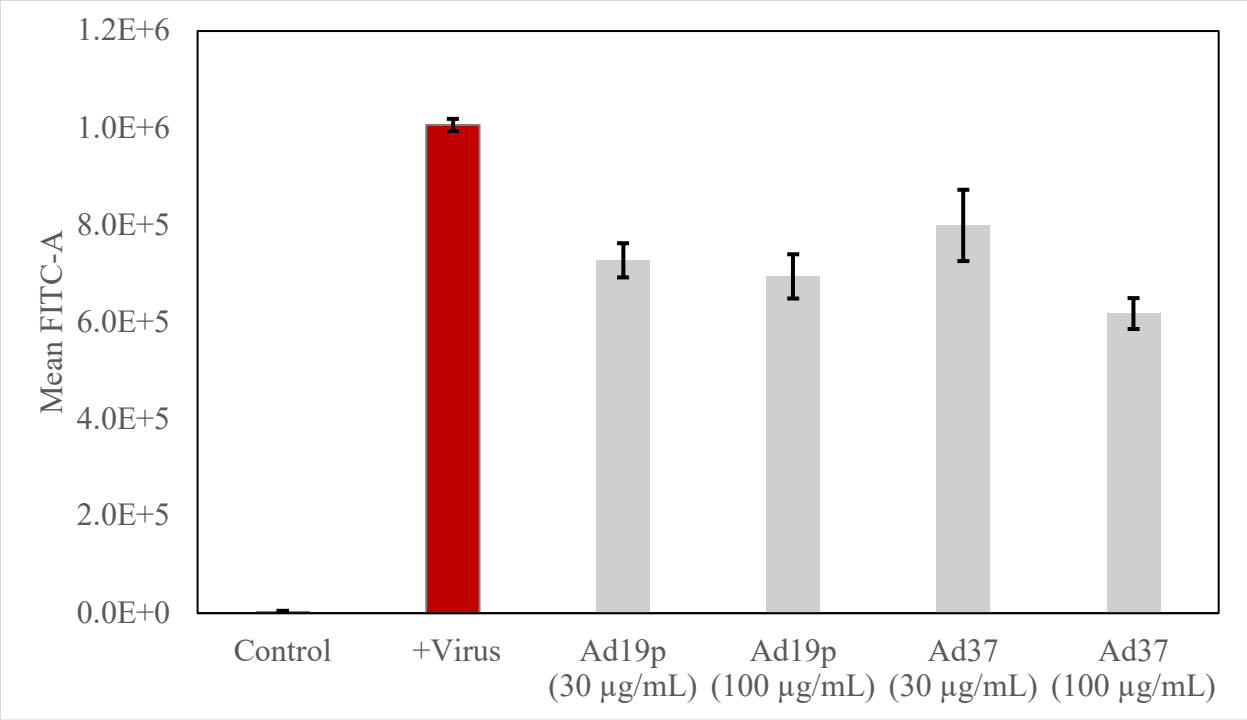

Figure 5. Ad64 mediated gene delivery into human conjunctival epithelial cells and fiber knob blocking experiments. Infections were done with the same concentration of virus, either with or without the respective concentration of fiber knob previously mixed before addition to cells. Averages represent quadruplicate and error bars represent standard error. Red colored bar denotes a significant difference ($p < 0.0001$).

Discussion

Viral-receptor identification in host cells remains a cornerstone step when investigating infection mechanisms and disease progression both in vitro and in vivo. HCjE cells, notoriously sensitive and delicate in culture, provided the first appropriate EKC model for Ad64 infection. Our results reveal that HCjE cells transcribe, translate, and actively employ all isoforms of CD46 at the cellular surface. Additionally, Ad64 infection of HCjE cells was shown to be blocked by soluble CD46, the cleaving of sialic acid, and by soluble Ad19p and Ad37 fiber knob. These results support the major role of CD46 in EKC infections, particularly those infections caused by Ad64.

To our knowledge, CD46 isoforms have not been quantified in this strain of human epithelial conjunctival cells. Previous studies involving adenovirus-receptor interactions used Chang C conjunctival cells in experiments, which proved to be heavily contaminated with HeLa

cervical cancer cells (5). CD46, discovered in 1986, has important functions in the complement system alongside acting as a receptor for several types of viruses and bacteria (1). Additionally, CD46 is present in all nucleated cells, often all four isoforms, though some specific cell types contain high concentrations of specific isoforms for unknown reasons (1, 10). Our results highlight that HCjE cells contain all four isoforms as expected with higher expression levels of both BC1 and BC2 isoforms compared to either C isoform. Further research in immunological or infectious disease fields may work to elucidate this difference in expression and its significance in ophthalmological function.

Our use of genetically modified CHO cells expressing various isoforms of CD46 led us to the conclusion that the presence of CD46 is both sufficient and necessary for Ad64 to infect eukaryotic cells. Replicates of this experiment confirmed these results consistently; however, differences in Ad64 infection across either isoform of CD46 varied. Future research should focus on investigating isoform binding specificity for Ad64 to better understand viral entry conditions and requirements.

Current anti-viral therapies employ a variety of mechanisms in-vivo. Typically, they inhibit at the nucleic acid, protein synthesis, viral entry, or immunological level (4). Our blocking experiments utilized methods that worked to inhibit viral entry by acting as either a competitive inhibitor or by cleaving the receptor from the cellular surface. The most successful anti-viral technique was blocking Ad64 infection with soluble CD46, where we saw an approximately 2.5x decrease in Ad64 infection of the cells. Cleaving sialic acid using neuraminidase achieved an approximately 1.5x decrease in Ad64 infection, indicating that Ad64 may use sialic acid or CD46 to gain entry into cells. Testing both conditions at the same time rendered a decrease in infection similar to that of soluble CD46, which may indicate that an upper limit of blocking has been reached while the virus uses alternative methods, such as binding integrins at the cell surface via Ad64 penton proteins, to gain entry into the cell (7).

Viral attachment typically occurs through the fiber knob protein, however there is evidence that viruses implicated in EKC and other diseases have the ability to bind via the hexon. Previous research on Ad56, another D-species adenovirus like Ad64, has suggested that the virus binds CD46 via the hexon, obtaining results similar to our experiments (6). Although competitive inhibition of Ad64 binding to CD46 with soluble fiber knob significantly decreased infection, the high remaining measure of fluorescence indicates that the fiber knob is less

impactful on viral entry than other methods. Further investigation attempting to block viral entry with the hexon capsid may help elucidate the differences in hexon and fiber knob binding between Ad64 and CD46 in conjunctival cells.

Conclusion

Human epithelial conjunctival (HCjE) cells function as a prime in-vitro model for epidemic keratoconjunctivitis. We show that HCjE cells express all four major isoforms of CD46 and that CD46 is both necessary and sufficient for adenovirus type-64 (Ad64) infection. Additionally, Ad64 infection can primarily be blocked with the addition of soluble CD46 and cleavage of sialic acid. The addition of soluble fiber knob decreases infection significantly, but more research must be done to investigate the binding of Ad64 to CD46 via the hexon instead of the fiber knob. We suggest that Ad64 serves as a strong candidate for gene therapies and vaccine vectors utilizing CD46 and that CD46 and sialic acid remain prime targets for anti-viral experiments.

References

(1) Andrews PW, Knowles BB, Parkar M, Pym B, Stanley K, Goodfellow PN 1985. A human cell-surface antigen defined by a monoclonal antibody and controlled by a gene on human chromosome 1. Ann Hum Genet 49:31-39. https://doi.org/10.1111/j.1469-1809.1985.tb01673.x

(2) Arnberg N, Edlund K, Kidd AH, Wadell G 2000. Adenovirus Type 37 Uses Sialic Acid as a Cellular Receptor. J Virol. 74. https://doi.org/10.1128/jvi.74.1.42-48.2000

(3) Burrow MK, Patel BC. Keratoconjunctivitis. [Updated 2023 Aug 7]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK542279/#

(4) Kausar S, Said Khan F, Ishaq Mujeeb Ur Rehman M, Akram M, Riaz M, Rasool G, Hamid Khan A, Saleem I, Shamim S, Malik A 2021. A review: Mechanism of action of antiviral drugs. Int J Immunopathol Pharmacol. https://doi.org/10.1177/20587384211002621

(5) Lavappa, KS 1978. Survey of ATCC stocks of human cell lines for HeLa contamination. In Vitro 14(5):469–75. https://doi.org/10.1007/BF02616110

(6) Persson BD, John L, Rafie K, Strebl M, Frängsmyr L, Ballmann MZ, Mindler K, Havenga M, Lemckert A, Stehle T 2021. Human species D adenovirus hexon capsid protein mediates cell entry through a direct interaction with CD46. Proc Nat Acad Sci. 118(3). https://doi.org/10.1073/pnas.2020732118

(7) Wickham TJ, Mathias P, Cheresh DA, Nemerow GR 1993. Integrins αvβ3 and αvβ5 promote adenovirus internalization but not virus attachment. Cell 73(2):309-319. https://doi.org/10.1016/0092-8674(93)90231-E

(8) Wu E, Pache L, Von Seggern DJ, Mullen T, Mikyas Y, Stewart PL, Nemerow GR 2003. Flexibility of the Adenovirus Fiber Is Required for Efficient Receptor Interaction. J Virol 77. https://doi.org/10.1128/jvi.77.13.7225-7235.2003

(9) Wu E, Trauger SA, Pache L, Mullen TM, von Seggern DJ, Siuzdak G, Nemerow GR 2004. Membrane cofactor protein is a receptor for adenoviruses associated with epidemic keratoconjunctivitis. J Virol. 78(8):3897-905. https://doi.org/10.1128/jvi.78.8.3897-3905.2004

(10) Yamamoto H, Francesca Fara A, Dasgupta P, Kemper C 2013. CD46: The 'multitasker' of complement proteins. J Inter Biochem & Cell Biol 45(12):2808-2820. https://doi.org/10.1016/j.biocel.2013.09.016

(11) Zhou X, Robinson CM, Rajaiya J, Dehghan S, Seto D, Jones MS, Dyer DW, Chodosh J 2012. Analysis of Human Adenovirus Type 19 Associated with Epidemic Keratoconjunctivitis and its Reclassification as Adenovirus Type 64. Invest Ophthalmol Vis Sci. 53(6):2804-2811. https://doi.org/10.1167/iovs.12-9656.