Co-infection of GFP, Ad2 & Ad12 in A549 & IMR90 cells to evade the interferon response

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Abstract Background: Over the years several experiments have been directed into understanding the interaction of adenovirus type 2 and the human hosts which it infects. **Aim of the study**: This was designed to investigate the host cell morphological changes during Ad2/GFP and Ad12/GFP infections in human cells to understand the rescuing potential of Ad12/GFP in infected cells in comparison with Ad2/GFP in the same cell lines. **Materials and methods**: Human diploid fibroblast cell lines (IMR-90) and human alveolar epithelial cells (A549) were infected with Ad2/GFP, Ad12/GFP and GFP only. Infected cells were countered after 24, 48, 60 and 72 hours post infection (h.p.i). Changes in cell morphology were also monitored at the specified times post infection. At each particular h.p.i cells were harvested and proteins extracted. The proteins expressed at each h.p.i were measured with electrophoresis technique run in SDS buffer. Ad2 and Ad12 DNAs were also extracted for PCR studies. **Results**: The study suggested that although GFP alone could not lead to any cell deformities in IMR-90 and A549 cells after 72h, Ad12 could but did so after a slow start. At 60 hours the number of infected cells by Ad12 had gone past that of Ad2 in both cells. **Conclusion**: The general growth of Ad2 was however revealed to be better in IMR-90 and A549 cells than Ad12. Thus, it is possible that the inability of Ad12 to evade the interferon response may have implications for the virus to establish persistent infections.

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Abbreviations: Ad2:adenovirus 2, Ad12: adenovirus 12, GFP: Green fluorescence protein

1. Introduction

A lot of research has been done about the adenovirus type 2 in the past. Some characteristic features of adenovirus type 2 is that it seem to be a nononcogenic virus while at the same time has a efficient way to reduce the effects of the immunes system to infection. As for the genes, adenovirus type 2 encodes several of the characteristic genes of adenoviruses. Genes of importance are E1A, E1B, E3 and the gene encoding virus associated RNA (VA RNA) among others. E1A is the one of the must multifunctional ones It is important since it in some adenoviruses has the potential to interfere with the host cell's production of interferon alfa (INF-α), which in turn is important for the immune responses to virus infection. E1A is also important for its other effects which are to promote the expression of other early genes and stimulate the cell to go into S-phase (ref).

Research on adenovirus type 12 has not been that comprehensive compared with adenovirus type 2. Regardless of this, a lot of central aspects of the virus have been documented. For instance, the virus seems to have some oncogenic effects in contrast to adenovirus type 2 [1]. Also, the E1A gene in adenovirus type 12 has been investigated, for example in comparison to E1A in adenovirus type 5. Interesting data from these studies speaks of different protein binding sequences for the gene products in the different viruses. Such results could explain different binding affinities for specific proteins involved in the effects of the E1A and therefore direct differences between two types of adenoviruses for the same gene [1, 2, 3].

Green fluorescent protein is a protein which gives off a green fluorescent light when exposed to blue UV-light. The protein was first found in a jellyfish, though there are a lot more marine animals which have similar florescent proteins. In cells GFP is often used as a reporter expression gene that attaches to another gene of interest for example in cell cultures. Compared to many other florescent molecules, GFP causes very little harm to the living cells. The most common instrument for analyzing cells with GFP is florescent microscopes with the ability to expose the cells with blue UV- light [3, 4, and 5].

For adenoviruses there are mainly two ways of how they can evade the immune defense. One way is with the help of VA RNA I where this RNA blocks protein kinas R (PKR). PKR prevents translation of viral mRNAs by binding to dsRNA produced in a

virus infection and thereby become activated. Once activated, it can phosphorylate the eukaryotic translation initiation factor 2 (eIF2). eIF2 has a central role in the signaling pathway for viral mRNA translation. Interferon signaling is responsible for the expression of PKR in cells susceptible to infection [3]. The other central way for adenovirus to evade the immune defense is by the effects of E1A. E1A can block the expression of interferon β in the infected cell as well as interferon stimulated genes (ISGs). This is carried out by E1As ability to sequester p300/CBP (CREB binding protein) and thereby inhibit these proteins from interacting with transcription factors [4,6].

Interferons play a very crucial role in the immune responses to viral infections. By blocking the effects of such responses, adenovirus possesses a great ability to survive the immune defense and cause infection. Focus in our experiments is to investigate if adenovirus type 12 induces interferon responses when infected in human cells. With knowledge about adenovirus type 2, it can be used as a reference. We use it as a reference for adenovirus type 12 by performing all the experiments on both viruses. We also co-infect with GFP so that detection is possible. Experiments performed are infection, cell count of infected cells, electrophoresis and PCR. The aim is to see if interferon responses are induced in adenovirus type 12 infections, and if that is the case, see if the virus turns it on or lack to shut it off. Worth to mention is that the experiment and results presented here isn't alone the only one performed to answer the question formulation. With this in mind, some things are already known from previous performed experiments.

2. Materials and Method

Two types of cells, A549 (lung cancer cells) and IMR90 (Fibroblast) cells where split into seven 35 mm plates each *(jag kommer ihåg att hon visade nått angående de små plattorna från en tabell hon hade I sitt rum men kan inte hitta det, vet du vad det var?)*. The A549 where grown in Dulbecco's Modified Eagle Medium with GlutaMAX (DMEM) complemented with 10% of fetal bovine serum (FBS) and 100 U/ml of penicillin (PE). The IMR90 cells where cultured in the Modified Eagle Medium with GlutaMAX (MEM) supplemented with the same things as the previous medium. 1,5 ml media and 0,5 ml of the cultured cell solution was added to all of the plates. The plates where then incubated at 37 -^{0}C in a CO_2 incubator. The reason the cells where split into 7 plates was because two infection series where done for each cell type with three different types of infections;

1. GFP alone 2. Co-infection with GFP and Adenovirus type 2 and 3. Co-infection with GFP and Adenovirus type 12. These where done it two sets because the cells where harvested both 24 hours and 48 hours. The last set of plates where made to be used on an automatic counter which is able to count the amount of living cells / µl

By using the numbers from the automatic cell count along with virus stock concentration and desired multiplicity of infection (MOI), the amount of virus stock (in µl) per plate can be calculated according to the following formula: **(MOI** (PFU/cell) **x amount cells) / (virus stock concentration** (PFU/µl)**)**.

The infection of the cells where done by adding the calculated amount of virus to the respective plate for subsequent incubation at 37˚C in a CO_2 incubator. Post infection they where counted manually using a florescent microscope which was able to transmit blue UV-light. Each plate where split into twelve sections which where separately counted and the total amount was then added together for each plate. This was done the same way for both the 24h and 48h infected cells.

When harvesting the cells each plate where first carefully scraped using a specific scraper depending on the infection (GFP, co-infection of GFP with Adenovirus type 2 and co-infection of GFP with Adenovirus type 12). The medium from each of the plates where then added to different Eppendorf-tubes which where then centrifuged. The centrifuge caused the development of pellets in the Eppendorf-tubes. The supernatant of the tubes where removed and each of the plates where scraped again and the medium was added to the Eppendorf tubes which where centrifuged again. The Eppendorf tubes where washed with PBS which quickly was removed and then centrifuged a last time before once again removing the last of the supernatant.

For the protein-separation using electrophoresis two agarose gels where prepared for each cell type, A549 (lung cancer cells) and IMR90 (fibroblast) cells. The wells of the both gels where then added with cells which had been infected for different length period of time (24h, 36h and 48h) together with loading dye. The the remaining wells where then added with the virus alone with the same loading dye and a ladder. The electrophoresis where then run for an hour. For the Real-time PCR genome material used from pellets of cells infected with Adenovirus type 2 and Adenovirus type 12 were used. These where run together on the Real-time PCR together with a standard marker with different concentrations and the same primer for each of the samples.

Infection of cells with Ad2 and Ad12

The number of cells per plate were initially countered by the *countless automated cell counter*

and found to be 7.2 *x* 10^5. In calculating the amount of virus needed for the desired m.o.i the equation below was used:

$$\text{Amount of virus stock used per plate} = \frac{\text{multiplicity of infection } \left(\frac{FFU}{cell}\right) \times \text{number of cells per plate}}{\text{concentration of virus stock } \left(\frac{FFU}{\mu l}\right)}$$

With a multiplication of infection (m.o.i) of 20 fluorescence-forming units (FFU of Green Fluorescent Protein (GFP) was pipetted into four plates each of the A549 and IMR-90 cells. Again using a m.o.i. of 1000 FFU for Ad2 and 1000 OPU for Ad12. Two plates each of both cells were also mock infected. All the plates were then incubated at 37 ºC.

Microscopy and cell harvesting

At 24, 48, 60 and 72 hours post infection (h.p.i.) cells were observed under the microscope for the number of infected cells. The total number of infected cells for 12 field counts per each h.p.i was recorded. At each particular h.p.i cells were harvested, centrifuged at 10,000 rpm for 3 minutes and the supernatant discarded. The process was repeated after washing cells in PBS.

Protein extraction

A volume of 260µ*l* of 0.5 *x* RIPA buffer was added to each of the tubes containing the pellets of A549 and IMR – 90 for 48, 60 and 72 hours to lyse the cells. The mixture was shaken gently for 15 minutes on ice, vortexed and centrifuged for 5 mints. The supernatant was then transferred into new set of tubes for further analysis such as proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes and blocked using 5% nonfat dry milk in Tris-buffered saline–Tween. As well the confirmative test such as molecular tool assay .

Six standard solutions were also prepared with each containing 0.5 *x* RIPA buffer and serially diluted amounts of purified Bovine Serum Albumin (BSA).

Identification and quantitation of Ad2 and Ad12 proteins

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique was used to identify the adenovirus proteins expressed in the infected cell extracts. Here the protein extracts were run in SDS buffer at a voltage of 150V for 1 hour. The gel was then stained in PageBlue protein staining solution, washed with water and left overnight after which it was analyzed.

In the estimation of the amount of adenovirus proteins expressed, protein samples from mock, Ad2 and Ad12 infected cells were pipetted into wells using the multi channel pipette. Dye reagent concentrate (Bio-Rad Protein Assay) was added to each well and mixed by pipette up and down. Titertek multiscan machine was then used to measure the total protein concentration

Identification of the DNAs of Ad2 and Ad12.

DNA was extracted from the cell extracts of Ad2 and Ad12 by the standard procedure. Polymerase Chain Reaction (PCR) constituents including 10 *x* thermopol buffer, SyBr green water, forward and reverse primers, dNTPs and Tag enzyme were put into the quick plate wells. Standards as well as serially diluted Ad2 and Ad12 DNA samples were then added in the wells in an appropriate order. The negative controls were then added and put into the Mini Opticon Real Time PCR machine for analysis .

3. Results

The following tables show the total of 12 field counts of infected cells at 24, 48, 60 and 72 hours post infection.

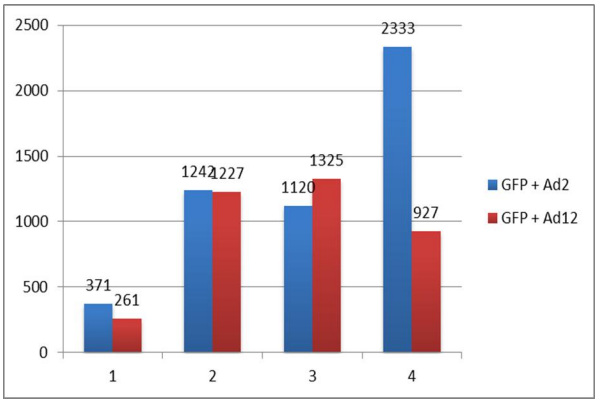

Fig:1 Time course of Human alveolar epithelial cells (A549 cells) infections GFP + Ad2 and GFP + Ad12

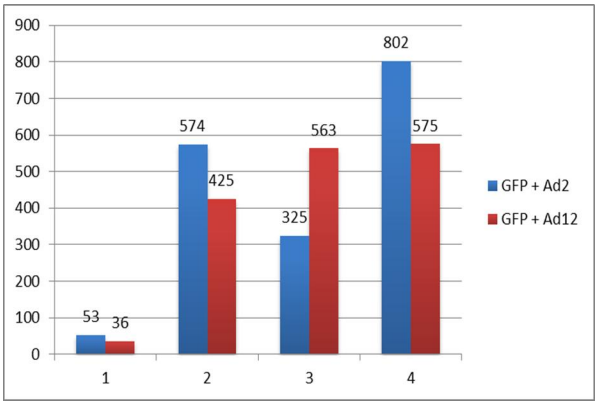

Fig:2 Time course of Human primary lung fibroblast cells (IMR90 cells) infections GFP + Ad2 and GFP + Ad12

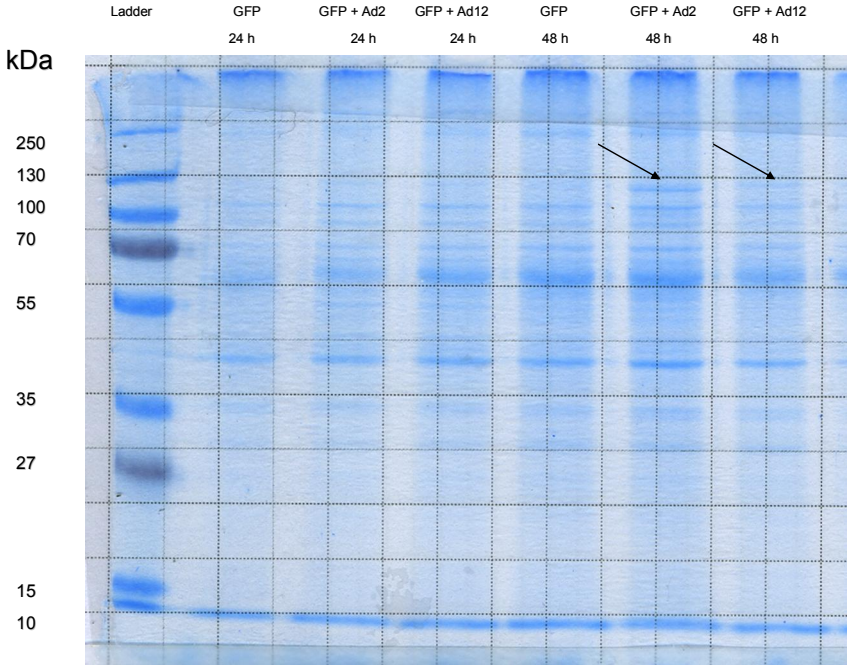

Figure 3. Gel from the protein-separation done by electrophoresis. The first wall is loaded with a ladder and the remaining ladders shown are loaded with cells infected with GFP, co-infection of GFP with Adenovirus type 2 and co-infection of GFP with Adenovirus type 12. Same infections are loaded with different infection time-series.

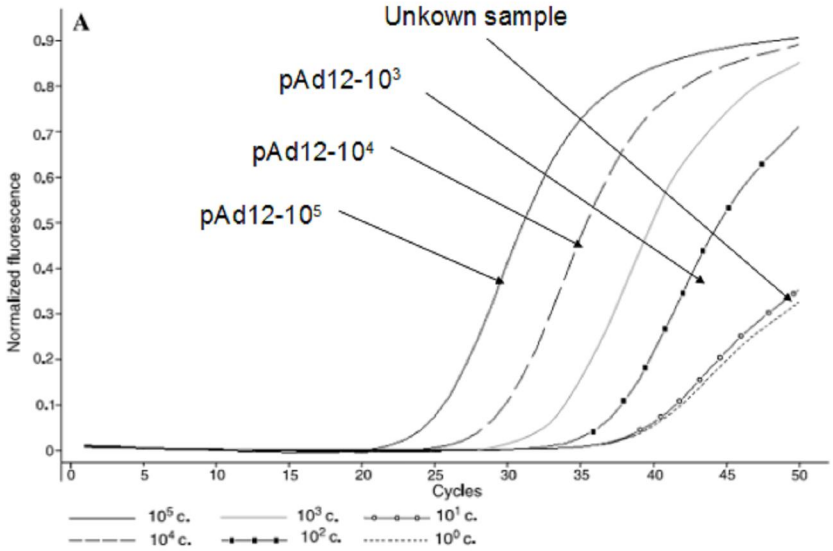

Figure 4. Diagram from Real-Time PCR. The three curves (pAd12-10^5, pAd12-10^4 and pAd12-10^3 are from the standard marker and the curve from the unknown sample is the curve used from one of the samples.

4. Discussion

In this study the results from the cell count, we can clearly see that infection in lung cancer cells seem to proceed faster than in fibroblasts independently of virus type with the exception for the day one count with GFP + adenovirus type 2 in fibroblasts (figure 1). To determine the period during which genes of different cell type was downregulated in the adenovirus12 life cycle. A possible reason for this is the fact that the lung cancer cells divide more

rapidly than the fibroblasts and therefore also provide more cells available for infection. A major difference can also be seen between the different cell types for day two where adenovirus type 2 infects the lung cancer cells more than adenovirus type 12 seems to do (figure 2). The opposite scenario seems to be the case for the fibroblast, where adenovirus type 12 is the more prevalent virus for infection on day two (figure 1). For the counting of cells we considered rounded cells as infected cells. But all rounded cells don't necessarily need to be infected cells; it could also be round due to apoptosis. For the samples of cells where GFP where added alone, round shaped cells also appears despite the fact that no virus is present. This can be explained by the fact that GFP alone can cause some little infection condition in the cells. Since we counted the cells manually we think this along with the difficulty to distinguish infected cells based on the shape could be a source of error.

The most interesting results from the electrophoresis is the difference in the samples for 48 hours where a band is present for adenovirus type 2 but not for adenovirus type 12 at a size of approximately 130 kDa (figure 3). This indicates that a protein of this size is present in cells infected with adenovirus type 2 but not with adenovirus type 2. With the knowledge that adenovirus type 12 causes a more slow infection than adenovirus type 2, it is plausible to consider the protein to be important for the infection. From previous experiments we also know that adenovirus type 12 only have the ability of VA RNA I to evade immune responses and therefore more or less ineffective to block interferon expression. This tells us that interferon responses are present in adenovirus type 12 and the thought of a lack to shut off these responses is very near at hand. If this is the case, the mRNAs from the E1A gene for adenovirus type 12 obviously don't translates into a protein product capable of sequestering p300/CBP and further prevent expression of interferon β [10,13]. According to the adenovirus life cycle, the expression of E1A gene, which is a trans activator for DNA-polymerase II-dependent viral early-gene expression, starts in the immediately during the early phase. The transcription of VA RNAs mediated by DNA polymerase III is independent of E1A-regulated transcription and, therefore, starts almost at the same time as E1A[10, 11]. The E1A gene in adenoviruses codes for two main protein products translated from either a 12S mRNA or a 13S mRNA [4, 5, 6, 7]. Whether the band in the gel for adenovirus type 12 is a protein product from some of the two different mRNAs of its E1A gene is harder to tell. Worth to mention here is that research about the E1A gene for adenovirus type 12 have showed some unique sequences in the protein that have some characteristic properties for binding to specific proteins. It's plausible that some of these sequences in these very same products prevent the ability for binding to p300/CBP and hence disrupt its further effects for interferon expression. It has been shown that p300/CBP interacts with the N-terminal region of E1A proteins [4,8,9]. Since one cannot exclude the possibility of a improper sequence in the N-terminal region for eventually disrupting the binding to p300/CBP, this could maybe serve as a good starting point for further investigation.

Since the PCR were performed for confirmation purposes the results doesn't give so much detailed information about the E1A in adenovirus type 12. What we can see though is that the primers didn't work for adenovirus type 2 but for adenovirus type 12 (figure 4). The only thing we can be sure of is that some genomic sequences in the E1A gene differ between the two virus types according to the gene sequencing.

The discussion is therefore more speculative than definitely facts. Our experiments could maybe give a hint about some aspects of how the E1A gene differs between adenovirus type 2 and adenovirus type 12.

Our results strongly suggest the ability of VA RNA I to evade immune responses and therefore more or less ineffective to block interferon expression. This tells us that interferon responses are present in adenovirus type 12 and the thought of a lack to shut off these responses, since VA RNAs expressed from FG AdVs may affect various cellular signaling pathways

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