Impaired antiviral response of adenovirus-transformed cell lines supports virus replication

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Activation of the innate immune response represents one of the most important cellular mechanisms to limit virus replication and spread in cell culture. Here, we examined the effect of adenoviral gene expression on the antiviral response in adenovirus-transformed cell lines; HEK293, HEK293SF and AGE1.HN. We demonstrate that the expression of the early region protein 1A in these cell lines impairs their ability to activate antiviral genes by the IFN pathway. This property may help in the isolation of newly emerging viruses and the propagation of interferon-sensitive virus strains.

The IFN pathway of the innate immune response plays an important role in the control of viral infections. Once a cell detects viral components via its intracellular pathogen recognition receptors, type I IFNs (IFN-α/β) are synthesized and secreted, which act in an autocrine and paracrine fashion and lead to the expression of IFN-stimulated genes (ISGs). Some of these ISGs, such as the myxovirus resistance A protein (MxA), possess direct antiviral activities (Haller et al., 2009). Viruses have developed efficient strategies to inhibit the IFN response (García-Sastre, 2011) and escape these antiviral mechanisms. Still, suppression of the antiviral response by chemical inhibitors, expression of viral antagonists or knockdown of antiviral proteins can improve permissiveness for emerging virus strains and increase yields in vaccine production (McSharry et al., 2015; Stewart et al., 2014; van Wielink et al., 2011; Young et al., 2003; Zhu et al., 2014). Inhibition of the IFN pathway can also support the propagation of IFN-sensitive viruses, like the influenza A virus (IAV) deletion strain that lacks its non-structural protein 1 (delNS1). The delNS1 strain is attenuated in IFN-competent cells and hence, is considered as a live vaccine for both animals (Richt & García-Sastre, 2009; Wang et al., 2008) and humans (Mössler et al., 2013; Wressnigg et al., 2009). So far, only a few cell lines, such as Vero cells, which are unable to produce IFN, support the propagation of IFN-sensitive viruses (Barrett et al., 2009; Emeny & Morgan, 1979; Stewart et al., 2014). However, not all viruses replicate in these African green monkey cells and some properties of the virus, such as the glycan structures of viral surface proteins, can be altered by propagation in cells that do not originate from natural host organisms (Dumont et al., 2015; Genzel et al., 2012; Schwarzer et al., 2009). Thus, there is a need to provide flexible host cell systems for the propagation of IFN-sensitive viruses.

In this study, we examined the effect of adenovirus early region protein 1A (E1A) and 1B (E1B) expression on the antiviral response and permissiveness for IFN-sensitive viruses in the adenovirus-transformed (Adt) cell lines, AGE1.HN (Niklas et al., 2011), HEK293 and HEK293SF (a derivative of HEK293 cells growing in serum-free (SF) medium and adapted to the proliferation in suspension (Côté et al., 1998)).

IAV efficiently replicates in adenovirus-transformed cell lines in the presence of IFN-β

To investigate the influence of adenoviral transformation on antiviral gene expression, Ad₄ and non-Ad₄ cells (A549) (Giard et al., 1973; Lieber et al., 1976) were cultivated in their respective media (A549 and HEK293, DMEM supplemented with 10% FCS; HEK293SF, FreeStyle 293 Expression Medium; AGE1.HN, Adenovirus Expression Medium supplemented with 2 mM glutamine) and stimulated with 1000 U ml⁻¹ IFN-β for 6 h. Relative MxA mRNA levels (for 5’-AGGTCAGTTACAGGACTAC-3’, rev 5’-ATGGCATTCTGGGCTTTATT-3’) were determined as a representative marker for the expression of antiviral ISGs at 6 h post stimulation (h p.s.) using SYBR Green-based quantitative reverse transcription PCR (RT-qPCR) and 18S RNA (for 5’-CGGACAGGATTGACAGATTG-3’,
rev 5′-CAAATCGCTCCACCAACTAA-3′) was used as a reference gene. Interestingly, only low MxA expression levels were detected in Adt cells after IFN stimulation, whereas non-Adt cells established a much stronger antiviral response (Fig. 1a). Next, we investigated the impact of IFN stimulation on influenza virus infection. To this end, we infected IFN-stimulated (6 h p.s., 1000 U ml⁻¹) and untreated Adt cells as well as non-Adt cells with influenza virus A/Puerto Rico/8/34 at an m.o.i. of 0.01. For infection, serum-free medium was used containing optimized trypsin concentrations (A549, 1 × 10⁻⁴ U per cell; HEK293, 1 × 10⁻⁵ U per cell; HEK293SF, 5 × 10⁻⁷ U per cell; AGE1.HN, 1 × 10⁻⁶ U per cell) for the activation of the viral haemagglutinin surface protein. MxA mRNA levels were determined at 72 h post-infection (h p.i.). In the course of an infection, Adt again showed significantly lower MxA expression levels compared with non-Adt cells, suggesting that the IFN-mediated antiviral response was generally impaired in Adt cell lines (Fig. 1b). In addition, virus titres were compared between IFN-stimulated and untreated groups using the haemagglutination assay (HA assay) according to a published protocol (Kalbfuss et al., 2008). While IFN-treated non-Adt cells showed reduced virus titres compared with untreated cells (Fig. 1c), Adt cell lines reached comparable virus titres with and without IFN stimulation at all time points (Fig. 1d–f).

**Adenovirus-transformed cells support propagation of IFN-sensitive viruses**

We next compared the permissiveness of Adt and non-Adt cells for the IFN-sensitive vesicular stomatitis virus (VSV) (Basu et al., 2006) and the influenza virus delNS1 (Garcı´a-Sastre et al., 1998). We infected IFN-stimulated or untreated Adt and non-Adt cells with an eGFP-tagged VSV (VSV-eGFP, m.o.i. 0.01) and analysed the fluorescence signals by microscopy at 17 h p.i. VSV-eGFP propagation was observed in all analysed cell lines in the absence of IFN-β, but only Adt cells supported VSV replication after IFN-β stimulation (Fig. 2a). Thus, the effective antiviral response of non-Adt A549 cells appears to completely abolish VSV-eGFP replication after IFN treatment.

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Fig. 1. Antiviral gene expression and IAV titres after IFN-β stimulation in Adt and non-Adt cell lines. (a) Relative MxA expression compared with untreated cells was determined after 6 h of IFN-β stimulation (1000 U ml⁻¹). (b–f) Cells were stimulated with IFN-β (1000 U ml⁻¹) for 6 h or left untreated, followed by an influenza virus A/Puerto Rico/8/34 infection at an m.o.i. of 0.01. (b) Relative MxA expression was determined at 72 h p.i. and compared with untreated, uninfected cells. (c–f) Virus titres of stimulated and untreated cells were measured by HA assay. Statistical significance compared with untreated, infected cells was tested using a two-tailed unpaired t-test (*P ≤ 0.05; ***P ≤ 0.001). Data shown represent the mean with SD of at least three independent experiments.
In addition, we performed infection studies with the influenza delNS1 mutant strain at an m.o.i. of 0.01 in serum-free medium and in the presence of trypsin (as mentioned before) and determined virus titres at 72 h p.i. using the HA assay. Even without IFN-β stimulation, delNS1 was unable to replicate in non-Adt A549 cells (Fig. 2b). Due to the deletion of the viral IFN antagonist NS1, this strain is known to be a strong inducer of the IFN pathway and is consequently attenuated in IFN-competent cells (García-Sastre et al., 1998; Seitz et al., 2010). However, delNS1 was able to replicate efficiently in Adt cell lines demonstrating their impaired antiviral response.

Adenoviral protein E1A inhibits the antiviral response

The adenoviral proteins E1A and E1B are required to act synergistically for efficient immortalization of primary cells (Endter & Dobner, 2004). To investigate whether these proteins were responsible for blocking the IFN response in Adt cells, we knocked down E1A and E1B in both AGE1.HN and HEK293 cells, using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s protocol, and using the following siRNA sequences: E1A, 5’-GGUCCGGUUUCUAUGCCAA-3; E1B, 5’-CCGACUGGUGUUUGCUCAU-3; non-targeting control, 5’-GUGCAGAGUAUGGUAUAGU-3FLU. At 48 h post-transfection, cells were stimulated with IFN-β and mRNA expression of MxA, E1A (for 5’-ATAGCTGTGACTCCGGTCCCT-3, rev 5’-ACTCTCACGGAACCTGTTT-3) and E1B (for 5’-AAGAAMCCATCTGAGCCGG-3, rev 5’-TGTTCTCACAACCGCCTC-3) were analysed by SYBR Green-based RT-qPCR. A knockdown of at least 70% was achieved. Knockdown of E1A partially recovered MxA expression (Fig. 3a, b), whereas knockdown of E1B did not increase MxA levels. These results are in agreement with earlier publications that showed an inhibition of the IFN response by E1A expression (Ackrill et al., 1991; Anderson & Fennie, 1987; Gutch & Reich, 1991; Reich et al., 1988). The knockdown of E1A in AGE1.HN cells restored MxA expression to a greater extent than for HEK293 cells, although similar E1A mRNA levels were observed in both cell lines and knockdown efficiencies were comparable. Since E1B knockdown led to the expected elevated levels of apoptosis (data not shown), delNS1 propagation (m.o.i. 0.01) was tested in HEK293 cells depleted of E1A. Analysis of MxA expression revealed that E1A knockdown cells established a stronger antiviral response after delNS1 infection, compared with the control (Fig. 3c). In addition, decreased virus titres measured by TCID₅₀ were observed for E1A knockdown cells (Fig. 3d), which may be explained by the restored antiviral response. In line with our results, Anderson and Fennie (1987) showed that VSV replication was strongly inhibited by IFN stimulation, but VSV propagation could be rescued by coinfection with adenovirus type 5, which expresses a functional E1A protein. Taken together, these results suggest that the expression of E1A leads to the impaired antiviral response and thereby supports the efficient replication of IFN-sensitive viruses.

Many efforts have been taken to suppress the antiviral response, to improve the propagation of IFN-sensitive viruses such as human cytomegalovirus (McSharry et al., 2015) and influenza A delNS1 mutant strain (van Wielink et al., 2011) that do not reach high titres in IFN-competent cells. To our knowledge, we showed for the first time that Adt cell lines are inherently non-responsive to IFN and therefore permissive for IFN-sensitive viruses. Adenoviral transformation can be applied to cells from different animal species (Endter & Dobner, 2004; Graham et al., 2015).

Fig. 2. Propagation of IFN-sensitive viruses in Adt cell lines. (a) Cells were stimulated with 1000 U ml⁻¹ IFN-β or left untreated for 24 h followed by a VSV-eGFP infection at an m.o.i. of 0.01. Cells were analysed by fluorescence microscopy at 17 h p.i. (bars, 200 µm). (b) Cells were infected with delNS1 virus at an m.o.i. of 0.01 for 72 h p.i. and virus titres were determined by HA assay. The data represent the mean with SD of three independent experiments.
1977; Jordan et al., 2009a, b; Schiedner et al., 2000) for the generation of versatile host cells for various viruses. Beside these advantages for basic research, some Ad cell lines such as PER.C6 and AGE.CR.pIX provide a potential platform for the manufacturing of viral vectors and viral vaccines already used in clinical trials (Koudstaal et al., 2009; Lohr et al., 2009, 2012; Pau et al., 2001; Subramanian et al., 2007). These cell lines were shown to be a good substrate for a wide spectrum of viruses, such as poliovirus (Sanders et al., 2015), modified vaccinia Ankara virus (Jordan et al., 2009b) and West Nile virus (Samina et al., 2007). In addition, several Ad cell lines can be cultivated in suspension allowing a better scalability and flexibility in virus production processes (Genzel & Reichl, 2009). Thus, Ad cell lines are a powerful system for virus cultivation, in particular IFN-sensitive virus strains, and may be considered as an alternative to already existing cell culture-based viral vaccine production platforms such as Vero, MDCK and MRC-5 (Barrett et al., 2009).

**Acknowledgements**

This work was supported by the Federal Ministry of Education and Research Germany, e:Bio project CellSys, 0316189D. We thank Stefanie Lützner and Nancy Wysskosky for excellent technical support and Susanne Fritzschke and Tanja Laske for helpful discussions. We are grateful to Volker Sandig and Thomas Rose (ProBioGen AG, Berlin, Germany) for providing the AGE1.HN cell line and technical advice, as well as to Amin Kamen (NRC, Montreal, Canada) for providing the HEK293SF cell line. In addition, we want to thank Dr Maria Mercedes Segura and Professor Francesc Gòdia for technical advice on cultivation of HEK293SF cells.

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**Fig. 3.** Knockdown of E1A and E1B gene expression in Ad cell lines. (a, b) AGE1.HN and HEK293 cells were transfected with siRNAs against E1A or E1B, or with a control siRNA. After 48 h, cells were stimulated with 500 U ml⁻¹ IFN-β for 6 h before relative MxA mRNA expression was measured in AGE1.HN (a) and HEK293 cells (b). (c, d) HEK293 cells were transfected with an siRNA against E1A or a control siRNA and 24 h later infected with the influenza A virus delNS1 at an m.o.i. of 0.01. (c) Relative MxA mRNA expression was measured at the indicated time points. (d) Virus titres were determined by TCID₅₀. Values were normalized to untreated control cells. Statistical significance compared with control cells was tested using a two-tailed unpaired *t*-test (*P < 0.05; **P < 0.01). The data represent the mean with SD of three independent experiments.
References


