Arch Virol (2008) 153:2097–2102
DOI 10.1007/s00705-008-0219-2

BRIEF REPORT

Acute effect of infection by adipogenic human adenovirus Ad36

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Received: 22 July 2008 / Accepted: 11 September 2008 / Published online: 2 October 2008

Abstract Human adenovirus Ad36 is causally and correlatively associated in animals and humans, respectively, with increased adiposity and altered metabolic profile. We inoculated rats with Ad36 or UV-inactivated Ad36, or mock-infected them. Four days later, Ad36-infected rats showed 23% greater epididymal fat pad weight and viral mRNA; the viral DNA could also be detected in tissues viz. the liver, brain, and adipose tissue. Intranasal or intraperitoneal routes of viral inoculation showed similar tissue affinity. The serum cytokine response was markedly downregulated. Ad36 acutely suppresses the systemic immune response and spreads widely. This information will help to determine Ad36 tissue tropism and its metabolic consequences.

Although obesity is associated with several health risks [9], not all obese individuals are adversely affected by it [24]. To better utilize health care resources, it would help immensely to selectively target prevention and treatment efforts by distinguishing "at risk" individuals from those who are relatively protected from the adverse effects of obesity. Recent emerging evidence from animal models [18] and human studies [17] indicates that some forms of obesity may indeed carry relatively low health risk. We reported that human adenovirus Ad36 infection appears to be one such marker of "low-risk" obesity. Experimental infection of animals with Ad36 induces obesity without increasing food intake and induces relative-hypolipidemia [6–8] and better insulin sensitivity [20]. In vitro, Ad36 induces adipogenic commitment, differentiation and lipid accumulation in adipocyte progenitors [21, 22, 25] and increases cellular glucose uptake [23, 26]. These causal effects of Ad36 infection are mirrored in humans naturally infected with Ad36, who have greater body weight and body fat [1] but relative-hypolipidemia [1], greater potential of preadipocyte differentiation [21], better glycemic control and lower inflammatory response of adipose tissue [23] compared to uninfected individuals. Thus, Ad36 induces obesity with a metabolically favorable profile in animals and is associated with such obesity in humans. Strong association of Ad36 infection with human obesity and a metabolic phenotype makes it an important candidate for evaluating its causative role in human obesity. Also, Ad36 proteins are promising novel candidates [22] as tools to reveal regulatory controls that could be therapeutically manipulated to alleviate cardiovascular risk factors in humans. Despite increasing data showing the significance of Ad36, fundamental information about the acute spread of Ad36 in a host, which is critical for determining the host-virus interaction in the future, is still lacking.

Ad36 belongs to serotype D of the 51 human adenoviruses identified and is antigenically unique [27]. Ad36 was isolated from fecal sample of a girl suffering from enteritis [27] and shows about 15–17% prevalence in the adult population in the United States [1]. About 30% of obese, but only 11% of non-obese adults are naturally infected with the virus [1]. While Ad36 DNA can be isolated from human adipose tissue [21], Ad36 tissue tropism or the host immune response to experimental infection in humans cannot be studied due to ethical considerations. Since Ad36 can successfully infect rats, we used this animal model to

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study acute host response to the viral infection as described in the following two experiments.

For experiment 1, 22 male Wistar rats (4-weeks old) were obtained from Harlan (Madison, WI). After one week of acclimatization to the new surroundings, rats were randomized into three weight-matched groups (control, $N = 8$; Ad-36 inactivated, $N = 7$; and Ad-36, $N = 7$) and housed individually in micro-isolator cages under bio-safety-level-two containment. Rats were inoculated intranasally with medium (mock) or 8×10^5 particles of either UV-inactivated Ad-36 or Ad-36. Ad libitum access to water and rat chow was offered. Protective clothing, gloves, shoes, hairnets and masks were used when entering the rooms, and care was taken to prevent cross-contamination. Four days post-inoculation, animals were sacrificed after an overnight fast. Trunk blood was collected and serum was separated. Lung, pancreas, heart, spleen, brain, stomach, epididymal fat, liver and spleen were carefully separated and flash frozen in liquid nitrogen. The various techniques that were used and assays that were conducted are described below.

The second experiment determined the effect of a different route of infection on the spread of Ad36. Four-week-old male Wistar rats were inoculated intraperitoneally (i.p.) with either 200-µl cell culture medium ($N = 6$) or 10^7 PFU of Ad36 ($N = 6$). Four days post-inoculation, the animals were sacrificed, and epididymal, visceral and retroperitoneal fat tissue, lung, liver spleen and skeletal muscle were harvested. DNA was isolated and a nested PCR was conducted as described below.

Ad-36 was obtained from American Type Culture Collection (catalog no. VR-913; American Type Culture Collection, Manassas, VA). The virus was plaque-purified as described previously [6].

Purified virus was UV irradiated with 250 mJ/cm^2 using a Stratalinker UV crosslinker (cat # 400079, Stratagene).

RNA was extracted using the RNeasy Mini Kit, as per the manufacturer's instructions (cat # 74104, Qiagen). Residual DNA was eliminated by using Amplification Grade Deoxyribonuclease I (cat # 18068-015, Invitrogen). One µg of total RNA was reverse-transcribed to cDNA using iScript^{TM} cDNA Synthesis Kit (cat # 170-8890, Bio-Rad) as per the manufacturer's protocol. Samples were stored at −80°C until used for real time PCR.

A standard was generated using DNA pooled from the experimental samples. At least three data points, representing tenfold dilutions, were required for generating a standard curve. Non-template control reaction mixtures contained water instead of sample. Real-time quantitative PCR was carried out in optical 96- or 384-well reaction plates using an ABI PRISM 7700 sequence detector (Applied Biosystems, Branchburg, NJ) with a SYBER Green detection system (cat # 170-8880, Bio-Rad). Both the samples and the standards were run at least in duplicate, and each transcript level was adjusted to the housekeeping gene used (β-actin). The reactions were performed using the following parameters: one cycle of 48°C for 30 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. For the detection of Ad-36 DNA, the following primers were used: forward 5′-GGCATAC TAACCCAGTCCGATG-3′ and reverse 5′-TGCCAGAAT CCCACCCATAC-3′.

PCR core system II (cat # M7665, Promega) was used for the amplification of cDNA, obtained as described above. Water was used a negative PCR control. The positive PCR control was DNA from Ad36-infected A549 cells. DNA was denatured for 2 min at 95°C and subjected to 35 cycles of PCR (94°C for 1 min, 58°C for 1 min, 72°C for 2 min) followed by extension at 72°C for 5 min. PCR products were visualized on a 1.2% agarose gel with a 100-bp DNA ladder (cat # G-2101, Promega). The following primers were used to detect Ad-36 E4Orf1: forward 5′-CATACTAACCCAGTCCGATG-3′ and reverse 5′-AATC ACTCTCTCCAGCAGCAGG-3′. The expected product size was 138 bp.

Total DNA was extracted from the tissue using DNeasy Tissue Mini Kit (cat # 69504, Qiagen). DNA samples were stored at −80°C until used for amplification. PCR core system II (cat # M7665, Promega) was used for the amplification of DNA. Negative PCR controls were water and DNA from uninfected rats. the positive PCR control was DNA from Ad36-infected A549 cells. DNA was denatured for 2 min at 95°C and subjected to 35 cycles of PCR (94°C for 1 min, 58°C for 1 min, 72°C for 2 min) followed by extension at 72°C for 5 min. PCR products were visualized on a 1.2% agarose gel with a 100-bp DNA ladder (cat # G-2101, Promega).

The following primers from the fiber protein region were used:

Ad-36 Outer forward primer (5′-GTCTGGAAAACT GAGTGTGGATA),

Ad-36 Outer reverse primer (5′-ATCCAAAATCAAAT GTAATAGAGT),

Ad-36 Inner forward primer (5′-TTAACTGGAAAAGG AATAGGTA),

Ad-36 Inner reverse primer (5′-GGTGTTGTTGGTTG GCTTAGGATA).

The expected product size was 650 bp.

A 14-plex assay using Luminex xMAP technology was used to determine serum cytokine levels (Millipore corporation, cat # RCYTO-80 K-PMX). Serum samples from 5 randomly selected animals from each group were used. The lowest detection limit for all assays was 24.5 pg/ml.

Upon termination of the experiments, we observed that the body weight of rats from all three groups remained almost identical 4 days after inoculation (Fig. 1a), but

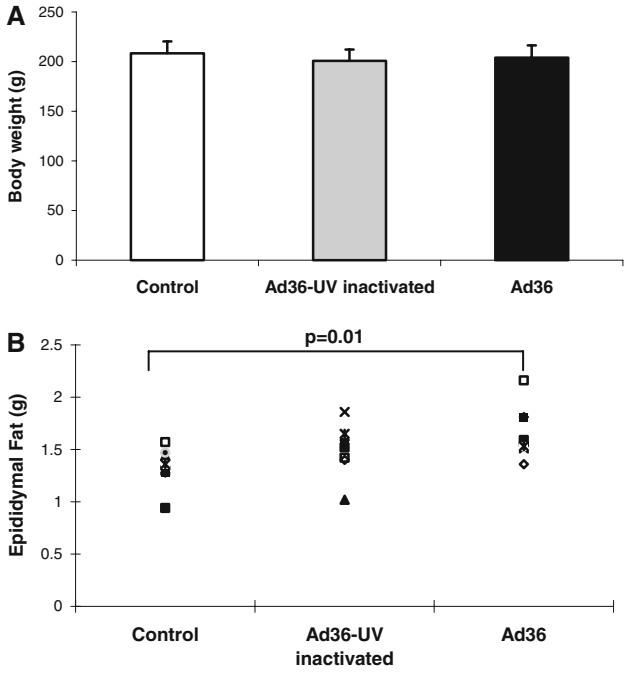

Fig. 1 **a** Body weight of rats in three groups on day 4 post-inoculation was not significantly different. **b** Epididymal fat pad weights of rats on day 4 post-inoculation

interestingly, epididymal fat pad weight of the Ad36-infected group was 23% heavier ($P = 0.01$) compared to that of the uninfected control group (Fig. 1b). As expected, UV-inactivated virus did not influence the fat pad weight.

Two experiments determined the spread of Ad36 to body tissues in rats inoculated via two separate routes. Samples from all harvested tissues showed the presence of viral DNA (Fig. 2a, b), but it was not found in the uninfected control animals (a representative sample is shown in Fig. 2b). The epididymal fat pad showed Ad36 E4orf1 mRNA, indicative of viral replication (Fig. 2c).

The following cytokines were below the detection level (24.5 pg/ml) for all animals in the group: Granulocyte-monocyte-colony-stimulating factor (GMCSF), IL1, IL4, IL18, IL10, IL12, IL5, TNFα. These were therefore excluded from the analysis. The rest of the cytokines were analyzed, and if values were below the detection levels, they were assigned the value 24.5 pg/ml. Overall, the live virus, but not the UV-inactivated Ad36, appears to reduce cytokine response 2 and 4 days post-inoculation (Fig. 3). Although the reduction in cytokine response to Ad36 infected reached statistical significance for only macrophage chemoattractant protein 1 (MCP-1) and IL-18, perhaps due a limited number of animals per group (n = 5 per group), many of the cytokine levels were reduced by 50 to 95% by the virus.

In humans, adenovirus infections are common and cause acute upper respiratory tract infections, enteritis or conjunctivitis [10]. Adenoviruses may persist asymptomatically in adult humans [13]. Although adenoviruses are not thought to integrate into host DNA, recent reports suggest this to be a possibility [28]. There are 6 major subgroups (A–F) among the 50 human adenoviruses. Each subgroup has a number of specific serotypes. Ad36 belongs to subgroup D, serotype 36. Ad36 is serologically different compared to other human adenoviruses [27]. Adenoviruses are non-enveloped DNA viruses that replicate in host cell nucleus.

Several medium- to long-term studies about the adipogenic effect of adenoviruses have been published [5–8, 20]. However, surprisingly little is known about the acute host response to these adipogenic adenoviruses, which is fundamental for understanding the role of these viruses in human obesity and for devising treatment or prevention strategies. While Ad36 is a strong candidate for determining its role in human obesity and is a relatively well-studied virus, sketchy information is available about its target tissue and the immediate response of its host. Changes in food intake or activity do not explain the metabolic effects of Ad36, and the animals do not show overt signs of infection or premature mortality even up to 7 months post-infection [6–8, 20]. Animals could be experimentally infected by intranasal or intraperitoneal inoculation of Ad36. Also, blood-borne, air or fecal-oral transmission of Ad36 has been observed [6–8, 20]. Ad36 DNA appears in the blood of experimentally infected chickens and their uninfected cage mates about 12 h post-inoculation and lasts in the blood for up to 4 weeks [7] Also, Ad36 DNA could be detected in the blood and adipose tissue of animals 5 weeks post-infection [6] by capillary electrophoresis. Ad36-infected marmosets excreted active virus in feces up to 2 months post-infection and harbored viral DNA in adipose tissue for up to 7 months [8], and hamsters did so up to 4 weeks post-infection when the experiments were terminated.

In the present study, an increase in epididymal fat pad weight just 4 days after Ad36 infection was surprising. The lack of change in the fat pad weight of the UV-inactivated group further supports the role of active virus in increasing the adipose tissue. Also, mRNA expression of Ad36 E4orf1, the adipogenic gene of the virus [22], in these fat pads is particularly noteworthy. This is the first demonstration of Ad36 gene expression in adipose tissue of an experimentally infected animal host, suggesting active viral replication and possible adipogenic effect on the tissue.

As determined by the presence of viral DNA, intranasally or intra-peritoneally inoculated Ad36 spreads rapidly to several tissues, including some known for adenoviral tropism, such as the liver. Although the spread of the virus to the brain may be somewhat unconventional,

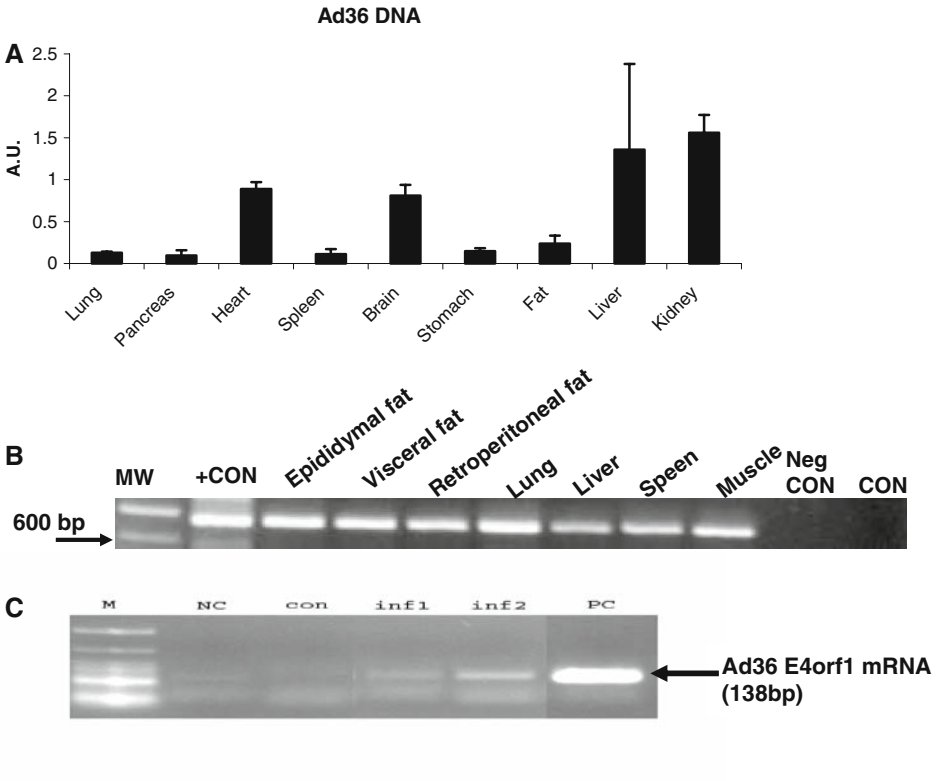

Fig. 2 **a** Presence of Ad36 DNA in various tissues of rats infected by an intranasal inoculation. The quantities were determined by quantitative real-time PCR. **b** Presence of Ad36 DNA in various tissues of rats infected by intraperitoneal inoculation. The quantities were determined by nested PCR. *MW* Molecular weight ladder. *Neg. CON* Water template, *CON* tissue from uninfected control animal. **c** Ad36 E4orf1 mRNA in epididymal fat pad of two representative animals (inf 1 and inf2) from the Ad36-infected group. *PC* Positive control: mRNA from Ad36-infected A549 cells. *NC* negative control: water template. *M* molecular weight ladder

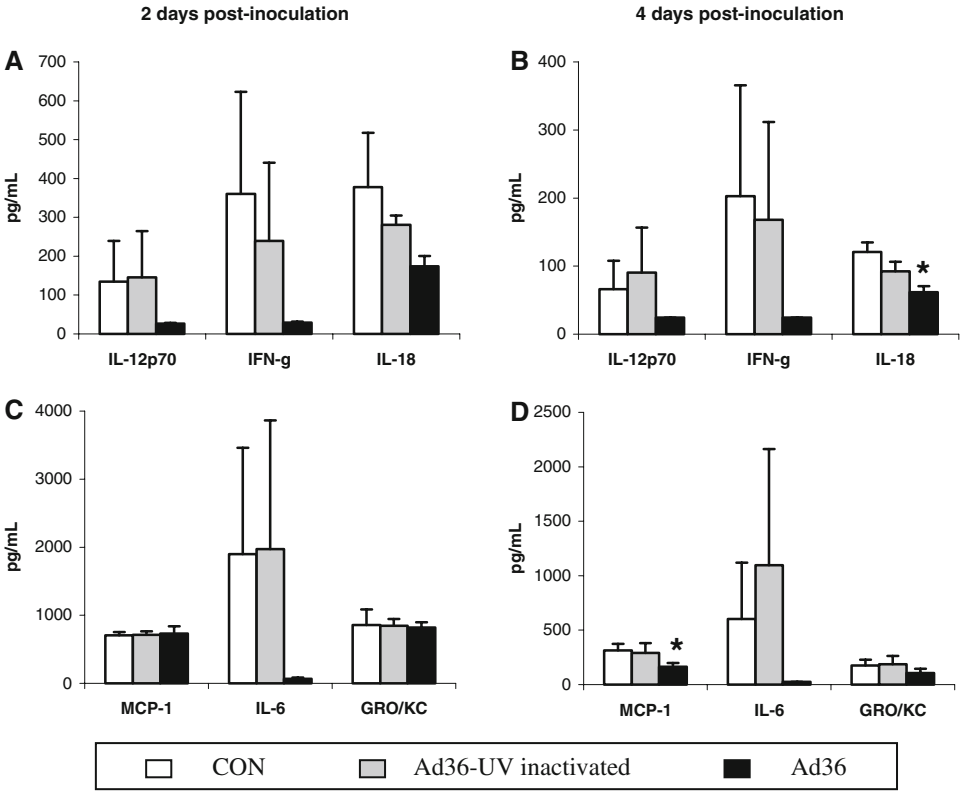

Fig. 3 Serum cytokine levels in five randomly selected animals from each group, 2 and 4 days post-inoculation. $*P < 0.05$. GRO/KC: growth-regulated oncogene

it may explain the changes in neurotransmitter levels observed in Ad36-infected rats [20]. Spread of the virus to adipose tissue and skeletal muscle is consistent with our previous findings [6, 8] and is important due to our recent in vitro findings in these tissues [21, 23, 26]. Given the effect of the virus on adipose tissue-derived stem cell

commitment and differentiation [21], spread of Ad36 to adipose tissue is particularly intriguing. About 27% of the Pima Indians screened harbored Ad36 DNA in their subcutaneous adipose tissue and showed greater adipogenic potential compared to their antibody-negative counterparts [21]. The functional effect, if any, of the presence of Ad36 viral DNA on these tissues remain to be determined.

Many viruses subvert the host immune system for successful infection, spread or persistence, and understanding these immune modulations may provide key information for defense against the microbe. Overall, Ad36 appears to down-regulate the host immune response in just 2 to 4 days post-inoculation. In response to infection, IL-12 induces IFNγ and, along with IL-18, mounts a Th1-type immune response. In early stages of infection, many viruses down-regulate the Th1 response by suppressing the production of these cytokines [4]. Down-regulation of this cytotoxic immune response may allow Ad36-infected cells to survive, which may be of particular relevance to cells from adipose tissue, which expands in response to Ad36 infection. A long-term and tissue-specific assessment of cytokine response to Ad36 is required to further understand the interaction.

Inflammatory cytokines are positively associated with obesity and insulin resistance [15, 29], and approaches to transgenically knock out inflammatory cytokines such as MCP1 and TNFα (tumor necrosis factor alpha) show improvement in insulin resistance [14], and knockout of cytokines such as IL-18 or the receptor of IL-1 increases adiposity [11, 19]. In vitro infection of human adipose tissue by Ad36 down-regulates inflammatory cytokines (unpublished observations). Thus, the down-regulation of inflammatory cytokines by Ad36 may contribute to its adipogenic effect and the paradoxical improvement in glycemic control. Long-term studies are required to test this hypothesis.

Although Ad36 appears to acutely down-regulate response of the inflammatory cytokines investigated, other intact human adenoviruses or replication-deficient adenoviral vectors are known to elicit a strong and acute inflammatory cytokine response [3, 12], which, in fact, is a serious limitation of otherwise efficient application of human adenoviruses as vectors [2]. Extensive research has been undertaken to overcome this shortcoming of human adenoviral vector use [2]. One of the alternative strategies is to use an adenovirus that does not elicit a strong inflammatory cytokine response. In response to such concerns, Iacobelli-Martinez et al. [16] reported human adenoviruses Ad16, Ad35 and Ad37, which down-regulate acute inflammatory response of cytokines such as IL-12, IFNγ and IL-6, and suggested the use of these adenoviruses for gene delivery and vaccine development. A pronounced anti-inflammatory cytokine response by Ad36, coupled with its ability to infect several host tissues, also suggests its possible role as vector.

In conclusion, acute effects of Ad36 in rats include greater fat pad weight and attenuation of host immune response, which may help in the widespread tissue distribution observed. This study provides the basis for studying tissue tropism and long-term modulation of the host immune response and its metabolic consequences. The data also provide candidate host tissues for the virus in humans naturally infected with Ad36.

Acknowledgments This work was funded by NIH1R01DK066164 awarded to NVD.

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