A Human Adenovirus Enhances Preadipocyte Differentiation

Sharada D. Vangipuram, Jonathan Sheele,* Richard L. Atkinson,§ Thomas C. Holland,‡ and Nikhil V. Dhurandhar*†*

Abstract

VANGIPURAM, SHARADA D., JONATHAN SHEELE, RICHARD L. ATKINSON, THOMAS C. HOLLAND, AND NIKHIL V. DHURANDHAR. A human adenovirus enhances preadipocyte differentiation.*Obes Res.* 2004;12: 770–777.

Objective: Adenovirus 36 (Ad-36) has been shown to increase adiposity in experimentally infected chickens, mice, and marmosets (nonhuman primates). Neutralizing antibodies to Ad-36 are associated with obesity in humans. The metabolic and molecular mechanisms responsible for Ad-36-induced adipogenesis are unknown. As a potential adipogenic mechanism, this study examined if Ad-36 enhanced differentiation of preadipocytes.

Research Methods and Procedures: To determine the suitability of 3T3-L1 cells (murine preadipocyte cell line) as a model, the first experiment determined if Ad-36 attaches and initiates replication in the cells. Next, effects of Ad-36 on the number of differentiated adipocytes, glycerol 3-phosphate dehydrogenase (GPDH) levels, and cellular lipid accumulation were determined. The last experiment determined the effect of Ad-36 on human primary preadipocyte differentiation. Ad-2, a known nonadipogenic human adenovirus, was used as a negative control in these experiments.

Results: Immunofluorescence studies showed adenoviral attachment to 3T3-L1 cells, and reverse transcriptase-polymerase chain reaction showed expression of the Ad-36 *E1A* gene in the infected cells. Ad-36, but not Ad-2, increased the number of differentiated adipocytes, GPDH enzyme levels, and the total cellular lipid content. Also, Ad-36, but not Ad-2, increased GPDH levels in human preadipocytes.

Discussion: Taken together, these experiments showed that Ad-36 enhanced differentiation of preadipocytes, which may be a contributory mechanism to its adipogenic effect in vivo. The lack of effect of Ad-2 on differentiation demonstrated that the observed findings were not a common characteristic of all adenoviruses. Future understanding of the molecular interactions of cellular and viral genes responsible for enhanced differentiation may reveal novel signaling pathways and controls of preadipocyte differentiation.

Key words: Ad-36, infectobesity, 3T3-L1, fat cells, Ad-2

Introduction

Although obesity is recognized as a disease of multiple etiologies (1), the possibility that viruses might be etiologic agents in obesity has received relatively little attention. However, published reports link at least five viruses to obesity in animals. These include several nonhuman viruses: canine distemper virus (2–5), Rous-associated virus 7 (6,7), Borna virus (8), and SMAM-1 (9,10). In addition, we have shown that a human virus, adenovirus 36 (Ad-36),[1] increases adiposity in experimentally infected chickens, mice, and marmosets (nonhuman primates) and causes a paradoxical decrease in serum lipid levels (11–13). Furthermore, neutralizing antibodies to Ad-36 are associated with obesity in humans. The prevalence of Ad-36 antibodies was 30% in the obese and 11% in the nonobese subjects screened ($p < 0.001$), and the antibody positive subjects had greater body weight but lower serum lipids, findings similar to those in the animal models (14).

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[1] Nonstandard abbreviations: Ad-36, adenovirus 36; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; pfu, plaque-forming unit(s); PBS, phosphate-buffered saline; RT, reverse transcriptase; PCR, polymerase chain reaction; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, insulin; MOI, multiplicity of infection; GPDH, glycerol 3-phosphate dehydrogenase; CPE, cytopathic effect; bp, base pair(s); I-D, infected donor; I-R, infected recipient; C-D, control donor; C-R, control recipient.

The metabolic and molecular mechanisms responsible for Ad-36-induced adipogenesis are not yet known. Nevertheless, we have found that increases in food intake alone cannot explain the observed increases in adiposity in infected animals, strongly suggesting that Ad-36 induces metabolic changes in the animals (11–13). Consistent with this, Ad-36 does not produce overt lesions in the hypothalamus of experimentally infected animals (11). Such lesions have been associated with obesity in the canine distemper virus model of murine obesity (15) and may alter responses to leptin or cause behavioral changes. It remains possible that Ad-36 induces other functional changes in the brain. However, other data have suggested that Ad-36 may affect adipose tissue directly. The amount of Ad-36 DNA in visceral adipose tissue of infected animals was correlated with adipose depot weight. In addition, in vitro studies of preadipocytes from Ad-36-infected chickens showed greater spontaneous differentiation vs. those of the uninfected controls (NV Dhurandhar and RL Atkinson, unpublished data). Taken together, these observations suggested that Ad-36 might have a direct effect on the differentiation of preadipocytes and/or the accumulation of lipid by adipocytes. To examine this possibility, we investigated the effect of Ad-36 on differentiation of the murine 3T3-L1 preadipocyte cells, a well-characterized preadipocyte cell line, and in primary human preadipocytes. Our findings indicated that Ad-36 has potent pro-adipogenic effects.

Research Methods and Procedures

Materials

Tissue Culture. A549 cells (human bronchial carcinoma cells) (catalog no. CCL-185, American Type Culture Collection, Manassas, VA) were used for harvesting adenoviruses, and 3T3-L1 cells (catalog no. CCL-92-1, American Type Culture Collection) were used for the differentiation experiments.

Media. Minimum essential media Eagle (catalog no. M-0643, Sigma-Aldrich, St. Louis) with nonessential amino acids, Earle's salts, and L-glutamine was used for growing A549 cells, and Dulbecco's modified Eagle's medium (DMEM) (catalog no. M-0643, Sigma-Aldrich) was used for 3T3-L1 cells. Ten percent of fetal bovine serum (FBS) was added to minimum essential media Eagle and DMEM.

Viruses. Ad-36 and Ad-2 viruses obtained from ATCC (catalog nos. VR-913 and VR-846, respectively) were used for the experiments. The titer of virus stocks was determined as described previously (12).

Experiments

Infection of 3T3-L1 Cells with Adenoviruses: Viral Attachment. To assess virus attachment, confluent monolayers of 3T3-L1 cells and A549 cells on precleaned coverslips were infected with 2×10^4 plaque-forming units (pfu) of either Ad-36 or Ad-2 at 37 °C for 1 hour with intermittent rocking. The medium was removed, and the cells were washed three times with phosphate-buffered saline (PBS) for 3 minutes per wash. The monolayers were fixed with acetone:methanol (70:30) for 10 minutes. The fixative was removed, and the coverslips were air dried. The coverslips were then incubated at room temperature for 1 hour with 1 mL of PBS containing 20% normal goat serum followed by 1 hour with rabbit anti-Ad36 antiserum diluted 1:80 in PBS. The coverslips were washed three times with PBS for 3 minutes per wash, followed by incubation for 1 hour with fluorescein isothiocyanate-conjugated antirabbit IgG (Sigma-Aldrich). After five PBS washes at 3 minutes/wash, the coverslips were mounted on glass slides with MOWIOL.

Infection of 3T3-L1 Cells with Adenoviruses: Expression of Viral Genes. To determine whether, subsequent to attachment, Ad-36 entered 3T3-L1 cells and began its replication cycle, we used reverse transcriptase (RT)-polymerase chain reaction (PCR) to test for the expression of *E1A* mRNA. To obtain RNA for analysis, confluent 3T3-L1 and A549 cells were inoculated with Ad-36 [3.82 multiplicity of infection (MOI)] or media. The permissive A549 cell line was used as a control. RNA was extracted from uninfected and Ad-36-inoculated A549 and 3T3-L1 cells at 3 hours postinoculation using TRIZOL reagent (Sigma-Aldrich catalog no. T-9424) followed by chloroform treatment and RNA precipitation by isopropyl alcohol. The *E1A* primary transcript may, by alternative splicing, produce three *E1A* mRNAs, designated the 10S, 12S, and 13S mRNAs. We designed the following primer pairs to detect these three mRNAs by RT-PCR using the one-step RT-PCR kit (BD Biosciences Clontech RT-PCR Kit, catalog no. K1403-2, BD Biosciences Clontech, Palo Alto, CA): 10S-1, 5′-CGCCTTCTACCTTCAACTGTGC-3′; 10S-2, 5′-TGTTCGTCCTCTGAATCGCTG-3′; 12S/13S-1, 5′-GCTGTGATTATGCTGGAGGACTTTG-3′; 12S/13S-2, 5′-GGTCTTCTTCTGAGGGTGATGACTC-3′; 13S-1, 5′-TGAGCAGCAGATGGCTCTAATCTC-3′; and 13S-2, 5′GGTCTTCTTCTGAGGGTGATGACTC-3′.

The following time and temperature conditions were used for the assay: reverse transcription, 1 hour, 50 °C; PCR, denaturation, 30 seconds, 94 °C; annealing, 30 seconds, 55 °C; extension, 1 minute, 68 °C; and final extension, 1 minute, 68 °C. Inactivated RT (by heating for 5 minutes at 95 °C) was used as a negative control for each sample. β-actin expression was used as a positive control for the assay.

Acceleration of 3T3-L1 Differentiation by Ad-36: Effect of Ad-36 on the Number of Differentiated Adipocytes. Only a fraction of 3T3-L1 cells differentiate into adipocytes after stimulation with differentiation agents. The following experiments were conducted to determine whether Ad-36 infection increased the number of 3T3-L1 cells that differentiated.

BODIPY Staining. Two plates of confluent 3T3-L1 cells were inoculated with media (control) or Ad-36 (2.6 MOI) and differentiated with 3-isobutyl-1-methylxanthine, dexamethasone, insulin (MDI) (MDI, DMEM media containing 10% bovine serum with 1× antibiotic-antimycotic, 10 μg/mL insulin, 39 μg/mL dexamethasone, and 115 μg/mL 3-isobutyl-1-methylxanthine). Cells were stained with lipid-specific BODIPY 493/503 fluorescent dye 5 days postinoculation as described by Gocze et al. (16).

Oil Red O Staining. After removing the media, cells inoculated with media (control), Ad-36, or Ad-2 were fixed with 10% buffered neutral formalin washed with 70% ethanol and then treated with 0.3% Oil Red O dye (lipid-specific nonfluorescent dye that stains adipocytes) for 10 minutes with gentle shaking. Next, the dye was removed, the cells were washed with 70% ethanol, and the number of Oil Red O stained in each well per 25-mm^2 area (five randomly selected areas/well; $n = 6$ wells per condition) were counted under the microscope.

Effect of Ad-36 on Preadipocyte Differentiation-Specific Enzyme Glycerol 3-Phosphate Dehydrogenase (GPDH): Differentiation of 3T3-L1 Cells. 3T3-L1 cells were grown to confluence in six-well plates. The media was removed from each well, and 3 mL of differentiation media was added to each well. After incubation for 48 hours at 37 °C, the differentiation media was replaced with 10% FBS-DMEM containing 0.001% insulin (maintenance media). Maintenance media was replaced every 2 days. Cells were used for GPDH assay.

Effect of Ad-36 on Preadipocyte Differentiation-Specific Enzyme GPDH: GPDH Assay. GPDH was measured using the method of Weiss and Green (17). The assay involves generation of glycerol 3-phosphate from dehydroxyacetone phosphate by GPDH, using NADH as coenzyme. GPDH from rabbit muscle (Sigma-Aldrich catalog no. G-6880) was used to construct a standard curve. The enzyme was expressed per milligram of protein extracted from 3T3-L1 cells (modified Lowry protein assay kit; Pierce catalog no. 23240, Pierce Chemical, Rockford, IL).

GPDH Time Course. Confluent 3T3-L1 cells in six-well plates were inoculated for 1 hour with 50 μL of media, Ad-36, or Ad-2 virus [3.5×10^5 pfu (3.8 MOI) and 4.0×10^5 pfu (3.3 MOI), respectively], immediately followed by MDI treatment. Two days later, the differentiation media was changed to DMEM with 10% FBS and 0.001% insulin (maintenance media), and the cells were incubated further. Plates were serially tested for GPDH on days −1 (day before inoculation), 0 (1 hour postinoculation), 1, 2, 3, 4, 7, 8, 11, and 14.

Quantitation of the Effect on GPDH. To confirm the acceleration of GPDH induction in Ad-36-infected 3T3-L1 cells, confluent cells were infected with Ad-36, Ad-2, or mock infected as described above, then immediately subjected to MDI treatment and maintenance medium, as before. GPDH was determined on day 4 postinoculation.

Effect of Ad-36 on Lipid Accumulation in 3T3-L1 Cells: Determination of Lipid Accumulation by Oil Red O Staining and Alcohol Extraction. This assay is based on the fact that the degree of staining of fat cells with Oil Red O is proportional to the extent of cell differentiation (18). Differentiation in confluent 3T3-L1 preadipocytes (six wells/group) was induced by MDI following their inoculation with Ad-36, Ad-2 [3.5×10^5 pfu (3.8 MOI) and 4.0×10^5 pfu (3.3 MOI), respectively], or media. On the 5th day postinoculation, cells were fixed for 1 hour with 10% formalin, then washed with water and stained for 2 hours with Oil Red O, followed by exhaustive rinsing with water. After evaporating the excess water at 32 °C, the dye was extracted with isopropyl alcohol, and its absorbance was determined at 510 nm, which was normalized to the amount of cellular DNA in the sample.

Effect of Ad-36 on GPDH Secretion by Human Fat Cells

Human primary preadipocytes obtained from Zen Bio, Inc. (Research Triangle Park, NC) were grown to confluency in three six-well plates and inoculated with media, 3.5×10^5 pfu (3.8 MOI) Ad-36, or 4.0×10 (5) pfu (3.33 MOI) Ad-2, followed by induction of differentiation with MDI. GPDH levels were determined 5 days postincubation.

Statistical Analysis

Effects of Ad-36 and Ad-2 were determined by comparing the group means with those of the uninfected controls, using Student's *t* test, followed by Bonferroni adjustment.

Results

Infection of 3T3-L1 Cells with Adenoviruses

Viral Attachment. Like most other adenoviruses, Ad-36 replicates well and causes cytopathic effects (CPE) on the human A549 tumor cell line. In contrast, preliminary studies showed that the mouse 3T3-L1 preadipocyte cell line exhibited little or no CPE after infection with Ad-36 or Ad-2. To determine whether these viruses were able to infect 3T3-L1 cells, we first examined their ability to attach to these cells. Attachment was examined by using indirect immunofluorescence to detect virions bound to the cell surface. As shown in Figure 1, both Ad-36 and Ad-2 attached to both 3T3-L1 and A549 cells.

Expression of Viral Genes. The adenovirus *E1A* genes are the first viral genes expressed during the virus replication cycle. They are expressed by alternative splicing of a single primary transcript. Based on its sequence homology with other *E1A* genes and the presence or absence of consensus splice sites, we expected that Ad-36 would produce 12S and 13S *E1A* mRNAs. The smaller 9S or 10S mRNA produced by some adenoviruses was not expected to be produced by

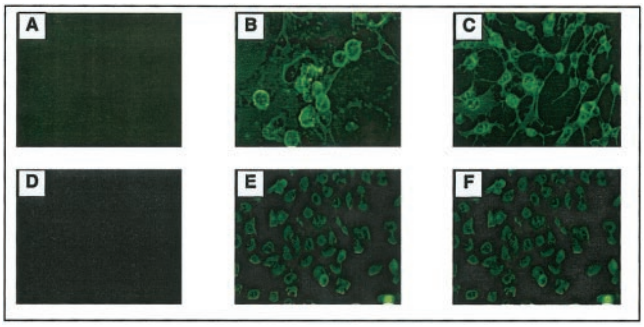

Figure 1: Immunofluorescence showing viral attachment. To assess virus attachment, confluent monolayers of 3T3-L1 cells and A549 cells on precleaned coverslips were infected with 2×10^4 pfu of either Ad-36 or Ad-2 and stained with fluorescein isothiocyanate-conjugated antirabbit IgG. (A) 3T3-L1 alone (No virus). (B) 3T3-L1 + Ad-36. (C) 3T3-L1 + Ad-2. (D) A549 alone (No virus). (E) A549 + Ad-36. (F) A549 + Ad-2. Ad-36 and Ad-2 attached to both 3T3-L1 and A549 cells. No fluorescence was detected in A549 or 3T3-L1 cells in absence of Ad-36 or Ad-2.

Ad-36 due to the absence of an appropriate consensus splice site. We designed three primer pairs for analysis of *E1A* mRNAs. The "10S" primer pair would produce a 182-base pair (bp) product from a possible 10S mRNA and a 350-bp product from 12S and 13S mRNAs. The "12S/13S" primer pair was expected to produce a 361-bp product from 12S mRNA and a 546-bp product from 13S mRNA. The "13S" primers were expected to produce a 227-bp product from 13S mRNA.

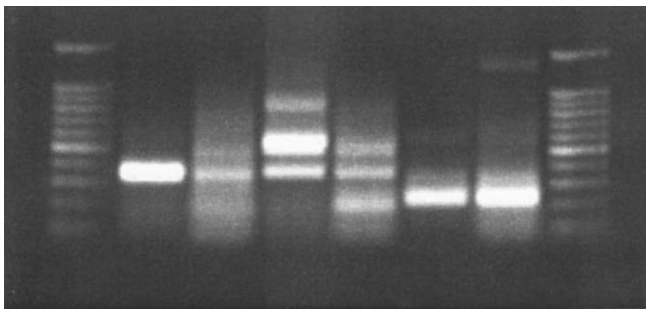

Figure 2: Expression of Ad-36 *E1A* in A549 and 3T3-L1 cells. RNA was extracted from A549 and 3T3-L1 cells 3 hours postinoculation with Ad-36 (3.82 MOI) and used in RT-PCR reactions. (Lane 1) 100-bp ladder. (Lane 2) A549 RNA with 10S primers. (Lane 3) 3T3-L1 RNA with 10S primers. (Lane 4) A549 RNA with 12S/13S primers. (Lane 5) 3T3-L1 RNA with 12S/13S primers. (Lane 6) A549 RNA with 13S primers. (Lane 7) 3T3-L1 RNA with 13S primers. (Lane 8) 100-bp ladder. The "13S" primers produced strong 227-bp bands from both infected A549 RNA and infected 3T3-L1 RNA, indicating the expression of 13S *E1A* mRNA in both cell types. The "12S/13S" primer pair, 546- and 361-bp products were produced from RNA from both infected A549 and infected 3T3-L1 cells. The "10S" primers produced a single product of 650 bp from Ad-36-infected A549 and 3T3-L1 cells. No 182-bp product was detected, indicating that Ad-36 did not produce a 10S mRNA.

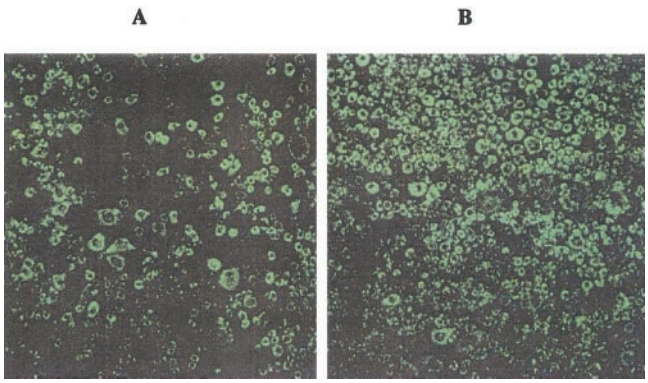

Figure 3: Differentiated 3T3-L1 cells stained with lipid-specific BODIPY stain. BODIPY 493/503 fluorescent dye staining of 3T3-L1 cells 5 days post-MDI/Ad-36 treatment. (A) Uninfected controls (MDI alone). (B) MDI + Ad-36. The figure shows much greater number of lipid-bearing cells in the Ad-36 group.

As shown in Figure 2, RT-PCR using the "13S" primers produced strong 227-bp bands from both infected A549 RNA and infected 3T3-L1 RNA, indicating the expression of 13S *E1A* mRNA in both cell types. With the "12S/13S" primer pair, 546- and 361-bp products were produced from RNA from both infected A549 and infected 3T3-L1 cells. This confirmed the production of 13S mRNA and demonstrated that 12S *E1A* mRNA was also expressed in both cell types. The "10S" primers produced a single product of 650 bp from Ad-36-infected A549 and 3T3-L1 cells. This again confirmed the expression of 12S and/or 13S *E1A* mRNA. No 182-bp product was detected, indicating that Ad-36 did not produce a 10S mRNA. No *E1A* products were seen in uninfected A549 or 3T3-L1 cells (data not shown).

Acceleration of 3T3-L1 Differentiation by Ad-36

Effect of Ad-36 on the Number of Differentiated Adipocytes: BODIPY Staining. Control (uninfected) 3T3-L1 cells and Ad-36-infected cells were stained with BODIPY on day 5 post-MDI treatment. As shown in Figure 3, highly fluorescent cells were much more abundant in the Ad-36-infected culture than in control cells, suggestive of increased number of adipocytes in Ad-36-infected cultures. This observation was quantitated and confirmed in the following experiment.

Effect of Ad-36 on the Number of Differentiated Adipocytes: Oil Red O Staining. Confluent cultures were infected with Ad-36, Ad-2, or mock infected, then subjected to differentiation using MDI for 7 days. As shown in Figure 4, the number of Oil Red O-stained cells in the Ad-36-infected cultures was almost triple the number in the control group

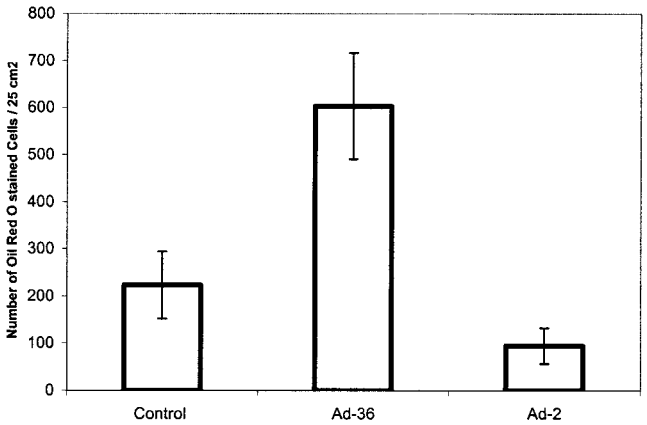

Figure 4: Number of Oil Red O-stained 3T3-L1 cells inoculated by Ad-36 or Ad-2. Confluent 3T3-L1 cells were inoculated with media, Ad-36, or Ad-2 followed by induction of differentiation by MDI treatment. Five days postinoculation, the cells were stained with Oil Red O dye (lipid-specific nonfluorescent dye that stains adipocytes). The number of Oil Red O stained in each well per 25-mm^2 area (five randomly selected areas/well; $n = 6$ wells per condition) was counted under the microscope and expressed in the figure as mean ± SD per group. The number of Oil Red O-stained cells in the Ad-36-infected cultures was almost triple the number in the control group ($p = 0.00,001$). Ad-2-infected cultures had fewer than one-half the number of the control group ($p = 0.009$).

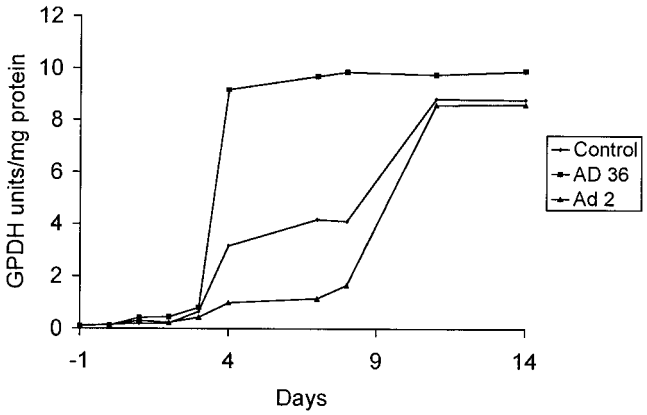

Figure 5: Time course of GPDH expression by 3T3-L1 cells inoculated by Ad-36 or Ad-2. Confluent 3T3-L1 cells were inoculated with media, Ad-36, or Ad-2 virus [3.5×10^5 pfu (3.8 MOI) and 4.0×10^5 pfu (3.3 MOI), respectively], immediately followed by MDI treatment. Two days later, the differentiation media was changed to maintenance media, and the cells were incubated further. Plates were serially tested for GPDH on days −1 (day before inoculation), 0 (1 hour postinoculation), 1, 2, 3, 4, 7, 8, 11, and 14. The GPDH expression was accelerated in Ad-36-infected 3T3-L1 cells, reaching maximal levels by day 4. In uninfected 3T3-L1 control cells, GPDH expression was slightly increased on day 4 but did not reach its maximal level until day 11. Expression of GPDH in Ad-2-infected 3T3-L1 cells was reduced in comparison with uninfected controls until day 11, after which enzyme levels were comparable.

($p = 0.00,001$). In contrast, the Ad-2-infected cultures had fewer than one-half the number in the control group ($p = 0.009$).

Effect of Ad-36 on Preadipocyte Differentiation-Specific Enzyme GPDH: GPDH Time Course. Differentiation of preadipocytes into adipocytes requires the induction of a number of genes, including those encoding enzymes directly involved in adipose tissue triglyceride synthesis. One of these is GPDH. Using GPDH as a marker of fat cell differentiation, we compared the time course of differentiation of Ad-36-infected 3T3-L1 cells with that of Ad-2-infected and uninfected control cells. As shown in Figure 5, the expression of GPDH was dramatically accelerated in Ad-36-infected 3T3-L1 cells, reaching maximal levels by day 4. In uninfected 3T3-L1 control cells, GPDH expression was slightly increased on day 4 but did not reach its maximal level until day 11. Expression of GPDH in Ad-2-infected 3T3-L1 cells was reduced in comparison with uninfected controls until day 11, after which enzyme levels were comparable.

Quantitation of the Effect on GPDH. Cells were harvested on day 4, the time of maximal difference in GPDH between Ad-36-infected and control cultures in the previous experiment. As shown in Figure 6, GPDH levels in Ad-36, but not in Ad-2-inoculated cells, were significantly greater ($p < 0.025$) compared with the uninfected control cells. This confirmed the observation that Ad-36 infection accelerated expression of this differentiation marker. In another experiment, we showed that formalin-inactivated Ad-36 had no effect on GPDH expression during 3T3-L1 differentiation (data not shown).

Effect of Ad-36 on Lipid Accumulation in 3T3-L1 Cells. Lipid accumulation in Ad-36 and Ad-2 infected groups was expressed as percentage of lipid in the uninfected control group (Figure 7). Ad-36-inoculated 3T3-L1 cells had significantly greater lipid accumulation (mean ± SD, 181 ± 17%, $p < 0.005$) than the uninfected controls. This effect was not seen with Ad-2. Taken together, these experiments established that Ad-36 promoted differentiation of the 3T3-L1 preadipocyte line.

Effect of Ad-36 on GPDH Secretion by Human Fat Cells

To determine whether Ad-36 has an adipogenic effect in human cells, cultures of human preadipocytes were infected with Ad-2, Ad-36, or mock infected, followed by induction of differentiation with MDI. As shown in Figure 8, on day 5 after induction of differentiation, GPDH levels were significantly higher in Ad-36-infected human preadipocytes than in control cells ($p = 0.0001$). The effect of Ad-2 on GPDH expression was not significant.

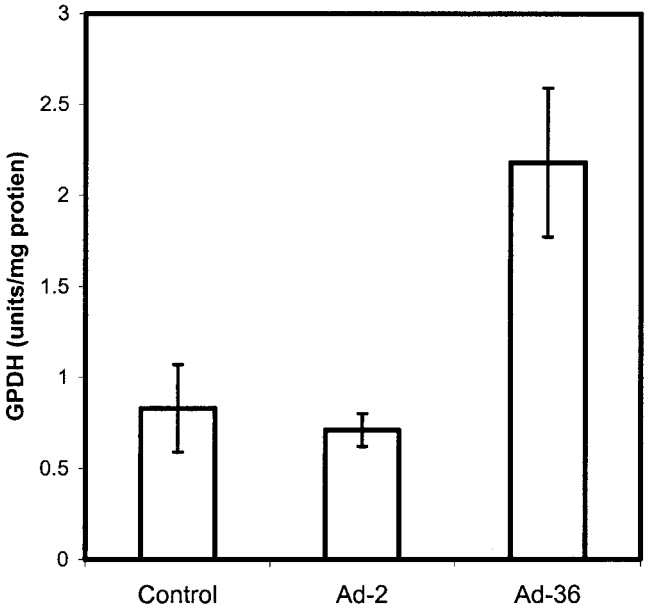

Figure 6: GPDH levels on the 5th day post-differentiation in 3T3-L1 cells inoculated by Ad-36 or Ad-2. Confluent 3T3-L1 cells were inoculated with Ad-36, Ad-2 [3.5×10^5 pfu (3.8 MOI) and 4.0×10^5 pfu (3.3 MOI), respectively], or media and immediately subjected to MDI treatment and maintenance medium. GPDH was determined on day 4 postinoculation. GPDH levels in Ad-36, but not in Ad-2-inoculated cells, were significantly grater ($p < 0.025$) compared with the uninfected control cells.

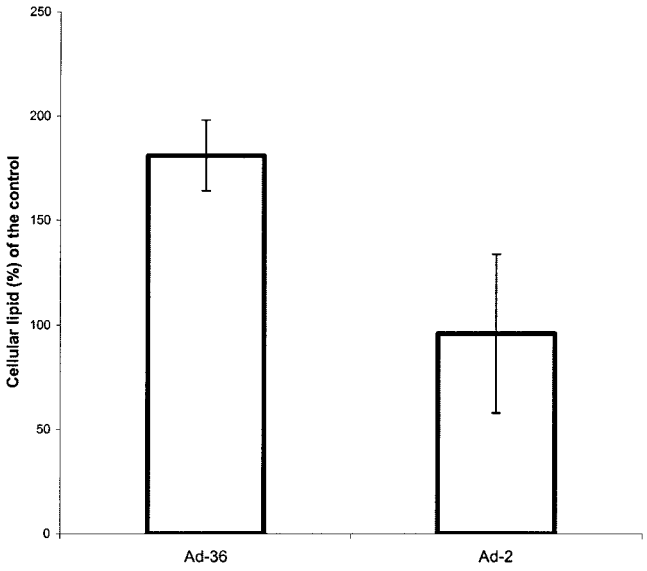

Figure 7: Lipid accumulation in 3T3-L1 cells inoculated by Ad-36 or Ad-2. Differentiation in confluent 3T3-L1 preadipocytes (six wells/group) was induced by MDI after their inoculation with Ad-36, Ad-2 [3.5×10^5 pfu (3.8 MOI) and 4.0×10^5 pfu (3.3 MOI), respectively], or media. On the 5th day postinoculation, cells were stained with Oil Red O, followed by extraction with isopropyl alcohol. The absorbance of the extract determined at 510 nm was normalized to the amount of cellular DNA in the sample. Ad-36-inoculated 3T3-L1 cells had significantly greater lipid accumulation (mean ± SD, 181 ± 17%, $p < 0.005$) than the uninfected controls. This effect was not seen with Ad-2.

Discussion

Adenoviruses are naked DNA viruses with icosahedral symmetry and a diameter of 65 to 80 nm. In humans, adenoviruses are frequently associated with acute upper respiratory tract infections and also may cause enteritis and conjunctivitis. Adenoviruses can easily be isolated from nasal swabs or from feces. Adenovirus infections are transmitted through respiratory, fomite, droplet, venereal, and fecal-oral routes. Not much is known about the pathogeneity of Ad-36.

Ad-36 infection increases adiposity in animals (11–13). This syndrome was not seen in the chickens inoculated with avian adenovirus chick embryo lethal orphan virus. In a subsequent experiment, four age- and weight-matched groups of chickens were used: infected donors (I-Ds) and infected recipients (I-Rs) and control donors (C-Ds) and control recipients (C-Rs) (12). Blood was taken from the I-D and C-D groups and injected into the recipient groups. The I-D and I-R groups had ~2.5 and 1.8 times more visceral fat compared with the C-D group. Ad-36 DNA was detected in the adipose tissues of I-D and I-R groups but not in controls. The two infected groups showed significantly decreased cholesterol levels, and the I-D group had a significant reduction in serum triglycerides. These data confirmed the previous findings that Ad-36 produces adiposity and paradoxical reductions in serum lipids. In addition, the study fulfilled a Koch's postulate that adiposity could be transmitted from infected animals (I-D group) to a new set of animals (I-R group). Next, two studies were conducted in nonhuman primates to investigate the adiposity-promoting potential of Ad-36 (13). In the first study, Dhurandhar et al. (13) observed spontaneously occurring Ad-36 antibodies in serum samples from adult male rhesus monkeys that were stored over a 7-year period at the Regional Primate Research Center located at the University of Wisconsin. The monkeys gained ~0.1 kg in the year preceding but ~1.8 kg in the year after seroconversion. Serum cholesterol fell ~35 mg/dL after the appearance of Ad-36 antibodies. In the second experiment, male marmosets inoculated with Ad-36 had a 4-fold gain in body weight, an increase in body fat of ~60%, and reduction in serum cholesterol of 34 mg/dL compared with uninfected controls over a period of about 6 months. These data demonstrated that Ad-36 is capable of increasing body fat in nonhuman primates.

A lack of overt hypothalamic lesion, preferential presence of Ad-36 DNA in the adipose tissue of the infected animals, and the preliminary data showing enhanced differentiation potential of preadipocyte derived from Ad-36-infected animals prompted us to use 3T3-L1 preadipocytes

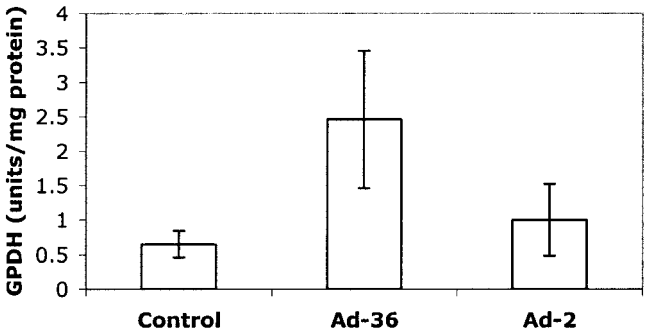

Figure 8: GPDH levels in human primary preadipocytes inoculated by Ad-36 or Ad-2. Human primary preadipocytes were grown to confluency and inoculated with media, 3.5×10^5 pfu (3.8 MOI) Ad-36, or 4.0×10 (5) pfu (3.33 MOI) Ad-2, followed by induction of differentiation with MDI. GPDH levels were determined 5 days postincubation. GPDH levels were significantly higher in Ad-36-infected human preadipocytes than in control cells ($p = 0.0001$). The effect of Ad-2 on GPDH expression was not significant.

to test the effect of Ad-36 on their differentiation. 3T3-L1 cells have been extensively used to conduct adipocyte differentiation studies and provide a cell line with relatively uniform characteristics in contrast to primary adipocytes obtained from animals or humans. In addition, they can be reliably differentiated on command by exposure to the differentiation-inducing cocktail MDI. Unlike the Ad-36-permissive A549 cells, which show CPEs and cell lysis in response to Ad-36 infection, 3T3-L1 cells did not show overt signs of infection. Therefore, before using 3T3-L1 cells for studying Ad-36-induced differentiation, it was necessary to confirm that adenoviruses attach to and enter the cells. Data obtained showed that Ad-36 attached to 3T3-L1 cells and initiated the viral replication cycle, as evidenced by expression of the viral *E1A* gene. Because it is likely that other Ad-36 genes are also expressed in infected 3T3-L1 cells, further research will be required to identify the Ad-36 genes responsible for the virus's adipogenic effects.

Ad-36 infection had multiple effects on the differentiation of 3T3-L1 cells. Infection with Ad-36 increased the number of cells that differentiated into adipocytes as judged by Oil Red O or BODIPY staining. In addition, the total amount of lipid that accumulated was also greater in Ad-36-infected cultures. A time course study of the expression of the adipocyte marker enzyme GPDH showed that this enzyme reached maximum levels of expression by day 4 in Ad-36-infected cells, whereas maximum levels of expression were not reached until day 11 in uninfected cells. This large effect suggests that Ad-36 has a powerful influence on expression of genes related to adipogenesis.

Ad-36-induced adiposity in mice (11) and 3T3-L1 cells are mouse embryonic preadipocytes. Therefore, the pro-differentiation effect of Ad-36 observed in 3T3-L1 cells is a significant finding that may contribute to Ad-36-induced adiposity in mice. We also observed that Ad-36 exerted a pro-adipogenic effect on human preadipocytes. This effect on human cells further suggests that Ad-36 may have a role in human obesity. This is particularly significant because Ad-36-neutralizing antibodies are correlated with human obesity (14).

There are ~50 types of human adenoviruses, but their adipogenic potential is largely unknown. To determine the specificity of the effect of Ad-36, we used Ad-2, the most common human adenovirus that does not induce adiposity in animals (19). A lack of effect of Ad-2 on differentiation demonstrated that the observed findings were not a common characteristic of all adenoviruses.

Like several other causative factors, viral infections may play a role in certain types of obesity. Although a causal relationship between Ad-36 and adiposity has been shown in animal models and Ad-36 shows an association with human obesity, the complete extent of the role of viruses in human obesity is not yet determined. Elucidating the mechanism of action of Ad-36 in 3T3-L1 cells may lead to the eventual understanding of the adiposity promoting effect of Ad-36 in vivo. However, it is possible that multiple mechanisms are involved in the adipogenic effect of Ad-36, and enhancement of adipocyte differentiation may be only one such mechanism. Nonetheless, future understanding of the molecular interactions of cellular and viral genes responsible for enhanced differentiation may reveal novel signaling pathways and controls of preadipocyte differentiation.

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