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Increased adiposity in animals due to a human virus

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BACKGROUND: Four animal models of virus-induced obesity including adiposity induced by an avian adenovirus have been described previously. This is the first report of adiposity induced in animals by a human virus.
OBJECTIVE: We investigated the adiposity promoting effect of a human adenovirus (Ad-36) in two different animal models.
DESIGN: Due to the novel nature of the findings we replicated the experiments using a chicken model three times and a mammal model once. In four separate experiments, chickens and mice were inoculated with human adenovirus Ad-36. Weight matched groups inoculated with tissue culture media were used as non-infected controls in each experiment. Ad-36 inoculated and uninfected control groups were housed in separate rooms under biosafety level 2 or better containment. The first experiment included an additional weight matched group of chickens that was inoculated with CELO (chick embryo lethal orphan virus), an avian adenovirus. Food intakes and body weights were measured weekly. At the time of sacrifice blood was drawn and visceral fat was carefully separated and weighed. Total body fat was determined by chemical extraction of carcass fat.
RESULTS: Animals inoculated with Ad-36 developed a syndrome of increased adipose tissue and paradoxically low levels of serum cholesterol and triglycerides. This syndrome was not seen in chickens inoculated with CELO virus. Sections of the brain and hypothalamus of Ad-36 inoculated animals did not show any overt histopathological changes. Ad-36 DNA could be detected in adipose tissue, but not skeletal muscles of randomly selected animals for as long as 16 weeks after Ad-36 inoculation.
CONCLUSIONS: Data from these animal models suggest that the role of viral disease in the etiology of human obesity must be considered.
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Introduction

Obesity is a serious disease that affects more than one third of adults and over 20% of children in the USA and produces major increases in morbidity and mortality.[1,2] There are multiple etiologies of obesity, but most scientists in the field have focused either on genetic factors or behavioral aspects. Isolated cases of obesity due to hypothalamic damage from accidents, tumors, bacterial infections and other unusual events have been reported, but such cases have been thought to be exceedingly rare.[3]

No consideration has been given to the possibility that human obesity might be due to viral infections, despite the evidence that viral infections may cause obesity in animals. Four different animal viruses have been identified that produce obesity syndromes in animals. Lyons *et al*[4] reported that canine distemper virus produced obesity in mice and later studies suggested that the mechanism was hypothalamic damage due to the virus.[5–7] Rous associated virus type 7 (RAV-7) was reported to induce obesity in chickens.[8,9] RAV-7 virus-induced obesity was associated with stunting of growth, hyperlipidemia, fatty liver, hepatomegaly, anemia and immuno-suppression.[8,9] Borna disease virus, a single stranded RNA virus, produced a syndrome of obesity in rats characterized by lympho-monocytic inflammation of the hypothalamus, hyperplasia of pancreatic islets, and elevated serum glucose and triglyceride levels.[10]

Dhurandhar *et al*[11] reported that SMAM-1, a chicken adenovirus isolated in Bombay, India, produced excessive fat accumulation in the visceral depots and a paradoxical reduction of serum levels of cholesterol and triglycerides in chickens. Of 52 obese humans tested by agar gel-precipitation test, 10 had antibodies to SMAM-1.[12] These 10 individuals had a higher body weight and lower serum cholesterol and triglycerides compared to antibody negative individuals.[12]

We wished to further study the adiposity promoting effect of SMAM-1 in the USA. However, the United States Department of Agriculture refused permission to import SMAM-1 from India. This prompted us to

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investigate the adiposity-promoting potential of a human adenovirus that is available in the USA. There are currently 50 types of human adenoviruses deposited in the American Type Culture Collection (ATCC) virus bank. Human adenovirus-36 (Ad-36) was first isolated in 1978 in Germany from the feces of a girl with diabetes mellitus.[13] Ad-36 is serologically different from at least 47 of the other 49 human adenoviruses, as there is no cross-reaction of anti-bodies between Ad-36 and these other adenoviruses.[13–18] Ad-36 was our first candidate to investigate its adiposity-promoting potential mainly due to its antigenic uniqueness.

In the current study, we performed four experiments in animals: three in chickens, and one in mice, that demonstrate that Ad-36 increases adiposity in animals.

Methods

Four experiments involving two different animal species were performed to demonstrate the cross-species reproducibility of Ad-36 induced obesity: assays used are described under 'Assays and techniques' at the end of the Methods section.

Experiment 1—chickens

One-day-old specific pathogen free (SPF) white leghorn broiler chickens ($n=39$) were obtained from Specific Pathogen-Free Avian Supply (SPAFAS, Roanoke, IL) and were housed in the National Wildlife Health Center, Madison, WI, under Biosafety level 3 conditions, with a separate air supply to each room. Protective clothing, shoes, gloves, hairnets and masks were used to enter the rooms and utmost care was taken to prevent cross contamination.

Access to food (Purina Starter Grow) and water was provided *ad libitum* throughout the study period and food consumption, corrected for spillage, was recorded for individual cages. For the first 3 weeks, the chicks were housed in a brooder with a 12 h light cycle and a temperature of 95°F that was reduced gradually to 70°F at the end of 3 weeks. At 3 weeks of age, the chickens were removed from the brooder and maintained at 70 ± 2°F thereafter. Chickens were weighed at 1 week, 3 weeks, and then every week until the termination of the study at 6 weeks.

After 3 weeks, the chickens were divided into three weight matched groups of 13 each (Ad-36, CELO and control) and were housed in three separate rooms (two chickens/cage). Blood was drawn from a wing vein for baseline measurements of serum cholesterol and triglycerides, and for measurement of adenovirus antibodies to insure that the chickens had not been previously exposed to Ad-36. All these assays were repeated at the time of sacrifice.

Group 1, the control group, was inoculated intranasally (i.n.) with 0.2 ml of media. Group 2 was inoculated i.n. with 0.2 ml of a suspension of human adenovirus Ad-36, representing a dose of 10^5 PFUs (plaque forming units) of Ad-36 and the third group was inoculated i.n. with 0.2 ml of CELO virus (10^4 PFU). Throat and rectal swabs were taken from all chickens one week after virus inoculation to confirm infection by virus isolation.

Three weeks after inoculation the animals were fasted overnight and sacrificed. The omental-mesenteric (visceral) fat was carefully dissected from each bird and weighed. Six birds from each group were randomly selected for total carcass fat determination.

Experiment 2—chickens

The above experiment was repeated in 32 male SPF chickens. In this experiment chickens were observed for a longer period of time after the virus inoculation. Housing conditions were similar to those described in experiment 1. Two groups of weight-matched animals ($n=16$ per group) were used for this experiment. Animals were inoculated intranasally at 3 weeks of age (1.8×10^6 PFU for the Ad-36 group and 200 µl sterile media for the control group). Food intake was measured for each cage. Blood was drawn from a wing vein 36 h after inoculation to determine viremia by virus isolation from the blood sample. Blood was also drawn 10 days post inoculation and at the time of sacrifice. Chickens were sacrificed 5 weeks post inoculation, body weight measured, visceral fat was separated and weighed. About 1 g samples of visceral adipose tissue and skeletal muscle from breast muscle area (keel) were removed and flash frozen in liquid nitrogen for viral DNA detection using capillary electrophoresis assay. Serum cholesterol and triglycerides levels were determined in the final serum obtained.

Experiment 3—chickens

This experiment assessed a different route of infection (intraperitoneal *vs* intranasal inoculation), a longer time period before sacrifice, and histopathological examination of the brains. Housing conditions were similar to those described above. Three-week-old male SPF chickens ($n=10$) were inoculated intraperitoneally with 10^5 PFU of Ad-36 virus media (Ad-36 group) or 0.2 ml of sterile media ($n=8$). Chickens were sacrificed 13 weeks post inoculation, body weight was measured, and visceral fat was separated and weighed. One gram samples of visceral adipose tissue were removed and flash frozen in liquid nitrogen for viral DNA detection using capillary electrophoresis assay. Randomly selected samples (three from each group) were tested for the presence of Ad-36 DNA with the help of capillary electrophoresis assay. At sacrifice the brains were carefully removed, preserved in 30 times the volume of 37% formalin, and sectioned for histopathological examination. Serum cholesterol and triglycerides levels were determined in the final serum obtained.

Experiment 4 — mice

This preliminary experiment was conducted to investigate the adipogenic effect of Ad-36 in a mammal model. Thirty-five 4-week-old outbred female ICR mice (Harlan Labs, Indianapolis) were received. Five mice were randomly chosen for sacrifice to draw blood for obtaining baseline serum. Baseline serum was obtained to ensure that the mice were free from Ad-36 antibodies at the beginning of the experiment. The remaining 30 mice were weight matched into two groups and injected i.p. with 0.2 ml of Ad-36 media containing 2×10^7 PFU ($n = 20$) or 0.2 ml of tissue culture media ($n = 10$, control group). Mice were housed under Biosafety level 3 containment, were offered *ad libitum* access to food and water, and food intakes and body weights were measured weekly. Blood samples for cholesterol, triglycerides and viral titers by serum neutralization were drawn at 10 weeks and at sacrifice 22 weeks post inoculation. At sacrifice, the visceral inguinal and retroperitoneal fat pads were dissected free and weighed. Body composition was performed on all animals.

Assays and techniques

Preparation of the virus suspension. Human adenovirus-36 (Ad-36, ATCC no. VR-913) and avian adenovirus CELO (ATCC no. VR 432) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The Ad-36 work stock was grown on A549 cells, a human bronchial carcinoma tissue culture line and the CELO work stock was grown using SL-29 cells (chick embryo fibroblasts). The titer of the work stocks that caused a cytopathic effect (CPE) in 50% of the wells containing A549 or SL-29 cells was calculated and expressed as tissue culture infectivity dose (TCID-50) units per ml. TCID-50 of the work stocks were determined using serial 10-fold dilutions of the virus work stock.

Plaque forming units assay. Titers of the plaque purified Ad-36 and CELO virus were determined in A549 and SL-29 cells, respectively, by this assay. The harvested and frozen virus suspensions were rapidly thawed at 37°C in a water bath. Starting with 100 µl of virus suspension and 900 µl of media, serial 10-fold dilutions were made. Cells were grown to confluence in six well plates and three wells were used for each dilution. Three wells were used as the blank control and were not infected with the virus suspension. The media was removed from each well and 100 µl of media containing serially diluted virus suspension were pipetted into the wells. The plates were incubated at 37°C, shaking gently every 15 min. After 1 h of incubation, viral suspension from the wells was removed and discarded. The wells were overlaid with about 3 ml of 1% agar in media per well, with 1 × antibiotic-antimycotic solution. The plates were inverted and incubated at 37°C for 8 days until plaques appeared. After 8 days, wells were stained overnight with about 1 ml of crystal violet per well. The next day, the number of plaques formed was counted after removing the agar. The number of plaques formed × dilution of viral suspension used gave PFU/100 µl of inoculum used. This was multiplied by 10 to express PFU/ml.

Serum neutralization assay for viral antibodies. Serum neutralization assays were performed using the 'constant virus-decreasing serum' method. Test serum was heat inactivated in a water bath at 57°C for 1 h and serially diluted (2-fold) in 96-well plates. The serum dilutions ranged from 1:2 to 1:512. One hundred TCID-50 of the respective adenovirus work stock was added to each of the wells. A549 cells (SL-29 cells were used for detecting CELO antibodies) were added to these wells after 1 h of incubation at 37°C. Each test serum was run in duplicate. Serum control (serum and cells, but no virus), cell control (cells alone, no virus, no serum) and virus control (cells and virus, no serum) were included with each assay. Plates were incubated at 37°C for 11 days and the presence of CPE was noted. Serum samples without CPE in dilutions of 1:8 or higher were considered positive for the presence of neutralizing antibodies to the respective virus. CPE in virus control wells, and its absence in serum control and cell control wells, was ascertained.

Virus isolation. A549 human bronchial carcinoma cells in tissue culture were used for isolation of Ad-36 and SL-29 cells were used for isolating CELO virus. Flasks containing A549 cells or SL-29 cells were inoculated with about 200 µl of sample (media from the oral-rectal swabs or whole blood) and were incubated at 37°C in media containing 2 × antibiotic−antimycotic solution. Culture media from the cells was collected after 8 days and transferred to cells in new set of flasks. This was repeated for a total of three passages. Infection was confirmed by observing if viral CPE occurred in the respective cells.

Assays for serum cholesterol and triglycerides. Serum cholesterol was assayed using the cholesterol oxidase-peroxidase method. Colorimetric determinations were made using Sigma kits (catalogue no. 352) and the absorbance read at 500 nm. Cholesterol calibrator (Sigma catalogue no. C 7921) and Cardiolipid control (Sigma catalogue no. C 4571) were used.

Triglycerides were determined using the glycerol phosphate−peroxidase method. Colorimetric determinations were made using Sigma kits (catalogue no. 339-50) and the absorbance was read at 540 nm. Glycerol (Sigma catalogue no. 339-11) was used as a standard.

Body composition analysis. Digestive tracts of the carcasses were cleaned and returned to the carcasses. After autoclaving and homogenization of the carcasses, aliquots were used for water, ash and fat contents. All measurements were performed in triplicate. Water content was determined by heating samples to a constant weight in a drying oven overnight at 90°C. Ash content was determined by incinerating the sample in a furnace at 600°C for 4 h. The Folch extraction method was used for body fat determination. Fat was extracted with methanol–chloroform.

Capillary electrophoresis. The assay has been previously described in detail.[19] Briefly, the assay was divided in three parts, namely Probe synthesis, DNA extraction and hybridization, and CE-LIF (capillary electrophoresis–laser-induced fluorescence) analysis.

Probe synthesis. We have the sequence of the entire Ad-36 genome. To ensure specificity of the DNA detection, only a segment with a sequence unique to Ad-36 was probed. The uniqueness of the sequence was verified by a gene bank search. A 5′fluorescein phosphoramidite labeled DNA probe for Ad-36 adenovirus was synthesized by IDT Laboratories (Coralville, IA).

DNA extraction and hybridization. The DNA from the sample was extracted with the Qiagen QIAmp Blood or tissue kit and quantitated spectrophotometrically. The genomic DNA was then digested with MboI by standard procedures to generate smaller DNA fragments for hybridization, and treated with RNAse One to remove any RNA contamination. This DNA was then hybridized with the DNA probe (1.0125 μg) in a buffer volume of 30 μl containing 10 mM Tris–HCI (pH 7.2), 1 mM EDTA (pH 8.0) and 50 nM NaCl. The mixture was heated at 85°C for 10 min, and then incubated at 42°C for 4 h. Following the incubation, samples were flash frozen at −80°C.

CE-LIF analysis. Separations were performed on a P/ACE 2050 system using laser-induced fluorescence in the reversed-polarity mode (anode at the detector side) at excitation of 488 nm and emission of 520 nm. Samples were introduced hydrodynamically by 10 s injections at 0.34 Pa across a 65 cm × 100 μm coated eCAP dsDNA capillary filled with replaced linear polyacrylamide. The capillary was conditioned with eCAP dsDNA 1000 gel buffer. Separations were performed under constant voltage at 7.0 kV for 15–30 min.

The hybridization procedure generates a DNA fragment comprising the fluorescently labeled probe and the target viral DNA. The negative control generates a DNA fragment of the probe bound to itself. The migration times of the positive and the negative control are nearly identical, however, the positive sample has an increased peak area indicating bound DNA and a slightly longer retention time due to a mass effect. Samples were quantitated by comparing the peak area obtained for the samples to a control from an identical tissue. The viral DNA is calculated based on the difference in peak areas. A linear relationship between the peak area and concentration was demonstrated from 0.072 to 21.46 pg ($r^2 = 0.99$, $y = 516.88 + 18.01x$). The intra-day and inter-day migration time precision was 0.18% ($n = 9$) and 0.22% ($n = 6$), respectively. The intra-day peak area precision was 7.3% ($n = 6$) and the inter-day peak area precision was 11% ($n = 9$). The minimal detectable level was 36 ag (signal-to-noise ratio 3 : 1).

DNA from A549 cells without virus, and DNA from A549 cells infected with Ad-36 were used as negative and positive controls, respectively.

Histopathology of brains

Specimens of brain were paraffin embedded, cut with a microtome, and stained with hematoxylin–eosin. One micron sections were taken to include the hypothalamus, and every fifth section was examined. About 15–30 sections were examined per chicken, of which at least two in each chicken were from the hypothalamus.

Statistical analysis

Means of groups were compared with those of the control using Student's *t*-test followed by Bonferroni adjustment for multiple comparisons. The difference in prevalence of obese animals in each group was tested by the chi-square test.

Results

Experiment 1

Confirmation of viral infection. Isolation of Ad-36 and CELO viruses was attempted using three passages on A549 or SL-29 cells. After three passages of oral–rectal swabs, Ad-36 and CELO viruses could be isolated from all animals of the respective groups. No virus could be isolated from the control group. Virus isolated from swabs from Ad-36 inoculated chickens demonstrated the presence of human adenovirus antigen by Adenoclone enzyme immunoassay (Meridian Diagnostics, Cincinnati), further confirming infection with this human virus. No EIA kit was available to detect CELO virus. Infection was also confirmed by a four-fold or more rise from baseline of specific neutralizing antibody titers in all animals from the Ad-36 and the CELO groups. No rises and no antibody titers were noted in the control group.

Body composition and prevalence of obesity. Post inoculation cumulative food intake per chicken was not different for the three groups (mean ± s.d., control group 1150.0 ± 157.0 g, Ad-36 group 1038.5 ± 249.1 g and CELO group 1282.5 ± 505.5 g; P = NS compared to the control group). Body weights were not different among the groups at the time of sacrifice, but visceral fat and total body fat were significantly greater ($P < 0.02$) in the Ad-36 chickens compared to controls (Table 1). Visceral fat of the Ad-36 group was 100% greater than that of the control group. Visceral fat and the carcass fat of the CELO group did not differ significantly from that of the control group (Table 1).

If obesity is defined as a visceral fat greater than the 85th percentile of the range of the control group, our data show that three chickens in the control group (23%) and nine in the Ad-36 group (70%) were obese ($P < 0.02$; Table 2). Six chickens from the CELO group (46%) were obese by the definition; this prevalence in the CELO group was not significantly different from that of the control group.

Serum lipids. Compared to the control, serum cholesterol was lower only for the Ad-36 group ($P < 0.02$), but serum triglycerides levels were significantly lower for the Ad-36 as well as the CELO group (Table 1).

Experiment 2

Confirmation of viral infection. Ad-36 virus could be isolated in tissue culture by repeated cell passages of the blood obtained 36 h post inoculation from the Ad-36 group and detected as a human adenovirus with the help of Adenoclone EIA kit in 12 out of 16 chickens. No virus could be isolated from the control group. Blood drawn 10 days post inoculation demonstrated neutralizing antibodies to Ad-36 in 10 of 16 chickens. None of the control chickens had such antibodies.

DNA isolated from the blood, visceral adipose tissue and skeletal muscle obtained from three infected and three control chickens were assayed for the presence of Ad-36 DNA by capillary electrophoresis.[19] Ad-36 DNA could be detected in all three blood samples drawn 36 h after infection and in two of three samples of visceral fat taken at sacrifice 5 weeks after infection, but not in the muscle samples taken at 5 weeks from the Ad-36 infected chickens. Ad-36 DNA was not detected in any tissues of the control chickens at any time.

Body composition and prevalence of obesity. Post inoculation cumulative food intake per chicken was not different for the two groups (mean ± s.d., control group 5401.6 ± 268.1 g, and Ad-36 group 5234.8 ± 338.3 g; P = NS). Body weights were not different among groups, but visceral fat was 128% higher in the Ad-36 chickens ($P < 0.0005$; Table 3). Total body fat was 46% greater in the Ad-36 infected group, compared to the control ($P < 0.0005$).

Using the definition of obesity as a total body fat greater than the 85th percentile of the range of the control group, 64% of Ad-36 chickens and 18% of control chickens were obese ($P < 0.02$; Table 2).

Serum lipids. Compared to the control group, serum cholesterol and triglycerides were lower ($P < 0.0005$ and $P < 0.02$, respectively) in the Ad-36 group (Table 3).

Experiment 3

Confirmation of viral infection. Serum neutralization assays of the kill serum confirmed the presence of antibodies to Ad-36 in all chickens in the Ad-36 group and none in the control group. Capillary electrophoresis demonstrated Ad-36 DNA in visceral fat of all three infected chickens tested, but in none of the controls.

Table 1 Experiment 1: infection of chickens with human adenovirus Ad-36

	Control	*Ad-36*	*CELO*
Number	13	13	13
Body weight (g)	502 ± 18.2	538 ± 18.2	527 ± 16
Visceral fat (%)	0.27 ± 0.05	0.54 ± 0.07*	0.37 ± 0.04
Total body fat (%)	5.8 ± 0.6	6.9 ± 0.1*	5.9 ± 0.6
Cholesterol (mmol/l)	7.14 ± 0.57	4.53 ± 0.31*	5.58 ± 0.61
Triglycerides (mmol/l)	0.84 ± 0.05	0.69 ± 0.03**	0.68 ± 0.02*

Mean ± s.e.m.; *$P \leq 0.02$; **$P < 0.05$ compared to control.

Table 2 Prevalence of obesity (%)

	Control	*Ad-36*	*CELO*
Experiment 1 (chickens)	23.07	69.23*	46.15
Experiment 2 (chickens)	18.1	63.6*	
Experiment 3 (chickens)	12.5	70.0*	
Experiment 4 (mice)	22.22	60.0**	

*$P < 0.02$; **$P < 0.05$. Obesity was defined as greater than the 85th percentile of adiposity of the control group.

Table 3 Experiment 2: replication of Ad-36 induced obesity in chickens

	Control	*Ad-36*
Number	16	16
Body weight (g)	1250 ± 28.4	1328 ± 39.4
Visceral fat (%)	1.08 ± 0.14	2.47 ± 0.23*
Total body fat (%)	7.8 ± 0.5	11.4 ± 0.6*
Cholesterol (mmol/l)	3.54 ± 0.10	3.03 ± 0.10*
Triglycerides (mmol/l)	1.13 ± 0.09	0.88 ± 0.04**

Mean ± s.e.m.; *$P \leq 0.0005$; **$P < 0.02$ compared to control.

Body composition and prevalence of obesity. Body weights and post inoculation cumulative food intakes were not different between Ad-36 and control groups, but visceral fat was 74% greater in the Ad-36 group ($1.74 \pm 0.2\%$ *vs* $1.01 \pm 0.2\%$, $P = 0.03$; Table 4).

Seventy percent of the infected chickens (seven out of 10) had visceral fat weights above the 85th percentile of the range of the control group, whereas 12.5% of control chickens (one out of eight) met this criteria ($P < 0.02$; Table 2).

Serum lipids. Compared to the control group, serum cholesterol and triglycerides of the Ad-36 group were lower by 20% and 18%, respectively. However, the values did not reach statistical significance.

Brain histopathology. The sections revealed no morphological abnormalities in either group, with the exception that one sample from each group had changes thought to be not relevant (as described below). One control chicken had a unilateral focus of mild gliosis and perivascular accumulation of lymphocytes in the pituitary isthmus of the hypothalamus and some vacuolization of the myelin sheath in some areas of white matter. In one Ad-36 infected chicken, a single dilated blood vessel with transmural localization of mononucleated cells was noted adjacent to the third ventricle.

Experiment 4

Confirmation of viral infection. Absence at baseline of antibodies to Ad-36 was confirmed in five randomly selected mice by serum neutralization assay. Ad-36 antibodies were seen in 12 of 20 Ad-36 inoculated mice after 10 weeks and in 19 of 20 mice after 22 weeks. None of the control mice had a positive Ad-36 antibody titer ($\geq 1:8$).

Body composition and prevalence of obesity. One animal from the control group, who was negative for antibodies to Ad-36, had a very high body weight (38.8 g) and visceral fat (2.43 g). This animal qualified as an outlier by two separate statistical tests for data rejection (Q Distribution Deviate and Maximum Normal Residual test). The data from this animal were discarded. Post inoculation cumulative food intake per animal was not different for the two groups (mean $\pm$ s.d., control group 467.0 ± 22.7 g, and Ad-36 group 480.3 ± 33.7 g; $P =$ NS). Compared to the control group, the mean body weight was 9% greater in Ad-36 mice ($P < 0.05$), total body fat was 35% greater ($P < 0.02$), and visceral fat was 67% greater (1.0 g *vs* 0.6 g; $P < 0.02$; Table 5). Retroperitoneal and inguinal fat pads were not different for the two groups.

Sixty percent of mice (12 out of 20) infected with Ad-36 and 22% of the control mice (two out of nine; $P < 0.02$) had total percent body fat weights above the 85th percentile of the range of the control group (Table 2).

Serum lipids. Serum cholesterol and triglycerides in the Ad-36 group were significantly lower than control by 38% ($P < 0.03$) and 31% ($P < 0.008$), respectively (Table 5).

Discussion

Four experiments demonstrated that Ad-36 increased visceral fat, total fat, and/or body weight compared to the control group. The first experiment tested the adiposity promoting effect of CELO virus along with Ad-36. CELO is antigenically similar to SMAM-1 and present in the USA and, therefore, we chose to use CELO in Experiment 1. Asymptomatic presence of CELO virus was reported from 64% to 100% of apparently healthy chickens 5 weeks of age and older.[20] The first experiment demonstrated that CELO, an avian adenovirus, did not produce adiposity similar to that produced by Ad-36 and that the adipogenic effect of Ad-36 may not be common to all adenoviruses.

Experiment 2 confirmed the findings that Ad-36 inoculation leads to increased visceral and total adiposity and paradoxically lower serum cholesterol and triglycerides. Experiment 3 was undertaken primarily to assess the effect of Ad-36 inoculation on the hypothalamus, as hypothalamic damage has been

Table 4 Experiment 3: intraperitoneal inoculation of Ad-36 in chickens

	Control	*Ad-36*
Number	8	10
Body weight (g)	1533.5 ± 59.1	1625.4 ± 53.8
Visceral fat (%)	1.01 ± 0.19	1.74 ± 0.25*
Cholesterol (mmol/l)	3.04 ± 0.36	2.51 ± 0.26
Triglycerides (mmol/l)	2.35 ± 0.24	1.92 ± 0.13

Mean $\pm$ s.e.m.; *$P \leq 0.04$ compared to control.

Table 5 Experiment 4: infection of mice with Ad-36

	Control	*Ad-36*
Number	9	20
Body weight (g)	27.9 ± 0.79	30.5 ± 0.95*
Visceral fat (%)	2.13 ± 0.27	3.16 ± 0.27**
Visceral fat (g)	0.60 ± 0.08	1.00 ± 0.11**
Inguinal fat (g)	0.22 ± 0.02	0.25 ± 0.02
Retroperitoneal fat (g)	0.30 ± 0.03	0.27 ± 0.03
Total fat (%)	7.2 ± 0.57	9.3 ± 0.6**
Cholesterol (mmol/l)	2.68 ± 0.35	1.67 ± 0.08**
Triglycerides (mmol/l)	2.26 ± 0.18	$1.56 \pm 0.06^{\dagger}$

Mean $\pm$ s.e.m.; *$P \leq 0.05$; **$P < 0.03$; $^{\dagger}P < 0.008$ compared to control.

postulated to be the cause of obesity induced by canine distemper virus and borna disease virus[4–7,10] Experiment 4 was a preliminary experiment to test the suitability of a mammal model for future studies involving adiposity promoting effect of Ad-36.

Previously, human and avian adenoviruses were not thought to infect across species. The isolation of Ad-36 from oral and rectal swabs taken from infected animals 7–10 days after infection, as well from the blood drawn 36 h post inoculation of the infected animal and the presence of viral DNA in adipose tissue as long as 16 weeks post-inoculation are unequivocal evidence of cross-species infection with this human virus. Four-fold or greater change in the antibody titer is considered an evidence of infection. Twelve mice from the Ad-36 group showed ≥4-fold rise in antibody titer between 10 and 22 weeks post inoculation. Although this observation suggests virus replication in animals, we cannot explain the observation at this time.

Ad-36 did not cause severe symptoms in any of the experiments. Some animals appeared to have reduced activity for 1–2 days post-inoculation, but no other overt signs or symptoms were noted and there was no premature mortality in any of the experiments.

Both intra-nasal and intra-peritoneal inoculation of Ad-36 increased adiposity, predominantly by increasing visceral adipose tissue stores. Individual susceptibility to increased adiposity was different among animals. Using the 85th percentile for adiposity of the control group as an arbitrary definition of obesity, 60–70% of all Ad-36 inoculated animals in each experiment became obese.

Different times of sacrifice post inoculation revealed that increased adiposity could be observed as soon as 3 weeks (experiment 1), was not of transient nature, and persisted for 13 weeks (experiment 3) to 22 weeks (mice—experiment 4). Durations selected in this study should be helpful for future work in the area.

The mechanisms of obesity with Ad-36 are unknown. Histopathological examination of the brains of chickens did not show any evidence of damage to the hypothalamus or other areas of the brain. Hypothalamic damage was postulated to be the etiology of the obesity after infection with canine distemper virus and borna disease virus[5–7,10] However, the initial report of canine distemper did not observe any hypothalamic damage,[4] and subsequent reports demonstrated that the timing of the sacrifice and examination of the brains was critical in demonstrating the presence of virus in the hypothalamus.[5–7] We used only one time point, 13 weeks after infection in chickens. It is possible that we missed the critical period, and more studies are needed with sacrifice at intervals after infection.

In addition to lesions of the brain, abnormalities of peripheral mechanisms may be postulated to produce obesity. Ad-36 DNA was detected by capillary electrophoresis in adipose tissue at 5 weeks and at 13 weeks after initial infection in chickens. The paradoxical reduction of serum cholesterol and triglycerides seems unlikely to be due to a central mechanism, but would support the postulate of an abnormality in adipose tissue. The presence of adiposity and the presence of the lower serum lipids appear to be linked and, if so, future research probably should focus on peripheral mechanisms. The liver would be the next obvious organ to investigate for a possible role in the observed syndrome. Conversely, it is also possible that the two conditions may not be linked, but may operate through separate mechanisms. It is unlikely that the reductions are due to non-specific mechanisms such as increased cytokines. For example, gamma-interferon can reduce serum cholesterol,[21] but such reductions are transient and are usually accompanied by increased, rather than decreased, serum triglycerides levels. Recently, we observed increased adipocyte differentiation due to Ad-36 *in vivo* as well as *in vitro*.[22] Three times as many 3T3L-1 preadipocytes differentiated when treated with Ad-36, compared to the uninfected control 3T3l-1 cells. Another human adenovirus, Ad-2, did not show increased differentiation of 3T3L-1 preadipocytes under similar conditions.[22] The effect of Ad-36 on adipocyte differentiation suggests that Ad-36 induces adipocity by targeting adipocytes. Effect of Ad-36 on adipocyte differentiation needs to be further characterized.

This study is the first report to demonstrate that a human virus can produce increased adipose tissue in animals. It is possible that other human adenoviruses may increase adiposity in animals, but the failure of CELO virus to significantly increase adiposity suggests that this property differs among adenoviruses. Regardless of the fact that the mechanism of action as well as the adipogenic potential of other human viruses is unknown, the finding that a human virus induces adiposity in animals is an unusual and potentially significant finding.

There is little information about the epidemiology, pathophysiology and the usual route of infection of Ad-36. Human adenoviruses are associated with infection of the respiratory tract, gastrointestinal tract, and conjunctiva. The initial isolation of Ad-36 from feces of a diabetic child with enteritis suggests that this virus may be spread by the oral–fecal route, but there is insufficient evidence to draw conclusions about other routes of entry.

The finding of low serum cholesterol and triglycerides provides a convenient marker for this syndrome. Patients previously reported to have antibodies to the avian adenovirus, SMAM-1, also had low serum lipids.[12] About 30% of the obese individuals but only 5% of non-obese individuals have antibodies to Ad-36 and the obese antibody positive individuals have significantly lower serum cholesterol and triglycerides.[23] These observations from different experiments, when taken together, suggest that there may be some fundamental property of some adenoviruses that

predisposes infected animals, and perhaps infected people, to obesity and altered serum lipids. Additional research using other adenoviruses is needed.

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