Intracellular Infections Enhance Interleukin-6 and Plasminogen Activator Inhibitor 1 Production by Cocultivated Human Adipocytes and THP-1 Monocytes

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Obesity is associated with a chronic inflammatory state, and adipocyte dysfunction is thought to play a crucial role in this. Infection of adipose tissue may trigger the production of inflammatory cytokines, leading to increased recruitment of macrophages into adipose tissue, which in turn may exacerbate the inflammatory state in obesity. Low-grade inflammation was mimicked in an in vitro coculture model with human adipocytes and THP-1 monocytes. Adipocytes and monocytes were infected with adenovirus, cytomegalovirus (CMV), or influenza A virus. After 48 h, transinfection was evaluated and interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), adiponectin, and plasminogen activator inhibitor 1 (PAI-1) were measured. IL-6 production was upregulated in cocultures of uninfected adipocytes and THP-1 macrophages in a THP-1 cell number-dependent fashion. IL-6 production by CMV-infected adipocytes was increased relative to that of uninfected adipocytes (P < 0.01). IL-6 production by CMV-infected cocultures was 16- to 37-fold higher than that of uninfected adipocytes (P < 0.001). IL-6 production in influenza A virus-infected cocultures was increased 12- to 20-fold (P < 0.05). Only CMV infection increased levels of PAI-1 in cocultures (fourfold; P < 0.05). Soluble factors produced by THP-1 macrophages rather than by adipocytes were responsible for the increased production of IL-6 in cocultures. Infection of cocultivated human adipocytes and THP-1 monocytes with CMV or influenza A virus led to increased production of IL-6 and PAI-1. Thus, infection of adipose tissue evokes an inflammatory response, leading to adipose tissue dysfunction and subsequent overproduction of IL-6 and PAI-1. This may further compound the atherogenic effects of obesity.

Abdominal obesity is an important risk factor for the development of insulin resistance, metabolic syndrome, diabetes mellitus type 2, and atherosclerosis (16, 26). Adiposity is also associated with a state of low-grade chronic inflammation. Adipose tissue is composed of different cell types, including adipocytes and macrophages (7), both of which are involved in lipid storage and cytokine secretion. Inflamed adipose tissue that has been invaded by macrophages produces interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), thereby contributing to the development of insulin resistance and cardiovascular diseases (2, 20, 33, 34). Moreover, the increase in adipose tissue mass may lead to hypoxia, which may stimulate the invasion of T lymphocytes (19) and macrophages (6, 32). Although the trigger for adipose tissue inflammation is not yet known, it may be viral infection. The inflammatory response of adipocytes to infection may stimulate the production of inflammatory cytokines, which in turn may exert paracrine effects on neighboring cells and attract more macrophages into adipose tissue. This further increases the capacity of adipocyte tissue to produce inflammatory mediators (36, 37). In addition, viruses and bacteria can also exert direct atherogenic effects following infection of the vascular wall (15, 22, 23, 25). Although a viral etiology of obesity remains unproven and controversial, adenoviruses may be causally related to obesity, suggesting a role for these pathogens in the etiology of obesity (1, 8–10, 29). Previously, we showed that human adipocytes could be infected by several microorganisms in vitro and that infection led to an increased production of IL-6. These results suggested that viral infections could contribute to the development of type 2 diabetes and atherosclerosis (3). In the present study, we investigated the effects of infection of cocultures of human adipocytes and macrophages by cytomegalovirus (CMV), influenza A virus, and adenovirus subtypes 2 and 36 on the production of inflammatory cytokines and plasminogen activator inhibitor 1 (PAI-1) and whether transinfection of the two types of cells occurred.

MATERIALS AND METHODS

Preparation of virus stocks. Adenovirus subtypes 2 and 36 were propagated on human HEp-2 larynx carcinoma cells (no. 03-108; ATCC CCL 23; Flow Laboratories/Amstelstad BV, Zwanenburg, The Netherlands). The adenovirus 36 isolate was kindly provided by the Department of Microbiology of the Erasmus University Rotterdam, Rotterdam, The Netherlands. Influenza A/H1N1/Netherlands/300/00 virus was propagated on LLC-MK2 rhesus monkey kidney cells (no. 03-200; ATCC CCL7; Flow Laboratories/Amstelstad BV, Zwanenburg, The Netherlands), and CMV was cultured on a human embryonic lung cell line. The nonadapigen adenovirus 2 subtype (control virus for adenovirus 36), influenza A, and CMV strains were clinical isolates from patients with common respiratory infections (Department of Virology, Diakonessen Hospital Utrecht, Utrecht, The Netherlands). Infection of cells in cultures with adenoviruses 2 and 36 and influenza A virus was established by indirect immunofluorescence staining with a pooled specimen screening reagent containing affinity-purified mouse monoclonal antibodies directed against respiratory viruses, among which were adenovirus and influenza A and B viruses (Barlets VRK anti-viral screening reagent; no. B1029-86; Trinity Biotech, Bray, Ireland). CMV infection was identified by

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indirect immunofluorescent staining with anti-CMV immediate-early antigen antibodies (clone E13; no. 11-003; Argene, Varilhes, France). The cytoplasmic (influenza A virus) and nuclei (adenoviruses, CMV, influenza A virus) of infected cells display apple-green fluorescence, whereas the cytoplasts of uninfected cells are red (counterstained with Evans blue). After propagation on the appropriate cell lines in tissue culture bottles, the inoculation materials were harvested, divided into aliquots, and frozen at −80°C. The 50% tissue culture infective dose of each isolate was determined using corresponding cell lines according to the method of Reed and Muench (27).

Cell culture. Adipocyte precursor cells were grown and stimulated to differentiate into mature adipocytes as described previously (3). Briefly, human subcutaneous preadipocytes (SF-3; Zen-Bio, Inc./Tebu-Bio BV, Heerhugowaard, The Netherlands) were seeded at passage 4 in 24-well plates (Nunc, Roskilde, Denmark) and grown to semiconfluence in preadipocyte medium (PM-1; Zen-Bio, Inc./Tebu-Bio BV, Heerhugowaard, The Netherlands) at 37°C and 5% CO₂. Cells were stimulated to differentiate by replacing PM-1 with differentiation medium (DM-2; Zen-Bio, Inc./Tebu-Bio BV, Heerhugowaard, The Netherlands), which is PM-1 supplemented with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, and a peroxisome proliferator-activated receptor γ agonist. After 1 week, the differentiation medium was replaced with adipocyte medium (AM-1; Zen-Bio, Inc./Tebu-Bio BV, Heerhugowaard, The Netherlands) at 37°C and 5% CO₂. Cells were harvested and frozen at 80°C. The 50% tissue culture infective dose of each isolate was determined using corresponding cell lines according to the method of Reed and Muench (27).

Infection of adipocytes. Cultures containing more than 75% differentiated adipocytes were infected 13 to 15 days after the start of differentiation. All infections were monitored by fluorescent immunostaining, and the severity or grade of infection was determined by counting the number of cells infected, expressed as a proportion of the total cell count. Optimal infection grades, expressed as the multiplicity of infection (MOI), were determined in preliminary experiments with serial dilutions of each stock (data not shown). The rationale for using low MOI values was to mimic chronic infection in vivo and to avoid causing cell death. One day prior to infection, the AM-1 medium was replaced and adipocytes and macrophages were incubated with 100 µl of each virus at an MOI of 0.01. As a negative control, cells were incubated with culture medium and added to freshly cultured cells, and 48 h later IL-6 was measured in duplicate. Optical density was measured with an automated plate reader (ElbioPath; BioTek Instruments, Inc.) at 450/630 nm, and data were processed with analytical curve-fitting software (KC Junior; BioTek Instruments, Inc.).

RESULTS

Infection of adipocytes and THP-1 cells. The percentages of infected adipocytes after 48 h of incubation with adenoviruses 2 and 36, CMV, and influenza A virus were 1.4% ± 0.2%, 2.8% ± 0.3%, 2.5% ± 0.1%, and 0.2% ± 0.1%, respectively. The percentages of infected THP-1 cells after 48 h of incubation with adenovirus 36, CMV, and influenza A virus were 4.8% ± 0.8%, 5.2% ± 0.4%, and 4.0% ± 0.6%, respectively. THP-1 cells were refractory to adenovirus 2. Transfection occurred from adipocytes to THP-1 cells and vice versa. The addition of THP-1 cells to infected adipocytes resulted in infection of THP-1 cells by adenovirus 36 (4.6% ± 1.0%), CMV (1.8% ± 0.7%), and influenza A virus (0.2% ± 0.1%) (Fig. 1); adenovirus 2 infection did not proceed to infect THP-1 cells. The addition of uninfected adipocytes to infected THP-1 cells led to subsequent infection of adipocytes by adenovirus 36 (1.4% ± 0.3%), CMV (1.4% ± 0.5%), and influenza A virus (0.2% ± 0.1%) (Table 1).

Cytotoxicity. Viral infection of cocultures did not evoke a substantial cytotoxic reaction, as determined by the release of LDH into the culture medium. Overall, cytotoxicity in infected cell cultures relative to uninfected (co)cultures varied between 0% and 7.4%. In cell cultures incubated for 48 h with 1 ng/ml IL-1, cytotoxicity was maximally 11.3%.

PAI-1 production in adipocyte/THP-1 cocultures. After 48 h, PAI-1 production by uninfected adipocytes was 199.8 ± 82.6 ng/ml. Neither uninfected THP-1 monocyes nor virus-infected THP-1 cells alone produced PAI-1 (<2 pg/ml) (data not shown). In uninfected cocultures with high THP-1 levels, PAI-1 production increased but was not significant (406 ± 74.9 ng/ml; P > 0.05). There were no significant effects of infection on PAI-1 production by adipocytes, THP-1 cells, or cocultures except in the cocultures infected with CMV and a high THP-1 cell/adipocyte ratio (788 ± 167.3 ng/ml; P < 0.05) (Fig. 2). Considering the fact that THP-1 cells did not produce PAI-1, it seems obvious that adipocytes were accountable for the increased PAI-1 production,
induced by CMV infection in combination with a high number of THP-1 cells.

Adiponectin and TNF-α production in adipocyte/THP-1 cocultures. Adiponectin production by uninfected adipocytes was 13,917 ± 850 pg/ml after 48 h. As expected, THP-1 monocytes did not produce adiponectin. Adiponectin production by adipocytes and monocytes alone or in coculture was not affected by infection (data not shown). TNF-α production by uninfected adipocytes was 24 ± 6.3 pg/ml; it was 5 ± 1.9 pg/ml by uninfected THP-1 monocytes. TNF-α production by adipocytes and monocytes alone or in coculture was not affected by infection (data not shown).

IL-6 production in THP-1 cultures. After 48 h, uninfected THP-1 cells did not produce IL-6 and neither did adenovirus 2- and 36-infected THP-1. CMV-infected THP produced IL-6 only marginally as follows: 40 ± 6 pg/ml, 45 ± 1 pg/ml, and 43 ± 1 pg/ml by 2,500, 12,500, and 50,000 THP-1 cells, respectively. IL-6 production by influenza virus-infected THP-1 was 558 ± 128 pg/ml, 627 ± 2 pg/ml, and 551 ± 43 pg/ml by 2,500, 12,500, and 50,000 THP-1 cells, respectively.

IL-6 production in adipocyte cultures. IL-6 production by adipocytes infected with adenoviruses 2 (148 ± 59 pg/ml; P > 0.05) and 36 (133 ± 67 pg/ml; P > 0.05) was similar to that of uninfected adipocytes (210 ± 49 pg/ml). Although IL-6 production by CMV- and influenza virus-infected adipocytes increased to 2,877 ± 1,130 pg/ml and 1,559 ± 442 pg/ml, this tendency was not statistically significant (both had P values of >0.05 compared to uninfected adipocytes).

IL-6 production in adipocyte/THP-1 cocultures. IL-6 production in uninfected cocultures with high THP-1 cell/adipocyte ratios (1:2.5) was 2,004 ± 882 pg/ml (P < 0.05) compared to IL-6 production in uninfected adipocytes (210 ± 49 pg/ml). IL-6 production by adenovirus-infected cocultures with high THP-1 cell levels (THP cell/adipocyte ratio, 1:2.5) was higher, but not significantly so, than that in uninfected cocultures as follows: 2,400 ± 1,075 pg/ml (adenovirus 2) and 2,608 ± 1,056 pg/ml (adenovirus 36) versus 2,004 ± 882 pg/ml (uninfected; both with P values of >0.05). In contrast, IL-6 production was significantly increased in CMV-infected cocultures of adipocytes and THP-1 cells (3,345 ± 715 pg/ml with a P value of <0.01 [ratio, 1:50]; 3,838 ± 430 pg/ml with a P value of <0.01 [ratio, 1:12.5]; and 7,768 ± 1,073 pg/ml with a P value of <0.001 [ratio, 1:2.5]). Influenza A virus infection increased only IL-6 production in cocultures with high THP-1 levels (4,081 ± 1,073 pg/ml; P < 0.05) (Fig. 3).

The increased IL-6 production in these cocultures could be attributable to either adipocytes or THP-1 cells. To determine the relative contribution of THP-1 cells versus adipocytes to increased IL-6 levels in cocultures, fresh adipocytes and THP-1 cells were incubated with medium recovered from previously infected THP-1 cells or adipocytes (conditioned medium).

IL-6 production in cocultures after incubation with conditioned media. In Table 2, ratios are given for the relative contribution of THP-1 cells versus adipocytes to increased IL-6 levels in cocultures, fresh adipocytes and THP-1 cells were incubated with medium recovered from previously infected THP-1 cells or adipocytes (conditioned medium).
in the conditioned medium of CMV-infected adipocytes was 870 ± 238 pg/mL, and it was 370 ± 95 pg/mL in influenza A virus-infected adipocyte-conditioned medium. After a 48-hour incubation of THP-1 cells with adipocyte CMV-conditioned medium, the measured IL-6 concentration in the culture medium was 1,762 ± 1,042 pg/mL (P = 0.36; ratio, 2.0); it was 550 ± 177 pg/mL (P = 0.33; ratio, 1.4) after incubation of THP-1 cells with influenza A virus-infected adipocyte-conditioned medium (Table 2).

The IL-6 concentration in the conditioned medium of CMV-infected THP-1 cells was 11 ± 2 pg/mL, and it was 117 ± 11 pg/mL in influenza A virus-infected THP-1-conditioned medium. After a 48-hour incubation of adipocytes with THP-1 CMV-conditioned medium, the measured IL-6 concentration in the culture medium was 1,038 ± 204 pg/mL (P = 0.01; ratio, 76.5); it was 639 ± 149 pg/mL (P = 0.03; ratio, 3.8) after incubation of THP-1 cells with influenza A virus-infected adipocyte-conditioned medium (Table 2). Conditioned medium from adenovirus 2- or 36-infected adipocytes and THP-1 cells did not induce any changes in IL-6 production.

Immunofluorescence on cells that were incubated with the respective conditioned media did not demonstrate any viable virus, ruling out the possibility that viral presence could have accounted for the observed effects.

**FIG. 2.** PAI-1 production by infected adipocytes and THP-1 cell cocultures. Y axis, PAI-1 production in nanograms/milliliters. Shown are control adipocytes without THP-1 cells (hatched bars), control THP-1 cells without adipocytes (white bars), and adipocytes cocultured with THP-1 cells. The number of THP-1 cells added to 125,000 adipocytes per well and the THP-1 cell/adipocyte ratio (between brackets) were as follows: 2,500 (ratio, 1:50) (light gray bars), 10,000 (ratio, 1:12.5) (dark gray bars), and 50,000 (ratio, 1:2.5) (black bars). X axis, adipocytes cocultivated with THP-1 cells and infected with adenovirus type 2 (AD2), adenovirus type 36 (AD36), CMV, and influenza A virus (INF A) as well as uninfected adipocytes (Uninfected). *, P < 0.05.

**FIG. 3.** IL-6 production by infected adipocytes and THP-1 cell cocultures. Y axis, IL-6 production in picograms/milliliters. Shown are control adipocytes without THP-1 cells (hatched bars), control THP-1 cells without adipocytes (white bars), and adipocytes cocultured with THP-1 cells. The number of THP-1 cells added to 125,000 adipocytes per well and the THP-1 cell/adipocyte ratio (between brackets) were as follows: 2,500 (ratio, 1:50) (light gray bars), 10,000 (ratio, 1:12.5) (dark gray bars), and 50,000 (ratio, 1:2.5) (black bars). X axis, adipocytes cocultivated with THP-1 cells and infected with adenovirus type 2 (AD2), adenovirus type 36 (AD36), CMV, and influenza A virus (INF A) as well as uninfected adipocytes (Uninfected). #, a P value of <0.05 compared to uninfected adipocytes; *, P < 0.05; **, P < 0.01; ***, a P value of <0.001 compared to uninfected (co)cultures.
Activated PAI-1 levels. which adipocytes in vitro can be stimulated to produce elevated PAI-1 levels.

Monocytes do not constitutively produce PAI-1. Nevertheless, IL-6 production may be explained by the ability of infected adipocytes to become insulin resistant but also enhances the hepatic production of various (proatherogenic) chemokines and other factors, including angiotensin, free fatty acids, leptin, and PAI-1, by adipose tissue may contribute to the pathogenesis of these diseases. In the present study, we showed that both human adipocytes and THP-1 monocytes can be infected by adenovirus 36, CMV, and influenza A virus. Adipocytes are also susceptible to adenovirus type 2, but THP-1 monocytes are refractory to this virus type. The percentage of infection in monocytes by adenovirus 36 and CMV was twofold higher than that in adipocytes. This difference was even more pronounced for influenza A virus, with virus infecting more THP-1 cells than adipocytes (4.0% ± 0.6% in THP-1 cells versus 0.2% ± 0.1% in adipocytes). Infected adipocytes transmitted the infection to monocytes and vice versa (transinfection). In general, the rate of transinfection was 50% of that of direct infection.

In this study, infections with CMV and influenza virus evoke an inflammatory response in cocultivated adipocytes and monocytes as measured by enhanced IL-6 and PAI-1 production. Circulating TNF-α and IL-6 plasma concentrations are mildly elevated in obesity (28). IL-6 not only predisposes cells to become insulin resistant but also enhances the hepatic production of acute-phase proteins, such as C-reactive protein or fibrinogen (12, 24). Therefore, obesity may represent a state of chronic low-grade inflammation accompanied by adipocyte dysfunction, as reflected by enhanced production of IL-6. This may also provide a link between obesity and the development of associated vascular complications such as atherosclerosis (4, 18, 21). Previously, we showed that virus-infected adipocytes stimulated IL-6 production (3). In the present study, infection with CMV and influenza virus further accelerates IL-6 production in cocultures of adipocytes and THP-1 cells. This extensive IL-6 production may be explained by the ability of infected adipocytes as well as monocytes to produce IL-6 and that both infection and monocyte levels significantly contributed to this.

Our results are consistent with those of recent studies with animal cell models. In a coculture model with murine 3T3-L1 adipocytes and murine macrophage-like cell line RAW264 (peritoneal macrophages), the production of IL-6, TNF-α, and monocyte chemotactic protein 1 was upregulated, whereas that of adiponectin was downregulated (30). Also, the duration of coculture influences the increase in TNF-α mRNA expression in cocultures of 3T3-L1 and THP-1 (31). Incubation with bacterial lipopolysaccharide in a coculture of 3T3-L1 and RAW264 did not influence TNF-α production but increased IL-6 production 100-fold, suggesting an exaggerated biological interaction between macrophages and adipocytes in persistent low-grade infection by gram-negative bacteria (35).

The increased production of IL-6 in cocultures may be mediated by cell-cell contact because the production of IL-6 increased with the number of THP-1 monocytes added to the cocultures. However, it is more likely that locally produced inflammatory substances produced by dysfunctional adipocytes and adipose tissue macrophages exerted autocrine/paracrine effects on neighboring cells. Conditioned medium from uninfected or virus-infected adipocytes did not stimulate IL-6 production by THP-1 cells or cocultures, whereas incubation of adipocytes and cocultures with conditioned medium from CMV-infected THP-1 cells led to a nearly 80-fold increase in IL-6 production. Infective virus was not detected in the conditioned medium, indicating that there was no lytic infection and that de novo IL-6 synthesis was not a direct effect of infection. Moreover, this suggests that the observed effects depended on cross-talk between cells that was orchestrated by soluble factors produced by infected cells in the culture environment. This is consistent with the observation that the release of inflammatory cytokines in adipose tissue samples from obese subjects is due mainly to the production of cytokines by cells other than adipocytes (13). Adipose tissue macrophages are able to produce extensive amounts of proinflammatory mediators, which could contribute to the development of insulin resistance (36). Here, we conclude that the paracrine effects of soluble factors (such as IL-6 or TNF-α) released by infected macrophages, rather than by infected adipocytes, were responsible for the increased production of IL-6 in cocultures.

The transinfection phenomenon we demonstrated may explain how infectious agents invade several parts of the body and infect cells of different organ systems, such as vessel walls and TABLE 2. IL-6 production after incubation with conditioned medium

| Type of infection | AD-CM THP | AD-CM coculture | THP-CM adipocytes | THP-CM coculture |
|-------------------|-----------|-----------------|------------------|-----------------|
| None              | 59 ± 12 (0.6) | 361 ± 4 (0.4) | 229 ± 38 (2.4) | 382 ± 65 (2.5) |
| Ad 2              | 66 ± 14 (1.3) | 310 ± 27 (1.0) | 266 ± 62 (4.7) | 388 ± 71 (2.9) |
| Ad 36             | 73 ± 11 (1.6) | 364 ± 31 (0.8) | 242 ± 41 (8.6) | 365 ± 22 (4)  |
| CMV               | 1,762 ± 1,042 (2) | 1,490 ± 583 (1.3) | 1,038 ± 204 (76.5) | 1,224 ± 509 (79.5) |
| INF A             | 550 ± 177 (1.4) | 772 ± 79 (1.2) | 639 ± 149 (3.8) | 802 ± 191 (3.9) |

Mean IL-6 concn ± SE (ratio)\(^{ab}\)  

\(^{a}\) IL-6 concentrations (mean ± standard error in picograms/milliliter; \(n = 2\)) after incubation with conditioned medium from adipocytes and THP-1 cells. Ad 2, adenovirus subtype 2; Ad 36, adenovirus subtype 36; INF A, influenza A virus; AD-CM THP, adipocyte-conditioned medium incubated with THP-1 cells; AD-CM coculture, adipocyte-conditioned medium added to cocultures; THP-CM adipocytes, THP-1 cell-conditioned medium added to adipocytes; THP-CM coculture, THP-1 cell-conditioned medium added to cocultures of adipocytes and THP-1 cells. Ratio, de novo IL-6 synthesis expressed as ratio of measured IL-6 concentrations against IL-6 present in the conditioned medium used for incubation after subtraction of baseline values.  

\(^{b}\) Statistically significant (\(P < 0.05\)) increase in de novo IL-6 production.
adipose tissue, thus contributing to local and systemic inflammatory states.

Microorganisms such as CMV or influenza A virus that infect adipose tissue may initiate and perpetuate a chronic inflammatory response, attracting more macrophages into the already inflamed tissue and exacerbating the total inflammatory response to the infection. The results of the present study further support the concept that both adipocytes and adipose tissue macrophages are involved in adipokine production and that their concerted actions synergistically contribute to increased production of IL-6 and PAI-1, reflecting the inflammatory state of adipose tissue. It is also conceivable that infected monocytes invade adipose tissue.

In conclusion, adipocytes and monocytes can become infected by several viruses in vitro, leading to an inflammatory response. Transfection occurs from adipocytes to monocytes and vice versa. The relative contribution of monocytes to the production of IL-6 in cocultures is likely to be greater than that of adipocytes.

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