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ORIGINAL RESEARCH

Regulation of adiponectin in adipocytes upon exposure to HIV-1

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Objectives

Adipose dysregulation, dyslipidemia, and insulin resistance are hallmarks of HIV-related lipodystrophy. The precise mechanisms behind these disturbances are unknown. In HIV-infected patients, we previously demonstrated a strong relationship between lipodystrophy and levels of adiponectin, an adipose peptide implicated in regulation of glucose and lipid metabolisms. In this study we investigated the effect of HIV on adipocytes, to determine whether HIV can directly infect adipocytes and/or alter the regulation and secretion of the adipocyte-derived hormone adiponectin.

Methods

Human subcutaneous preadipocytes and adipocytes were exposed to HIV-1 under various conditions. Adiponectin was measured in supernatants and cell lysates.

Results

Although adipocytes expressed CD4, the major HIV receptor, they could not be infected *in vitro*. However, exposure to HIV dramatically increased the secretion of adiponectin from human adipocytes, in the absence of infection. This was exacerbated with sustained exposure to HIV in a transwell assay. Further, human peripheral mononuclear cells also produced adiponectin, but this was largely dependent upon T-cell activation.

Conclusions

We propose that the stimulation of adiponectin production by HIV can perturb adiponectin regulation, leading to substantially decreased levels upon viral suppression by antiretroviral therapy. These data suggest a potential molecular mechanism of adiponectin regulation in HIV-infected patients.

Keywords: adipocyte, adiponectin, HAART, HIV, insulin resistance, lipodystrophy

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Introduction

HIV-related lipodystrophy is a fat dysregulation syndrome in HIV-infected patients, particularly those undergoing highly active antiretroviral therapy (HAART), characterized by accumulation of visceral and dorso-cervical adipose tissue, breast enlargement and accompanying loss of facial and limb subcutaneous fat [1–6]. This syndrome also involves metabolic pathologies such as dyslipidemia and severe insulin resistance leading to type 2 diabetes and potentially to cardiovascular complications. HIV-related lipodystrophy has become a major cause of concern in HAART as it represents a significant source of morbidity resulting from diabetes and heart disease and also negatively impacts on patients' quality of life. However, the exact cause of HIV-related lipodystrophy remains unclear. Lipodystrophy is often regarded as toxicity attributed to various antiretroviral drugs used in the

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treatment of HIV infection. In particular, certain classes of drugs such as the protease inhibitors are associated more frequently with HIV-related lipodystrophy. Anti-HIV drugs have been implicated in adipose tissue redistribution [2,3], adipocyte differentiation and metabolism [7–9], glucose homeostasis [10,11] and dyslipidemia [12]. The direct effect of drugs used in HAART is supported by data obtained from cultured adipocytes [7,9,13,14] and hepatocytes [15], but HIV-related lipodystrophy may also occur, albeit rarely, in treatment-naive patients. It is therefore possible that the virus itself may also be involved in the pathogenesis of HIV-related lipodystrophy and that viral suppression exacerbates the syndrome.

We [16] and others [17–20] recently showed that adiponectin, an adipose-derived regulator of glucose and lipid metabolisms, was significantly reduced in HIV-infected patients with lipodystrophy but not in otherwise matched HIV-infected patients. This, together with data suggesting a possible infection of adipocytes by HIV [21], led us to investigate the direct role that HIV might have in targeting adipocytes and regulating adiponectin secretion.

In this study, we investigated the effect of HIV on adipocytes, to determine whether HIV can directly infect adipocytes and/or alter the regulation and secretion of the adipocyte-derived hormone adiponectin. We have demonstrated that HIV stimulates the secretion of adiponectin from human adipocytes, although this effect does not result from direct infection of adipocytes by the virus. We have also demonstrated that peripheral blood mononuclear cells produce adiponectin, and its production is largely dependent upon activation. Our data suggest that HIV significantly induces adiponectin production but possibly in a reciprocal fashion in different cells. We propose that this might severely affect adiponectin endocrine regulation and that, when the virus is suppressed by HAART, adiponectin levels can no longer be physiologically sustained. This could potentially contribute to the HIV-related lipodystrophy syndrome commonly observed in patients undergoing HAART.

Materials and methods

Cell culture and viruses

Human subcutaneous preadipocytes and adipocytes were purchased from Zen-Bio (Research Triangle Park, NC, USA). To assay for infection, cells were treated with phytohemaglutinin A (PHA) (5 μg/mL) plus interleukin (IL)-2 (50 U/mL), IL-2 alone (50 U/mL), IL-6 (1 ng/mL) plus tumor necrosis factor α (TNF-α) (2 ng/mL) or medium alone for 2 days prior to viral inoculation. PHA was washed out prior to infection. Maintenance medium, AM-1 and PM-1 for adipocytes and preadipocytes, respectively, provided by the supplier of the cells (Zen-Bio), was supplemented with the cytokines used in pretreatment.

In an additional experiment, peripheral blood mononuclear cells (PBMCs) from normal blood donors were cultured in the upper chamber of a *trans*-well system. They supported HIV replication for the duration of the culture. In this case, the 2-day pretreatment was PHA plus IL-2 and cultures were maintained with medium AM-1 and PM-1 for adipocytes and preadipocytes, respectively, augmented with IL-2 (50 U/mL) and up to 20% fetal bovine serum (FBS).

All viral stocks were propagated in PBMCs from normal donors, harvested at the peak of infection, and stored at −75 °C. Infectivity titers were determined with serial 4-fold dilutions in PBMCs from one donor. Cell cultures were inoculated with 6000 tissue culture infectious dose 50 ($TCID_{50}$) of HIV-1JRCSF (CCR5-using strain) or HIV-1IIIB (CXCR4-using strain) marking day 0 of the experiment [22]. On day 2, cells were washed 10 times with a total of 20 times the culture volume. Cultures were followed for up to 15 days to detect infection. HIV-1 replication was assessed by p24 enzyme-linked immunosorbent assay (ELISA) in culture supernatants and by PCR using the cell lysate at the end of the culture, as previously described [22,23]. Media and cell lysates were also harvested and tested for adiponectin expression every 2 or 3 days for the duration of the culture. Human PBMCs were PHA-stimulated in the presence of 50 U/mL IL-2 for 2 days and cultured for 10 days with or without infection with the two strains of HIV-1. Supernatants were assayed as for the adipocytes/preadipocytes cultures.

RT–PCR

Human adipose tissue RNA and adipocyte and preadipocyte RNA were kindly provided by Drs Peter Arner (Karolinska Institute, Stockholm, Sweden) and Steven O'Rahilly (Cambridge University, Cambridge, UK). Synthesis of cDNA was performed with a cDNA cycle kit (Invitrogen, Carlsbad, CA, USA) and PCR was performed with the following primer sets:

CD4 (5′-TGCTGCCAACTCTGACACC-3′, 5′-CACCTGTTCCCCCTCTTTCT-3′)

CCR5 (5′-CTGTCGTCCATGCTGTGTTT-3′, 5′-TCCTGGAAGGTGTTCAGGAG-3′)

CXCR4 (5′-TTACCATGGAGGGGATCAGT-3′, 5′-TCGGTGATGGAAATCCACTT-3′)

Adiponectin measurements

Adiponectin levels in cell lysates and supernatants were compared using quantitative immunoblot analyses. For this, 5 μL of samples was resolved with 10% sodium-

dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Adiponectin levels were detected by a rabbit anti-human adiponectin antibody (a gift from Dr Philipp E. Sherer, Albert Einstein College of Medicine, New York, NY, USA) and ^{125}I-labeled anti-rabbit immunoglobulin G (IgG) secondary antibody (NEN, Boston, MA, USA), and quantified by a phosphoimager (Bio-Rad, Hercules, CA). For analysis of the PBMCs, the adiponectin levels were measured using a Radio-Immuno-Assay (RIA) kit (Linco Research, Inc., St Charles, MO, USA) as previously described.

Results

Expression of HIV receptors in adipocytes

We examined the expression of the HIV receptor and HIV coreceptors in human adipose tissue samples as well as in cultured human preadipocytes and adipocytes. As shown in Fig. 1a, RT-PCR analysis revealed the presence of CD4 mRNA expression in human adipose tissue. In cultured primary cells, CD4 mRNA expression was readily detectable in isolated human mature adipocytes and in human preadipocytes. The levels of CD4 expression in adipose tissue were comparable to those observed in Jurkat cells. However, we failed to detect the expression of CCR5 or CXCR4 in human adipose tissue or cultured human adipocytes or preadipocytes (Fig. 1b). This is in contrast to previous studies [21,24], which have reported CCR5 expression in human adipose tissue. In mouse adipocytes and preadipocytes, both CD4 and CCR5 expression were clearly evident in differentiated adipocytes but were quite low in preadipocytes (data not shown).

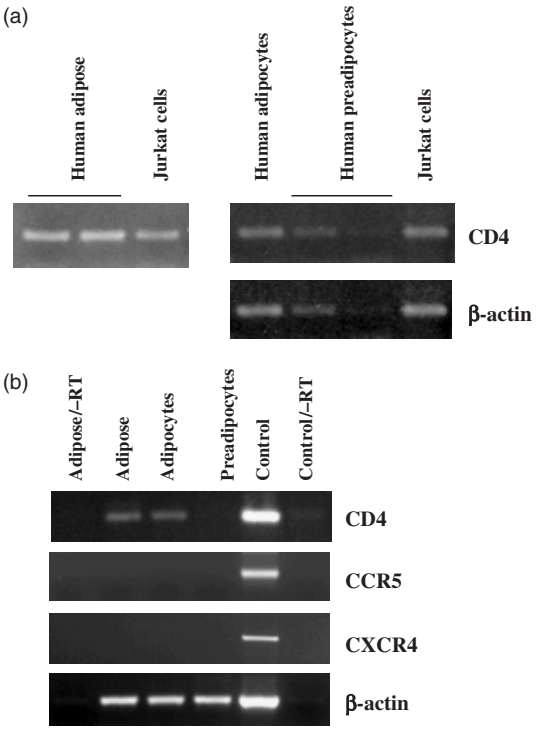

Fig. 1 Detection of CD4, CCR5 and CXCR4 mRNA expression in human adipose tissue, isolated adipocytes and preadipocytes by reverse transcriptase–polymerase chain reaction (RT-PCR). (a) Jurkat cells were used as positive controls for CD4 expression. β-actin was used as a control gene for the RT-PCR. Reverse transcriptase-negative (−RT) samples were used as negative controls. (b) U87CD4/CCR5 cells were used as positive controls for CD4 and CCR5 expression. The Jurkat human T-lymphocyte cell line was used as positive control for CXCR4 expression. −RT samples were used as negative controls. β-actin was used as a control gene for the RT-PCR.

Lack of HIV infection of human adipocytes

Given the fact that adipocytes express at least some components of the virus entry system, we tested whether HIV could directly infect human preadipocytes and adipocytes. Cultured primary human preadipocytes and differentiated adipocytes in maintenance medium were inoculated with 6000 $TCID_{50}$ of the JRCSF or IIIB strain of HIV-1 and cultured for up to 15 days. Conditioned media were collected at days 2, 4, 7, 9, 11 and 14 to detect the presence of p24 antigen by ELISA. Proviral DNA integration into the host genome by PCR analysis was assayed on cellular extracts at day 15. Based on these two assays, no evidence for HIV entry or amplification could be detected in either preadipocytes or adipocytes.

We further assayed infection in cultured primary human preadipocytes and adipocytes pretreated with IL-2, TNF-α and IL-6 or PHA and IL-2 with the same strains of HIV-1. Again, no evidence for HIV entry or amplification could be detected in either cell type. In duplicate cultures, activated PBMCs were cocultivated with the adipocytes or preadipocytes after day 15 to attempt to rescue/amplify low-level virus replication that might have been present but undetected. Still, no virus could be detected with the cocultivated PMBC by p24 antigen or proviral DNA PCR.

All these experiments demonstrated that, at least under the experimental conditions used in this study, HIV-1 cannot productively infect human adipocytes or preadipocytes.

The effect of HIV virus on human adipocytes

We sought to test whether the presence of HIV itself could affect the function of adipocytes by engaging the cell surface receptors. For this we first evaluated the capacity of

preadipocytes to differentiate into mature adipocytes upon exposure to HIV at different times during the differentiation process. We did not detect any change in growth rate, lipid accumulation, or the robustness of the differentiated phenotype, as judged by gross morphology, between control cells and those that were exposed to HIV at any time during the experimental protocol. Hence, these data suggest that the presence of HIV alone does not interfere with the adipogenic capacity of these cells. We also did not observe any gross morphological differences or signs of cytopathic effects in any of the cultures exposed to HIV for the duration of the experiments, i.e. 15 days.

Stimulation of adiponectin production by HIV

Finally, we asked whether HIV exposure alters the hormone production of adipocytes, without infection. For this, we measured cellular adiponectin protein levels and adiponectin secretion from adipocyte and preadipocyte cultures to evaluate potential functional alterations in these cells after exposure to HIV. In differentiated adipocytes, adiponectin was readily detected in both the cell lysates and the conditioned media. In the absence of HIV exposure, TNF-α and IL-6 treatment did not significantly influence cytosolic or secreted adiponectin protein levels (Figs 2a and b). Treatment with PHA and IL-2 resulted in a slight increase in adiponectin protein levels. Interestingly, exposure to both T- and M-tropic HIV-1 isolates dramatically stimulated adiponectin production in these cells (Fig. 2). More strikingly, upon stimulation by PHA and IL-2 and continuous exposure to HIV through infected PBMCs in a transwell system, adiponectin was abundantly produced. Experiments on PBMCs alone treated under the same conditions (PHA and IL-2), as described below, strongly suggest that the source of adiponectin was the adipocytes in this system. Adiponectin secretion from adipocytes was stimulated by HIV exposure even in cells that were not treated by cytokines and/or PHA. These experiments demonstrated that, despite lack of entry, HIV exposure alters the production of adiponectin from human adipocytes.

Production of adiponectin by PBMCs

As adipocytes share common features with cells of myeloid origin, we next asked whether T cells and macrophages express adiponectin and/or regulate its expression. To this purpose, human PBMCs were PHA-stimulated in the presence of IL-2 for 2 days and cultured for a further 9 days. We measured the secretion of adiponectin in the conditioned media by RIA. As shown in Fig. 3a, there was a temporary increase of adiponectin production until day 2 after PHA and IL-2 stimulation. However, the adiponectin

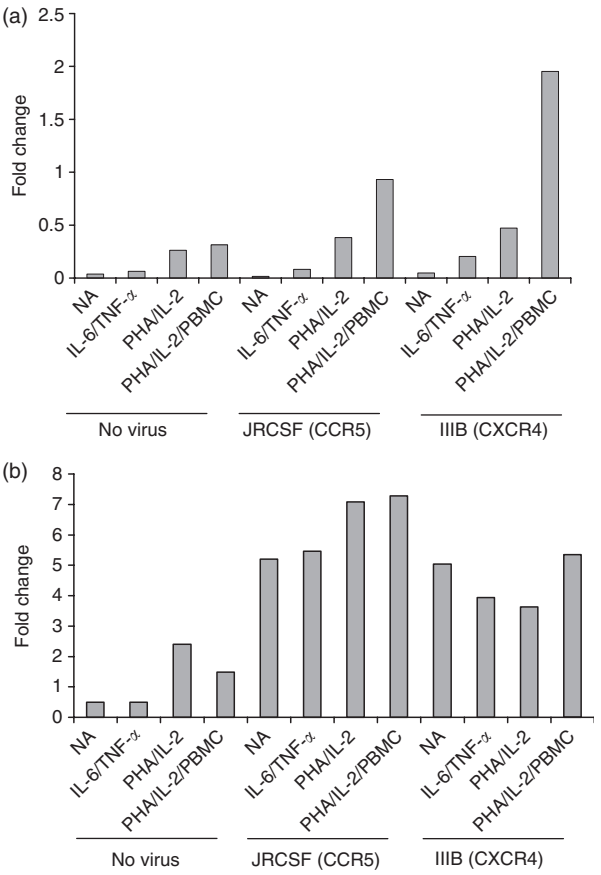

Fig. 2 Effects of cytokines and HIV on the adiponectin production of human adipocytes. Human subcutaneous adipocytes were incubated with CCR5 or CXCR4 viruses for up to 15 days, following pretreatment with various cytokines [interleukin (IL)-6 plus tumor necrosis factor α (TNF-α), or phytohemaglutinin A (PHA) plus IL-2] or with peripheral blood mononuclear cells (PBMCs) in transwells for 2 days. Adipocyte lysate (a) and adipocyte conditioned media (b) were harvested and tested for adiponectin by western blot analysis. The results were quantified with a phosphoimager and expressed as fold change compared with the basal culturing conditions without cytokines and virus exposure. NA, nothing added.

levels decreased later. We next examined the effects of HIV infection in primary PBMCs. Infection by either the JRCSF or the IIIB strain of HIV-1 did not increase adiponectin production in these cells (data not shown). These experiments demonstrated that, as in adipocytes, adiponectin is also expressed by lymphocytes, but unlike in adipocytes it is not stimulated by HIV infection. This indicates that PBMCs are not the source of the burst of adiponectin seen in the transwell experiment described above.

Discussion

In this study we investigated the regulation of adiponectin by HIV in adipocytes and lymphocytes. Despite the absence

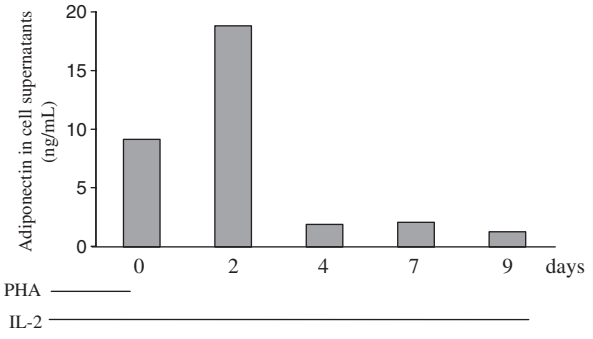

Fig. 3 Effects of cytokines and HIV on T-cell adiponectin production. Human peripheral blood mononuclear cells (PBMCs) were phytohemaglutinin A (PHA)-stimulated in the presence of 50 U/mL interleukin (IL)-2 for 2 days and cultured in IL-2-containing medium for a further 9 days. Media were harvested at various time-points and measured for adiponectin by Radio-Immuno-Assay (RIA) analysis.

of productive infection, HIV-1 induced adiponectin production by adipocytes under all conditions assayed.

Here, we confirmed the presence of at least one HIV receptor in human adipocytes. However, despite the presence of receptors, we were unable to show evidence of HIV replication in either human adipocytes or preadipocytes under the experimental conditions used. This is in contrast to a previously published study suggesting infection of adipocytes by HIV-1 [21]. In that study the authors were able to demonstrate a weak infection or a capture of viral particles by adipocytes and preadipocytes. Core antigen measured in the culture supernatant peaked within 48 h and rapidly became undetectable. Low levels of infectious virus could be detected with an indicator cell line up to 10 days after infection. In a more recent study by the same authors, HIV infection could not be established in primary adipose cells *in vitro* [25]. Another study looked at signs of HIV infection in adipose tissue of HIV-infected individuals with HIV-related lipodystrophy and failed to find infected adipocytes [26]. Collectively, these data suggest that preadipocytes and adipocytes do not readily support sustained and productive infection by HIV-1.

Nonetheless, our data illustrated the striking impact of HIV on human adipocytes through regulation of a metabolically significant hormone, adiponectin, even in the absence of productive infection of these cells. In the presence of virus, human adipocytes secreted high levels of adiponectin and this occurred at a similar rate with both T- and M-tropic HIV-1 isolates. Although it is not clear how HIV influences the production or secretion of adiponectin, we would postulate that the binding of HIV to the surface receptors on adipocytes might elicit a signal that leads to the alteration of adiponectin production. The level of increase of adiponectin in supernatants after 15 days was significantly higher than the level of increase in cell lysates, suggesting that adiponectin is readily secreted and accumulates in supernatant. The lack of significant cell death and alteration in the assay indicates that adiponectin in the media does not originate from the dead cells.

Previous studies have not reported adiponectin expression in cells other than adipocytes. In this study, we demonstrated that adiponectin is also expressed by PBMCs and is acutely regulated in the activation state. HIV infection did not augment adiponectin expression in these cells. Hence we observed a differential regulation of adiponectin in two critical cells types, lymphocytes and adipocytes, *in vitro*. The degree to which this differential regulation of adiponectin results in altered systemic concentrations of adiponectin under physiological or pathological conditions is still unclear.

We have previously hypothesized that abnormal regulation of adiponectin may contribute to the metabolic abnormalities in HIV-infected patients [16]. We showed that adiponectin levels were inversely correlated with HIV-related lipodystrophy. Circulating adiponectin concentrations were reduced in HIV-infected patients with fat redistribution compared with age- and body mass index-matched HIV-infected patients without fat redistribution or healthy subjects. These data suggested that changes in adiponectin contributed to the lipodystrophy syndrome. Alternatively, the possibility cannot be excluded that changes in adiponectin could be a result of changes in fat levels.

Our data suggest a model to potentially explain the differential regulation of adiponectin with institution of HAART and its contribution to the development of lipodystrophy. Early in HIV infection, the presence of HIV may stimulate adiponectin production from adipocytes. At this stage, systematic adiponectin levels may remain stable or even increase. HIV might become the main trigger for adiponectin production during this phase. The reduction in circulating adiponectin in HIV-infected patients with lipodystrophy develops during a later stage of the disease process. This might follow institution of HAART with suppression of viral load and removal of the adiponectin stimulus. However, it has to be noted that no acute change in adiponectin following acute treatment of HIV was observed in our previous study [16], suggesting that a prolonged infection with sustained viral load might be necessary to affect adiponectin production. The dysregulation of adiponectin in HIV infection can therefore be a contributing factor in HIV-related lipodystrophy. This is combined with adipose tissue dysregulation exacerbated by adiponectin deficiency and subsequent metabolic pathologies. Such a hypothesis remains to be tested in

longitudinal studies in patients infected by HIV and subsequently undergoing HAART, as lipodystrophy is a complex syndrome, the etiology of which is likely to be multifactorial, with contributions from drug type, age, sex, ethnicity, race and, as proposed here, viral factors. In particular, the role of drugs themselves in adiponectin regulation might be an important factor that remains to be further explored in conjunction with the role of the virus [27,28]. Our study has not definitively addressed the role played by HIV itself in metabolic abnormalities associated with HIV lipodystrophy; nonetheless, we have shown that HIV might play a role in the regulation of adiponectin.

In this study, we investigated the effect of HIV on adipocytes, to determine whether HIV can directly target adipocytes and/or alter the regulation and secretion of the adipocyte-derived hormone adiponectin. We have demonstrated that HIV stimulates the secretion of adiponectin from human adipocytes, although this effect does not result from productive infection of adipocytes by the virus. We also demonstrated that PBMCs produce adiponectin, and that its production is largely dependent upon activation. Our data suggest that HIV significantly induces adiponectin production from adipocytes. We propose that this might severely affect adiponectin endocrine regulation and that, when HIV is precipitously suppressed by HAART, adiponectin levels can no longer be physiologically sustained, therefore contributing to the induction of HIV-related lipodystrophy syndrome commonly observed in patients undergoing HAART. These are the first data to suggest a potential mechanism by which adiponectin is specifically regulated in HIV-infected patients. Further studies investigating the regulation of adiponectin in HIV-infected patients are necessary.

Acknowledgements

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