Immunohistochemical analysis of the expression of TNF-alpha, TGF-beta, and caspase-3 in subcutaneous tissue of patients with HIV Lipodystrophy Syndrome

Sandro Henrique de Souza Dantas Oliveira a,b, Tinara Leila de Souza Aarão b, Leonardo da Silva Barbosa a, Paulo Guilherme Souza Lisbôa a, Claudia Daniele Tavares Dutra c, Lorenza Margalho Sousa a, Juarez Antônio Simões Quaresma b, Rosana Maria Feio Libonati a,b,*

a Lipodystrophy Clinic, João de Barros Barreto University Hospital, Faculty of Medicine, Federal University of Pará (UFPA), Rua dos Mundurucus 4487, 66073-000 Belém, Pará, Brazil
b Laboratory of Immunology, Center for Tropical Medicine, Federal University of Pará (UFP), Avenida Generalissimo Deodoro 92, 66055-240 Belém, Pará, Brazil
c Lipodystrophy Clinic, João de Barros Barreto University Hospital, Faculty of Nutrition, Federal University of Pará (UFPA), Rua Augusto Corrêa 01, 66075-110 Belém, Pará, Brazil

A R T I C L E   I N F O
Article history:
Received 28 October 2013
Received in revised form 8 February 2014
Accepted 10 February 2014
Available online 28 February 2014

Keywords:
HIV Lipodystrophy Syndrome
Etiology
Tumor necrosis factor alpha
Transforming Growth factor beta
Caspase 3

A B S T R A C T
Introduction: HIV Lipodystrophy Syndrome (HIVLS) is a multifactorial clinical expression that presents alterations in the metabolism and distribution pattern of body fat via immunological changes capable of disrupting homeostasis. This study aimed to analyze the degree of inflammatory, anti-inflammatory, and apoptosis activity in the subcutaneous tissue of patients, based on the expression of Tumor Necrosis Factor-α (TNF-α), Transforming Growth Factor-β (TGF-β), and caspase-3, respectively, and correlate them with clinical data and with each other.

Methods: This is a cross-analytical study. The biopsy of subcutaneous cellular tissue was performed on the right thigh of 19 patients with HIVLS who were attended to at a university hospital, and four people without HIV and lipodystrophy, for comparison. The type of lipodystrophy and the estimation of body fat were obtained during the consultation or obtained from medical charts. The cytokine expression was observed in the adipose tissue through the streptavidin-biotin peroxidase method, and analyzed by optical microscopy.

Results: Despite the mixed clinical form having been prevalent in both genders, men were more lipomatrophic and women were more lipohypertrophic. Men showed higher expression of TNF-α and caspase-3 than women. Patients with lipodystrophy had higher expression of TNF-α and caspase-3 and lower TGF-β, compared to the control group. The percentage of body fat was negatively correlated with the expression of TNF-α and caspase-3. Longer durations of infection and use of antiretroviral therapy (ARTV) were positively associated with the levels of TNF-α. The expression of caspase-3 and TGF-β was associated with higher levels of TNF-α.

Conclusion: Regardless of the clinical form, HIVLS is characterized by a chronic inflammatory process associated with the male gender, the percentage of body fat, and lipodystrophy manifestations. There is increased apoptotic activity in more inflamed tissues and there is correlation between TNF-α and TGF-β, which suggests a possible negative feedback mechanism between the inflammatory and anti-inflammatory activity.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Access to antiretroviral therapy (ARTV) has substantially improved the indicators of morbidity, mortality, quality of life, and life expectancy of patients living with HIV [1,2]. However, ARTV has...
also led to a clinical manifestation characterized by alterations in the metabolism and body distribution of fats, as well as increased risk factors for systemic arterial hypertension, diabetes mellitus, and cardiovascular accidents, despite good viral control [3], the HIV Lipodystrophy Syndrome (HIVLS) [4–6].

HIVLS has a multifactorial etiology involving the HIV infection itself, ARVT, and environmental and personal factors of the patient [5,7,8]. Although not yet fully clarified, the physiopathogenic mechanisms proposed suggest a stimulation of the immunological inflammatory profile where inflammatory cytokines, especially the Tumor Necrosis Factor Alpha (TNF–α), have important roles [9–11].

In order to aid understanding of the physiopathogenic mechanisms involved in HIVLS, and to understand the biochemical changes involved, the inflammatory, anti-inflammatory, and apoptosis activity in the subcutaneous cellular tissue of patients with HIVLS was analyzed by the immunohistochemical expression of TNF–α, TGF-β, and caspase-3, respectively.

2. Material and methods

2.1. Outline of the study

A cross-analytical study was conducted. The subjects consisted of 19 patients with HIVLS who had been sent for referral services at the Lipodystrophy Clinic of a university hospital in Belém, Pará state in Brazil, and 4 people without HIV and lipodystrophy, as the control group.

2.2. Financing

The study is part of the research project entitled Terapia Antiretroviral e Lipodistrofia: uma abordagem clínico-terapêutica e etiopatogênica (Antiretroviral Therapy and Lipodystrophy: A clinical-therapeutic and etiopathogenic approach), supported by the Fundação de Amparo à Pesquisa do Estado do Pará (Pará State Foundation for Research Support). There were also funds from the Programa de Bolsas de Iniciação Científica (Program for Scientific Initiation Scholarships) through the Federal University of Pará. It was done after approval by the Research Ethics Committee, protocol 068/2006 and 010/2011.

2.3. Participants

The study included 19 patients of both genders who: are more than 18 years of age, have serology positive for HIV, are using ARVT (for at least 2 years), have a clinical manifestation suggestive of lipodystrophy, and who consented to participate in the research. Samples of four people without HIV infection and without lipodystrophy were used as the control group for comparison of immunohistochemical results.

2.4. Characterization of the clinical form

As there is not yet consensus about an objective method for the evaluation of lipodystrophy in clinical practice [12], the diagnosis and classification are done via the subjective complaints by patients regarding body changes perceived from the start of the ARVT and associated with the clinical observation by the examiner during the physical examination.

The patients were clinically diagnosed by physicians and classified into three groups, in accordance with the lipodystrophic manifestations displayed:

- Lipodystrophic form: lipoatrophy in the face, upper limbs, lower limbs, and/or buttocks;
- Lipohypertrophic form: lipoaccumulation in the dorsocevical, breast, and/or abdominal region;
- Mixed form: association between lipoatrophy and lipoaccumulation.

The skinfolds were measured with the aid of a caliper in triplicate, always from the right side of the body and directly on the skin in the following locations: biceps, triceps, subscapular, and suprailiac. The percentage of body fat was estimated from the sum of these skinfolds using the table of values of Durnin and Womersley [13].

2.5. Collection of biopsy

The subcutaneous tissue biopsies were obtained from the proximal inner face of the right thigh of the patients through an incision with a scalpel and local anesthesia. The material obtained was fixed in 10% formalin and sent to the Pathological Anatomy laboratory of the hospital to be dehydrated in increasing concentrations of alcohol, diaphanized in xylene, soaked in paraffin by a Tissue Processor (LUPE, model PT03) and embedded in liquid paraffin at a temperature of 60 °C using a LEICA EG1150H modular tissue embedding center. The paraffin blocks were forwarded to the Immunology Laboratory of the Center for Tropical Medicine, Federal University of Pará, for the immunohistochemical analysis of: TNF–α, for correlation with inflammatory and lipolysis activity; TGF-β, to research anti-inflammatory activity; and caspase-3, for evidence of cellular apoptosis.

2.6. Immunohistochemical processing

The immunohistochemical processing was performed by the streptavidin-biotin peroxidase method, in which 4 mm thick histological sections of paraffinized material obtained in a manual microtome were collected on slides prepared with an adhesive solution of 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO/USA, code A3648) and left in an oven overnight at 60 °C to improve the adhesion of the tissue. Then, they were deparaffinized by xylene bath at room temperature for 20 min and 10 min, respectively, and subsequently hydrated in ethanol in a descending sequence (100%, 90%, 80%, and 70%) for 2 min each and then washed under running water, distilled, and placed in Phosphate Buffered Saline 1× (PBS1×) for 5 min. The blocking of the endogenous peroxidase was done in a dark room with three incubations of 15 min in 3% hydrogen peroxide, and then the slides were again washed with running water, distilled, and placed in PBS1×. Antigen retrieval was performed with a 0.1 M citrate buffer and Tween 20 in a bain-marie in a Pascal Chamber, at 90 °C for 20 min. After being washed again, the sections were permeabilized in a 0.1% saponin solution (Sigma Chemical Co., St. Louis, MO/USA, code S7900) for 10 min, followed by 2 washes in PBS1×. The blocking of specific proteins was performed with a solution of skimmed milk (Molico, Nestlé®) for 30 min at room temperature. The next step consisted of incubation of the sections with the primary antibodies for each cytokine, diluted in a solution of 1% bovine albumin fraction V (BSA 1%) (SERVA code 11930) in a humid dark chamber and kept overnight at 4 °C. The next day, after two 5 min washes with PBS1×, the incubation was performed with the secondary antibody marked with biotin and then with the streptavidin-peroxidase complex, both for 30 min in a moist chamber dark under greenhouse conditions at 37 °C, interspersed by 2 washes of PBS1×. The sections were exposed to diaminobenzidine chromogen solution (3,3'-diaminobenzidine, DAKO, code K346811-2) for 20 s, washed in running water for 5 min, counterstained with Harris hematoxylin for 1 min, washed in running water and dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, and 100%), each for 2 min.
After drying, the slides were mounted with coverslips using the Entellan® mounting medium.

2.7. Analysis of the slides

The data related to the cytokine expressions were obtained from the average reading of 10 random fields using a ZEISS AXIO IMAGER Z1 optical microscope composed of a binocular microscope and an automatic photographic system with a 400× magnification Axio-cam attached, observing the immunohistochemical response of the caspase-3 (Fig. 1), TGF-β (Fig. 2), and TNF-α (Fig. 3). The expression of TGF-β in the control sample can be seen in Fig. 4.

2.8. Data analysis

The quantitative results of the immunohistochemical analysis and clinical evaluation were stored in electronic spreadsheets using Microsoft Office Excel 2007 software and analyzed with the BioEstat 5.0® software, establishing the alpha rejection level of the null hypothesis at 5% (p ≤ 0.05).

The average of the data was noted along with its standard deviation in the format “average ± standard deviation”. The Mann–Whitney test was used to compare the levels of certain quantitative variables between samples, rejecting or accepting the null hypothesis, in accordance with the “p” values obtained. The Kruskal–Wallis test was used for comparison of more than two samples. The Pearson or Spearman linear correlations were used for correlation between independent variables, measured by the coefficient degree (r or rs, respectively), which ranges from −1 to +1, with the association close to these extremes being stronger. The results were presented in the form of tables, histograms, and trend curves.

3. Results

3.1. Description and epidemiological analysis

Of the 19 patients who underwent biopsies, 57.9% (n = 11) were men, aged 45.5 ± 9.6 years, diagnosed with HIV for 9.8 ± 5.2 years, and have been using ARVT for 7.8 ± 3.9 years.

The bicep, tricep, subscapular and suprailiac skinfolds made it possible to estimate the body fat of these patients — it was observed that women have a higher percentage than men (Fig. 5).

The distribution of the clinical forms was in majority for the mixed form (63.2%, n = 12). Men showed more signs of lipoatrophy...
than women, regardless of the area affected (Average: 84 vs. 92.5, p = 0.05), with no difference in the presence of lipoaccumulation.

### 3.2. Immunohistochemistry

Men showed expression of caspase-3 and TNF-α that was significantly larger than for women, but with no difference in the expression of TGF-β (Table 1).

There was no statistical difference for the cytokines between the clinical forms (p > 0.05), although there was a trend for the lipoatrophic group to have higher apoptotic activity (16.4 ± 5.7 vs. 7.9 ± 2.7, p = 0.054) and greater TNF-α expression (6.12 ± 1.5 vs. 4 ± 1.1, p = 0.07) than lipohypertrophic. Regardless of the clinical form, in comparison with the control sample, lipoatrophic patients showed higher expression of caspase-3 (p < 0.01) and TNF-α (p < 0.01). The control group possessed higher expression of TGF-β than the group with lipoatrophy (p = 0.03 — see Fig. 6).

### 3.3. Correlations of the cytokines with clinical data

The TNF-α was positively correlated with the time of HIV diagnosis (rs = 0.6, p < 0.01), duration of the ARVT (rs = 0.5, p = 0.03), and the presence of lipoatrophic manifestations (rs = 0.7, p < 0.01). The percentage of body fat was inversely associated with the expression of caspase-3 (r = −0.8, p < 0.0001, 95% CI = −0.94 to −0.43), TNF-α (r = −0.51, p = 0.02, 95% CI = 0.78 to 0.08) and with the appearance of lipoatrophic manifestations (rs = −0.46, p = 0.047). The Simple Linear Regression Test showed that 62.17% of the changes in body fat were due to adipocytic apoptosis (F = 30.58, t = −5.53, p = 0.0001) and 22.09% were due to the TNF-α activity (F = 6.1, t = −2.47, p = 0.02).

### 3.4. Relationship between the cytokines

A positive correlation was observed between caspase-3 and TNF-α, TGF-β and TNF-α (Figs. 7 and 8). The Adjustment of Curves showed linear regression between caspase-3 and TNF-α (R² = 30.27%, p = 0.01), and geometric regression between TGF-β and TNF-α (R² = 33.94%, p = 0.009) and between TGF-β and caspase-3 (R² = 23.34%, p = 0.04).

### 4. Discussion

#### 4.1. HIVLS is a chronic process which inflammatory activity worsens over time, increasing lipoatrophy manifestations

The initial characteristic of lipoatrophy would be lipolysis, but with the chronicity of the infection and the medications, the tendency is that a dominance of the lipoatrophy manifestations occurs over the lipohypertrophic ones [14]. Vidal et al. [15] also observed that the time of infection, regardless of the degree of viral suppression, correlated inversely with the expression of PPAR-γ and the increase of TNF-α, regardless of the class of drugs of the ARVT. The results confirm these observations and indicate that the inflammatory stimulus in the lipoatrophy is chronic and tends to worsen over time, favoring lipoatrophy by lipolysis and adipocytic apoptosis.

#### 4.2. Men have more inflammatory and apoptotic activities than women

Immunohistochemical analysis showed inflammatory activity mediated by TNF-α and the presence of more intense apoptosis in
the subcutaneous adipose tissue of men, suggesting that they may develop the forms displaying lipoatrophy manifestations with greater frequency while women would be more lipohypertrophic [12,16–18]. The increased expression of TNF-α in their tissues translates into greater biological action of this cytokine which can induce cellular apoptosis by the binding of the Fas-Associated protein with Death Domain (FADD) to the TNF Receptor Associated Death Domain (TRADD) protein; or promote inflammation and lipolysis by way of the MAP kinases and/or NFκB, especially [19–21]. However, where to start and end the signaling for the TNF-α to generate apoptosis or inflammation, is still to be clarified.

Interestingly, women peripheral adipocytes are more resistant to apoptosis induced by TNF-α [22]. Women naturally have a protection factor for lipoatrophy that displays a higher percentage of body fat in comparison with men [23]. This, associated with the low expression of TNF-α and apoptosis, as well as the resistance of the tissue to this cytokine, promotes the trend of lipocaccumulation, which, at least initially, is classified as isolated lipohypertrophy and possibly due to not having the protective peripheral resistance to the TNF-α presented in women, men would develop more pronounced lipoatrophy manifestations.

4.3. Lipodystrophic patients have more inflammatory/apoptotic activities and less anti-inflammatory activity than control subjects

Lipodystrophic patients had higher apoptotic and inflammatory activity than the healthy control subjects, as proposed by Jan et al. [24] and Lihn [25]. The study of Lindegard et al. [23] also observed that the circulating serum levels of TNF-α did not differ between the clinical forms, being higher even in HIV-infected patients without lipodystrophy. It is proposed that during infection, viral proteins already stimulate the synthesis of TNF-α and its receptors [10,26], and this production is aggravated with the use of ARVT by its action on the immunological reconstitution by blocking the apoptosis of TNF-α-secreting T lymphocytes [10]. Unlike the lipodystrophy group, the control group showed higher expression of TGF-β, suggesting that lipodystrophic patients had a reduction in the efficiency of the anti-inflammatory response in containing the inflammatory stimulus.

4.4. Local inflammatory and apoptotic activities in adipose tissue are associated with changes in body fat

The subcutaneous adipose tissue, which is the main target and source of TNF-α, responds to its actions by increasing the rate of apoptosis of adipocytes and lipolysis; however, the visceral adipose tissue is transcriptionally and metabolically different [27] and proliferates, similar to Cushing Syndrome, in response to inflammatory cytokines such as TNF-α, or resulting from its activation as IL-1 and IL-6. There is disagreement about whether the local production of TNF-α in the subcutaneous region can reach significant levels in the bloodstream to promote its actions at a distance [3,28]. It is possible that the action of TNF-α in these cases is more indirect by the stimulation of the M1 profile [29,30], and the synthesis of other inflammatory cytokines that may trigger their effects in distant tissues, despite the restricted local production of TNF-α.

Good et al. [31] showed that obese patients with increased central accumulation exhibit greater expression of TNF-α in the visceral compartment than in the subcutaneous compartment. It may be possible that at the beginning of lipodystrophy there is an increase in inflammatory activity in all the adipose compartments, and those with greater body fat, especially from central accumulation, would feel the proliferative actions more on the visceral-abdominal fat. As the trend over time is the worsening of the inflammatory effects, these pure lipohypertrophic patients evolve into a mixed framework, with high expression of TNF-α in both compartments, while in the lipoatrophic patients one would expect a more significant increase only in the subcutaneous tissue. So it would be interesting to evaluate, if possible, the expression of the cytokine in the visceral tissue of the patients with HIVLS to identify the extent to which TNF-α affects, or does not affect, the increase in that risk.

The more inflamed the subcutaneous adipose tissue, the more susceptible it is to lipolytic and apoptotic stimuli [32]; and with increased adipocyte death, a reduction in the subcutaneous tissue of patients is expected, resulting in the reduction of total body fat; however, it may be that the apoptosis of the adipocytes occurs by other means, not only by the TNF-α stimulus.

4.5. Inflammatory activity is the major factor to induce adipocyte apoptosis although not the only one

The apoptotic activity increased in tissues with higher inflammatory activity — the action of the TNF-α accounts for approximately one third of the adipocytic apoptosis, suggesting that it is one of the main cytokines involved, though not the only one. Lauber et al. [33] suggested that apoptosis, despite being a process that does not trigger an inflammatory process, when it is present in great intensity, the macrophage responsible for the phagocytosis and release of TGF-β cannot contain the post-apoptotic necrosis in a timely manner, thus favoring the installation of the inflammation. It is suggested that HIVLS may occur in the same way, where inflammatory cytokines that are present due to use of the medication and infection, trigger cellular apoptosis so strongly that the secretion of TGF-β is not sufficient to contain the apoptotic necrosis that perpetuates the inflammatory state in these patients. However, further studies are still needed to clarify the actual mechanisms involved in the physiopathogenesis of HIVLS.

4.6. Negative feedback between TNF-α and TGF-β

The positive association between the inflammatory activity mediated by TNF-α and anti-inflammatory activity mediated by TGF-β suggests the presence of a negative feedback mechanism in an attempt to maintain tissue homeostasis. Oxidative stress, and the action of the ARVT on mitochondrial toxicity and in the inhibition of PPAR-γ, favor the maintenance of the M1 profile or the inflammatory type of tissue macrophages [34]. The activation of these macrophages induces the secretion of inflammatory cytokines, including TNF-α. The cytokine with autocrine and paracrine
action leads other local macrophages and adipocytes to secrete more TNF-α, which favors the establishment of an inflammatory environment suitable for lipolysis. In an attempt to balance and contain the inflammatory process, there is the synthesis and release of anti-inflammatory cytokines; for example, TGF-β and IL-10. However, in patients with HIVLS, there is less anti-inflammatory activity compared to healthy people, with the resulting increase insufficient for completion of the chronic inflammatory stimuli of the lipodystrophy, whose causal factors (HIV and ARVT) remain, thus maintaining a continuous circle of inflammatory and anti-inflammatory manifestations, which were observed by Capeau et al. [3] and Jan [24] as an increase in apoptosis, fibrosis, vessel density, and macrophage infiltration forming lipogranulomas. Therefore, it is possible that the interactions between distinct immunological activities have a role in the different clinical expressions of HIVLS.

The study does not attempt to establish cause and effect relations, which is impossible due to the design, but it does suggest more hypotheses and relationships that serve as a foundation for further studies, prospective studies, and studies with paired samples/subjects, in order to unravel the mechanisms responsible for this condition. Also, the study seeks methods to reduce morbidity and improve the quality of life of patients with HIVLS.

5. Conclusion

HIVLS involves a chronic and progressive inflammatory process that worsens as the time of infection and ARVT use increases, thus increasing the occurrence of lipatrophy manifestations. Men had higher expression of TNF-α and caspase-3 than women, with no difference for TGF-β. Lipodystrophic patients, regardless of the clinical type, showed greater expression of TNF-α and caspase-3 than the control group which presented higher TGF-β activity. Local inflammatory and apoptotic activity in the adipose tissue is associated with changes in the percentage of body fat, especially in relation to lipolysis. The apoptotic activity was highly related to the expression of TNF-α and to a lesser degree with TGF-β. Patients with increased expression of TNF-α had higher expression of TGF-β, suggesting the existence of a negative feedback mechanism.

References


Glossary

**ARVT:** Antiretroviral Therapy. It is a conjunct of several antiretroviral drugs in attempt to control HIV infection.

**Caspase-3:** It is a member of cysteine–aspartic acid protease family. Sequential activation plays a central role in the execution-phase of cell apoptosis.

**FADD:** Fas-Associated protein with Death Domain. It is an adaptor molecule that bridges the Fas-receptor to caspase-8 through its death domain to form the death-inducing signaling complex during apoptosis.

**HIV:** Human Immunodeficiency Virus. It is a lentivirus that causes acquired immunodeficiency syndrome, a condition in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive.

**HIVLS:** HIV Lipoystrophy Syndrome. It is a clinical manifestation characterized by alterations in the metabolism and body distribution of fats, as well as increased risk factors for systemic arterial hypertension, diabetes mellitus, and cardiovascular accidents associated to HIV infection.

**IL-1:** Interleukin-1. It is a group of cytokines, which plays a central role in the regulation of immune and inflammatory responses to infections or other insults.

**IL-6:** Interleukin-6. It is a cytokine secreted by T cells and macrophages to stimulate immune response during infection, trauma or other tissue damage leading to inflammation.

**IL-10:** Interleukin-10. It is a cytokine with anti-inflammatory activity.

**M1:** Proinflammatory Macrophages. They are immune effectors cells that are aggressive against microbes and also produce lymphokines.

**M2:** Anti-inflammatory Macrophages. They are alternatively activated macrophages involved to wound healing, tissue repair and to turn off immune system activation by producing anti-inflammatory cytokines.

**Map kinases:** Mitogen Activated Protein Kinases. They are serine/threonine-specific proteins kinases involved in directing cellular response to a diverse array of stimuli, such as mitogen, osmotic stress, heat shock and proinflammatory cytokines. They regulate proliferation, gene expression, differentiation, mitosis, cell survival and apoptosis.

**NFkB:** Factor Nuclear Kappa B. It is a protein complex that controls the transcription of DNA involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL and bacterial or viral agents.

**PPARγ:** Peroxisome Proliferator-activated Receptor Gamma. It is a nuclear receptor that regulates the fatty acid storage and glucose metabolism.

**TGF-beta:** Transforming Growth Factor Beta. It is a protein that controls proliferation, cellular differentiation and other functions in most cells.

**TNF-alpha:** Tumor Necrosis Factor Alpha. It is an adipokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction.

**TRADD:** TNF Receptor Associated Death Domain. It is an adaptor molecule that interacts with TNF receptors and mediates programmed cell death signaling and NFκB activation.