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Soluble CD26 and CD30 Plasma Levels in HIV Infected Patients with and Without GB Virus Type C Coinfection

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Abstract: GB virus type C (GBV-C) probably influences HIV infection associated disease by either directly inhibiting HIV replication or enhancing the immune competence to cope with HIV. Still the definitive mechanisms of this inhibitory effect need to be identified. To address the possibility of immune modulating effects of GBV-C coinfection in HIV patients we evaluated plasma levels of soluble (s) CD26 and CD30 in HIV infected patients with and without GBV-C. Cross-sectional comparison of sCD26 and sCD30 levels among 6 HIV/GBV-C coinfected, 11 HIV mono-infected and 13 healthy controls was carried out. We used a commercial EIA to evaluate sCD26 and scD30 and a RT-PCR assay to detect active GBV-C infection. The Mann-Whitney U test was used for statistical analysis. No statistically significant differences were observed in levels of sCD26 and sCD30 in plasma of HIV infected patients with and without GBV-C viremia. GBV-C infection does not appear to influence the sCD26 and sCD30 levels.

Key words: GB virus C; HIV; sCD26; sCD30; coinfection

INTRODUCTION

A recently discovered flavivirus, GBV-C (Simons *et al.*, 1995) is frequent in human immunodeficiency virus infected persons due to similar transmission routes of these viruses (Rmia *et al.*, 2004). Over the past several years, there has been substantial controversy regarding the interactions between GBV-C and HIV *in vivo*. A number of studies have demonstrated a surprising survival benefit among patients who are coinfected, as compared with those who are infected with only HIV (Sabin *et al.*, 1998; Tilman *et al.*, 2001; Williams *et al.*, 2004; Xiang *et al.*, 2001). Other studies, however, have not shown such a survival advantage (Birk *et al.*, 2002; Bjorkman *et al.*, 2004; Van Der Bij *et al.*, 2005). To address the possibility of immune modulating effects of GBV-C coinfection in HIV patients, we evaluated the sCD26 and sCD30 in subject with HIV and GBV-C coinfected patients.

Dipeptidyl peptidase IV (DPPIV; CD26; EC 3.4.14.5) is a 110-KD cell surface glycoprotein expressed on different cell types, including T cells. It is an enzyme that cleaves NH2-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position. It has two physiological forms: One is the soluble form found in plasma, whereas the other is the membrane-bound form preferentially expressed on the CD4+ helper/memory T cell population. CD26 is a multifunctional molecule involved in regulation of many key aspects of lymphocyte function. It delivers a potent co-stimulatory T cell activation signal and is strongly up-regulated following T cell activation (Boonacker and Van-Noorden 2003; Ohtsuki *et al.*, 2000). Strong up-regulation of this molecule on T cell by IL-12 confirms its role as a marker of Th1-type cells (Salgado *et al.*, 2000). Loss of CD26 significantly impairs the production of IL-2 and the proliferative capacity of T cell, these CD26 negative T cells have been shown to display a Th2-like cytokine pattern (Che-Chun *et al.*, 2004). sCD26 is taken up by monocytes and exerts its enhancing effect on T cell proliferation by altering the antigen presenting function of monocytes through the up-regulation of CD86 expression (Ohnuma *et al.*, 2001). Besides many other functions, CD26 has been implicated with HIV infection. There is a correlation between CD26 expression and HIV entry, replication and cytopathicity. After cleavage

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by DPP IV, SDF-1 alpha and SDF-1 beta lose their chemotactic and anti HIV activity but antiviral effects of RANTES are enhanced after truncation by DPP IV. CD26 interacts with HIV-1 Tat protein, thus mediating Tat's immunosuppressive effects (Boonacker and Van-Noorden, 2003; Ohtsuki *et al.*, 2000). Specific DPP IV enzyme activity is correlated with the levels of CD4+ cells and has an inversed correlation with HIV-1 RNA (Hosono *et al.*, 1999). The HIV-1 gp120/gp41 complex has been shown to be responsible for the initiation of cell killing by apoptosis in CD4+ cells. CD26, independent of its DPP IV activity, appears to be implicated in this process and the level of CD26 may determine the rate of HIV-envelope-induced apoptosis (Jacoto *et al.*, 1997).

CD30 is a member of the tumor necrosis factor-nerve growth factor superfamily and is expressed by T cells producing Th2 type cytokine. An 85Ka soluble form of the CD30 molecule has been shown to be released by CD30+cell *in vitro* and *in vivo*. Plasma sCD30 detection can be regarded as a marker of the amount of CD30+ cells present in the body. Previous studies have suggested that Th2 lymphocyte activation is related to increased sCD30 plasma levels. Soluble CD30 (sCD30) concentration increase in different pathologic conditions in which a pathogenic role for Th2 cells has been suggested, such as atopy, Omenn's syndrome, systemic lupus erytmatosus as well as following infection with measles virus or HIV (Horie and Watanabe, 1998; Bengtsson *et al.*, 2001). A recent paper on a CD30 homologue encoded by Ectromelia virus also coincides with function of the human CD30. This CD30 protein abrogates T-cell proliferation and type I cytokine-mediated inflammation *in vivo* but has no effect on type II cytokine-mediated inflammation (Saraiva *et al.*, 2002). Plasma levels of sCD30 were increased in the early phase of HIV infection and patients with higher sCD30 levels demonstrated a faster progression to AIDS (Rizzardi *et al.*, 1996).

True that the action of cytokine is short distanced but the primed cells circulation is not, analysis of some molecules such as CD26 and CD30 and their soluble forms in plasma has been proposed as a simple and useful tool in discriminating Th1 and Th2 responses in state of plasma levels of T-helper cells cytokine files. Several studies of sCD30 levels in plasma have shown correlations with Th2 associated conditions and also a number of information was found about the levels of sCD26 in Th1 associated conditions (Boonacker *et al.*, 2002; Hoshimoto *et al.*, 2000; Keane *et al.*, 2001; Nakao *et al.*, 2002), plasma levels of these markers have also been used to assess disease activity in different disorders (Ajdary *et al.*, 2006, 2007).

Coinfection with GBV-C may be confers relative resistance to the progression of HIV disease, however not all studies of co-infection with HIV and GBV-C have found a beneficial effect on HIV disease progression and there has been substantial controversy regarding the interactions between GBV-C and HIV *in vivo*. It may be that direct studies of GBV-C infection on HIV surrogate markers will be the only way to resolve this issue.

Because of the informativeness of sCD26 and sCD30 for characterization of Th1 and Th2 immune response and their correlation with disease activity in different disorders and also their implication with HIV infection we evaluated the levels of sCD26 and sCD30 among HIV infected patients with and without GBV-C coinfection, to find the possibility of immune modulating effects of GBV-C coinfection in HIV patients. To our knowledge, plasma levels of sCD26 and sCD30 in HIV/GBV-C coinfected patients have not been reported previously.

MATERIALS AND METHODS

Study subjects: Study subjects were patients being seen at the Iranian Blood Transfusion (IBTO) and Infectious Disease Department of Imam Khomeini Medical Complex during July and October 2006 in Tehran. The study was carried out in a group of 17 HIV infected subjects (2 women and 15 men) aged 29-54 (mean age 36.8 years), with no HBV and HVC infection sign who were at various stage of HIV infection, ranging from asymptomatic to those fitting the CDC case definition of AIDS, majority of them were IV drug abuser. HIV serology was determined by ELISA and confirmed by western blot in the research centre laboratory at Iranian Blood Transfusion Organization (IBTO). CD4+ lymphocyte counts of patients ranged from 123 to 754 μL^{-1}. The patients were stratified on the basis of the CD4+ lymphocyte counts: Patients with absolute CD4 count $< 200\ \mu L^{-1}$ of whole blood (7 patients) and patients with CD4 count $\geq 200\ \mu L^{-1}$ (10 patients). Six HIV infected subjects were positive for GBV-C RNA, 2 of them had CD4 count $< 200\ \mu L^{-1}$ of whole blood. Plasma from 13 age and sex matched HIV uninfected healthy individuals served as controls. All controls plasma were also negative for HCV antibody and hepatitis B surface antigen (HBS-Ag). After acquisition of informed consent blood samples were obtained from all these individuals and separated plasma were stored at -20°C until use.

Laboratory procedures
Lymphocyte subpopulations: The CD4+ and CD8+ T-cell counts were obtained for the subject samples by CD3/CD4+ and CD3/CD8+ double staining of lysed whole blood followed by flow cytometric analysis after gating on the lymphocyte population.

Detection of GBV-C: Plasma GBV-C RNA was detected by in –house Reverse-Transcription-Polymerase Chain Reaction (RT-PCR): Total RNA extracted from 250 µL of EDTA -anticoagulant plasma with TRIzol LS reagent, followed by organic extraction with chloroform, precipitate with isopropanol-ethanol. Five microliter RNA extract was add to RT mix containing of dnTP, random hexamer primer, Rnase inhibitor and murin leukaemia virus reveres transcriptase. CDNA synthesis was performed at 40°C for 40 min. After RT, CDNA was add to PCR mix containing of Taq polymerase, nucleotides mix and primer pairs located in the NS5a region of the viral genome (Forward: 5'-TAAGAGAAGGTTAAGATTCC-3' and Reveres: 5'-CAAGAGAGAACATTGAAGG-3') or primer pairs located in the NCR region of the viral genome reported previously (Kupfer *et al.*, 2005). Amplification was performed at 95 for 5 min, then 45 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec with final extension step of 5 min at 72°C. The amplicons were detected by gel electrophoresis on an ethidium bromide-stained 2% agarose gel under UV light. A plasmid containing GBV-C cloned sequences (Biodiversity Italy) used as positive control. Positive cases were reconfirmed by GBV-C detection kit (Roche Germany) according to the manufacturer's instruction. Briefly, the PCR products were labeled with digoxigenin during the amplification process. The labelled PCR products were analyzed by solution hybridization to a specific capture probe that was complimentary to the inner part of the amplification product. This capture probe was biotinylated to allow immobilization of the hybrid to a streptavidin-coated micro plate surface. The bound hybrid was detected by an anti-digoxigenin-proxidase conjugate and by use of colorimetric substrate.

Determination of plasma sCD26 levels: Plasma levels of sCD26 were measured by ELISA (Chemicon International, USA) a sandwich type immunoassay which can be used to measure sCD26 in plasma. Briefly, this assay utilizes an anti-CD26 monoclonal antibody coated to polystyrene microtiter wells. After addition of samples, or standards and rinsing, a biotinylated second monoclonal CD26 antibody was added and allowed to bind to sCD26, captured by the coating antibody. After rinsing, horseradish-peroxidase-conjugated streptavidin was added, followed by a biotinylated second antibody and addition of a chromogen, resulting in the formation of a colored product. Absorbance was measured at 450 nm to quantify color development. Levels of sCD26 in samples are determined by comparison with a standard curve prepared from six sCD26 standards, ranging from 500 to 15.6 ng mL^{-1}.

Determination of plasma sCD30 levels: Plasma levels of sCD30 were measured by ELISA (Biotest, Germany) a sandwich type immunoassay which can be used to measure sCD30 in plasma. Briefly, standards and patient specimens were added to peroxidase-conjugated mouse anti-CD30 in polystyrene microtiter wells precoated with another CD30 monoclonal antibody. After 3 h incubation and washing to remove unbound material, a chromogenic substrate was added to the wells. The reaction was then stopped and absorbance at 450 nm was measured. Levels of sCD30 in samples are determined by comparison with a standard curve prepared from 6 sCD30 standards, ranging from 100 to 1.6 U mL^{-1}.

PCR for HCV-RNA and HBV-DNA: These tests were performed at IBTO Iranian blood transfusion organization on the basis of routine procedures elsewhere.

Statistics: Because of small sample sizes, nonparametric statistics were used. The Mann-Whitney U-test in SPSS software was used for group comparisons. A p-value of 0.05 or less considered to represent significant.

RESULTS

The level of sCD26 were analyzed in relation to CD4 lymphocyte counts. The results are presented in Table 1. In contrast to finding of Pawlowska *et al.* (2003). The levels of sCD26 in these groups did not show statistically significant difference. The plasma concentration of sCD26 in 16/17 HIV infected patients ranged from 116-254 ng mL^{-1}, but one case the sCD26 value was 310 ng mL^{-1}, such concentration was found in a male patient with CD4+ lymphocyte counts over 200 µL^{-1} (627 µL^{-1}) without GBV-C coinfection. The comparison of sCD26 levels in HIV infected and HIV/GBV-C coinfected subjects did not show statistically significant difference. The results are presented in Table 2. Present suggest that GBV-C infection does not appear to influence the level of sCD26. Plasma sCD26 in HIV infected individuals revealed a wider range of levels (116-310 ng mL^{-1}) than in uninfected controls (102-194 ng mL^{-1}) However, there was no significant difference in the levels of sCD26 between HIV infected individuals and uninfected controls (Table 3). Present Finding was in agreement with those of Hosono *et al.* (1999).

Plasma levels of sCD30 were increased in HIV infected patients in comparison with controls (Table 4); this is a support for the suggestion of a bias toward Th2 type responses during HIV infection. The levels of sCD30 were analyzed in relation to CD4 lymphocyte counts. The

Table 1: sCD26 and levels in relation to CD4 lymphocyte counts

Parameters	sCD26 (ng mL^{-1})	Mean±SD	p
CD4<200 μL^{-1} (n = 7)	116-254	176.00±74.80	
CD4≥200 μL^{-1} (n = 10)	128-310	178.20±51.02	NS

Table 2: sCD26 in HIV – and HIV/GBV-C-infected patients

Parameters	sCD26 (ng mL^{-1})	Mean±SD	p
HIV (n = 11)	116-310	181.45±58.33	
HIV and GBV-C (n = 6)	148-196	169.66±22.78	NS

Table 3: sCD26 levels in the studied subjects

Parameters	sCD26 (ng mL^{-1})	Mean±SD	p
HIV infected patients (n = 17)	116-310	177.29±48.19	
Healthy controls (n = 13)	102-194	158.92±30.10	NS

Table 4: sCD30 levels in the studied subjects

Parameters	sCD30 (μ mL^{-1})	Mean±SD	p
HIV infected patients (n = 17)	5.2-93.0	29.35±26.82	<0.0001
Healthy controls (n = 13)	4.6-30.5	8.10±6.830	

Table 5: sCD30 levels in relation to CD4 lymphocyte counts

Parameters	sCD30 (μ mL^{-1})	Mean±SD	p
CD4≥200 μL (n = 10)	8.2-93.0	35.91±30.96	NS
CD4<200 μL (n = 7)	5.2-55.9	20.00±17.52	

Table 6: sCD30 HIV-and HIV/GBV-C-infected patients

Parameters	sCD30 (μ mL^{-1})	Mean±SD	p
HIV (n = 11)	5.2-90.5	27.13±25.98	NS
HIV and GBV-C (n = 6)	8.2-93.0	33.43±30.36	

NS = Not Significant

results are presented in Table 5. The concentration of sCD30 in patients with CD4 lymphocyte counts over 200 μL^{-1} were higher than patients with CD4 lymphocyte counts blow 200 μL^{-1} (35.91±30.96 vs 20±17.52 μ mL^{-1}) and revealed a wider range of levels (8.2-93 vs 5.2-55 μ mL^{-1}) we suggest that sCD30 levels may decline in the advanced stages of HIV disease. However, there was no significant difference in the levels of sCD30 between two groups.

The comparison of sCD30 levels in HIV infected and HIV/GBV-C coinfected patients did not shown statistically significant difference. The results are presented in Table 6.

DISCUSSION

We chose GBV-C RNA as a marker for active coinfection. We did not perform serological studies of GBV-C in our study because, in pervious studies the positive influence of GBV-C on the HIV prognosis was most marked among patients with active replication (Tilman *et al.*, 2001; Williams *et al.*, 2004; Xiang *et al.*, 2001). To reduce the heterogeneity HIV positive patients with no HBV and HCV infection sign were included.

Plasma levels of soluble markers of immune activation have prognostic capabilities which are different from HIV viral load and CD4 T cell levels, for instance plasma sCD30 concentration in patients with early HIV infection portends a rapid progression to AIDS (Pizzolo *et al.*, 1994; Rizzardi *et al.*, 1996). To explore the effects of GBV-C coinfection among HIV infected patients, we evaluated sCD26 and sCD30 because they have been implicated with HIV infection, moreover in a number of reports, it has been reported that regulation of CD26 cell surface expression correlate with production of Th1-like cytokine and CD30 is expressed by T cells producing Th2 type cytokine. It is therefore possible that the analysis of Th1/Th2 cytokines by measuring plasma sCD26 and plasma sCD30.

There was no significant difference between the plasma levels of sCD26 in 17 HIV infected individuals and in 13 uninfected controls. Further studies are necessary to clarify to explain the conflicting results on the role of CD26 in HIV infection. Plasma levels of sCD30 were increased in HIV infected patients in comparison with controls (Table 4); this is a support for the suggestion of a bias toward Th2 type responses during HIV infection but trend towards declining levels of sCD30 with advanced HIV disease may be due to destruction of Th2 cells by direct or indirect HIV mediated cell killing and Th1 to Th0 shift in a proportion of T, cells. The comparison of sCD26 and sCD30 levels in HIV infected and HIV/GBV-C coinfected subjects did not show statistically significant difference. The plasma concentration of sCD30 in HIV infected patients ranged from 5.2 to 93 μ mL^{-1}. The highest concentration of sCD30 was 93 μ mL^{-1}, such concentration was found in a male patients with CD4 lymphocyte counts over 200 μL^{-1} (532 μL^{-1}) with GBV-C coinfection. The highest sCD26/sCD30 ratio was found in a male patient with CD4 lymphocyte counts below 200 μL^{-1} (148 μL^{-1}) without GBV-C infection, in this case the sCD30 value was 5.2 μ mL^{-1}.

Present results suggest that GBV-C infection does not appear to influence the level of sCD26 and sCD30 and also these finding suggest the *in vivo* involvement of the Th2 cells in the regulation of immunological process in HIV/GBV coinfected patients.

Infection with both GBV-C and HIV has been shown to lead to stable serum levels of T-helper 1 (Th1) cytokine files during follow-up compared to patients who are GBV-C RNA negative in which Th1 cytokine profiles fall (Nunnari *et al.*, 2003). IL4 and IL10 levels rose over time and IL2 and IL12 levels fell over time in GBV-C RNA negative, HIV positive people whereas GBV-C RNA positive subjects had stable IL2, IL12, IL4 and IL10 levels, suggesting that GBV-C helps to maintain a Th1 cytokine profile (Nunnari *et al.*, 2003). Sathar *et al.* (2004). Showed, patients who tested positive for GBV-C RNA had

significantly lower CD30 cell counts, compared with those who did not, which reflects an increase in Th1 response.

Present results do not rule out significant difference in Th1 and Th2 cytokine related to GBV-C coinfection but our study suggest that there are not very large differences in sCD26 and sCD30 related to GBV-C infection. Although the use of sCD26 and sCD30 as surrogates for Th1 and Th2 are not widely supported in the literature and even though this cross-sectional study does not clearly reveal the associated between GBV-C coinfection and change in Th cytokine in people with HIV infection, the role of GBV-C in change of cytokine profiles seems controversial. Additional longitudinal studies that include more cases are needed to resolve this important issue. True that the action of cytokine is short-distanced, it would be more appropriate to directly analysis of constitutive cytokine mRNA expression in lymphoid tissues from HIV/GBV-C coinfected individuals to confirm an *in vivo* Th1/Th2 switch.

In summary, we found that there are not very large differences in sCD26 and sCD30 related to GBV-C infection among HIV infected patients in this cross-sectional study.

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