ZAP-70 POSITIVE CELLS IN TREATED AND UNTREATED HIV-1 INFECTED PATIENTS

Surangrat Srisurapanon[1], Suchitra Sukwit[2], Thippawan Chuenchitra[3]
and Somchai Santiwattanakul[1]

[1]Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Bangkok; [2]Army Institute of Pathology, Bangkok; [3]Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

Abstract. ZAP-70 is a critical protein tyrosine kinase in T-cell activation and proliferation processes. Defective recruitment of ZAP-70 molecules results in termination of the T-cell receptor (TCR) signal transduction pathway. Impairment of this pathway is one of the early markers of disease progression in HIV-1 infected individuals. T-cell dysfunction in HIV infected patients may be connected to a defect in the proximal TCR signaling cascade. To evaluate this presumption, the numbers and mean fluorescence intensity (MFI) of ZAP-70 positive cells in patients with treated and untreated HIV-1 infection and healthy controls were analyzed by flow cytometry. A correlation between the MFI in ZAP 70 molecules and the viral load was evaluated. A total of 41 HIV-1 infected patients, 30 patients on HAART and 11 untreated patients, and 11 healthy controls were enrolled. The data show ZAP-70+/CD4+ cells in treated and untreated HIV-1 infected individuals had a greater MFI of ZAP-70 molecules than those from healthy controls ($p<0.001$). The inverse correlation between the percentage of CD4+cells and the MFI of ZAP-70+/CD4+ T-cells was significant ($r =-0.5$; $p <0.01$). A stronger correlation between the percentage of CD4+/CD25+ cells and the MFI of ZAP-70+/CD4+ cells was observed ($r =-0.6$; $p<0.01$). However, no significant correlation was seen between the MFI of the ZAP-70+/CD4+cells and the viral load in patients with untreated HIV-1 infection ($r =-0.4$, $p = 0.16$). For HIV-1 treated patients, the viral loads were too low to detect so it was not possible to calculate the correlation. Elevated MFI levels of ZAP-70 molecules in CD4+cells in HIV infected patients may be associated with an inability to further activate T-cells.

Key words: HIV-1, ZAP-70, mean fluorescence intensity

INTRODUCTION

Human immunodeficiency virus (HIV) infection results in both intense and chronic immune activation (Sol-Foulon *et al,* 2007*).* Even with a vigorous response to HIV, a quantitative and qualitative decline in T- cells is seen (Kanner and Haffar 1995). Several studies have found evidence of multiple defects in the signaling pathways of T-cell activation (Roilides *et al,* 1991; Groux *et al,* 1992; Meyaard *et al,* 1992; Cayota *et al,* 1996; Geertsma *et al,* 1999; Giovannetti *et al,* 2000). Alteration in cellular signaling, especially at the level of ZAP-70, which plays an important role

Correspondence: Dr Surangrat Srisurapanon, Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Sukumwit 23, Sukumvit Road, Wattana, Bangkok 10110, Thailand.
E-mail: surangra@swu.ac.th

in T-cell activation, has been reported (Chan *et al,* 1992; Weiss, 1993; Schweneker *et al,* 2008).

ZAP-70 is an intracellular protein-tyrosine kinase, normally expressed in T cells and natural killer cells and plays a critical role in the initiation of T-cell activation. Upon T-cell activation, the tyrosine kinase Lck becomes activated and phosphorylates the intracellular portions of the CD3 complex (called ITAMs). The most important member of the CD3 complex is CD3-zeta, to which ZAP-70 binds and subsequently phosphorylates. Activated ZAP-70 can then phosphorylate the linker of the activated T-cell (LAT) allowing recruitment and activation of other critical signaling molecules, such as phospholipase C1γ (PLC γ) and guanine nucleotide exchange factor (Vav) (van Leeuwen *et al,* 1999). These reactions are required for Ras/Map kinase pathways. Activated PLCγ and Ras initiate important signaling pathways that culminate in the activation of transcription factors in the nucleus. Together, NFκB, NFAT, and AP-1 act on the T-cell chromosomes, initiating new gene transcriptions. Tou final outcome of T cell activation is the transcription of several gene products, which allow T cells to differentiate, proliferate and secrete a number of cytokines. When activated through T cell receptors (TCR), T cells have the ability to destroy healthy host tissue as well as infected cells. Therefore, immune activation not only inhibits but enhances HIV replication (Ostrowski *et al,* 1998; Scales *et al,* 2001; Sousa *et al,* 2002; Deeks *et al,* 2004; Catalfamo *et al,* 2008). Activation of T cells through the TCR is tightly regulated (Sloan *et al,* 2006). Nevertheless, T-cell dysfunction in HIV infected patients may be connected to a defect in the proximal TCR signaling cascade. The aims of this study were to investigate the numbers of activated T-cells, ZAP-70+cells and the association between the MFI of ZAP-70+/CD4+ cells and the viral load.

MATERIALS AND METHODS

Patients and controls

A total of 41 HIV-1 infected patients were enrolled in this study: 30 patients (13 males, 17 females; mean age, 41.3 years; mean CD4+cell count of 16.9) were treated with stavudine, lamivudine and nevirapine (GPO-vir) and 11 were untreated patients (5 males, 6 females; mean age, 43.8 years; mean CD4+cell count of 10.1). Eleven healthy controls were included (4 males, 7 females; mean age, 39.9 years; mean CD4+cell count of $41.4 + 1.6$). This study was approved by the institutional ethics committee (approved document SWUEC No 22/2550) and informed consent was obtained from each subject.

All EDTA blood samples were processed within 4 hours. Mononuclear cells were isolated using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), washed twice in phosphate-buffered saline, stained with specific monoclonal antibodies and then analyzed by flow cytometry.

Monoclonal antibodies and reagents

All monoclonal antibodies, fluorescein isothiocyanate (FITC) conjugated anti-ZAP-70 Clone**:** 1E7.2, allophycocyanin (APC) labeled anti-CD4, phycoerythrin (PE) labeled anti-CD25, corresponding isotype controls and fix-perm reagents were purchased from e-bioscience (Research Laboratories).

Immunofluorescent staining

The cells ($0.5\text{-}1\text{x}10^6/100\mu l$) were simultaneously stained with 3 surface markers: allophycocyanin (APC)-labeled anti-CD4 and phycoerythrin (PE) labeled CD25, as described previously (Sukwit *et al,* 2005). Anti-ZAP-70 was not included in the

initial cell surface stain because this antibody stained reasonably well after fixation and permeabilization. Fluorescein isothiocyanate (FITC)-conjugated anti-ZAP-70 was used according to manufacturer protocols. Briefly, after the last wash, cells were fixed, permeabilized, stained, washed and analyzed by flow cytometry (Becton Dickinson, CA). For each analysis 10,000 lymphocytes were acquired and analyzed using CellQuest software. Expression of ZAP-70 in the lymphocyte subpopulation was analyzed using two approaches: (1) by determining the number of ZAP-70+ cells using a lymphocyte gate and (2) by quantifying the mean fluorescence intensity (MFI) on ZAP -70+ cells. The MFI of ZAP-70+cells were compared with the same sample treated with an isotype control and this was used to define the negatively stained cells.

Quantification of HIV-1 RNA copy numbers in plasma

HIV-1 RNA levels were measured in EDTA plasma by quantitative reverse PCR (Amplicor HIV Monitor, Roche Diagnostic Systems, Branchburg, NJ). The limit of detection was 200 copies/ml plasma.

Statistical analysis

The statistical significance of the flow cytometry results were tested by means of the Kruskal-Wallis test. The Pearson correlation was used to assess the correlation between the variables. Significance was considered when the *p*-value was <0.05.

RESULTS

By triple immunofluorescence staining with anti-CD4, anti-CD25 and anti-ZAP-70, the numbers of CD4+, CD25+, ZAP-70+ cells were quantified by percentage of lymphocytes ± SEM (standard error mean). The relative number of ZAP-70 molecule was measured as the MFI of the anti-ZAP-70. These molecules were determined for ZAP-70 positive cells which had fluorescence within the detectable range (as described above). Among the 3 groups of subjects, (treated HIV-1 infected patients, untreated HIV-1 infected patients and healthy controls) the percentages of the lymphocyte subpopulations,

Table 1

Comparison of laboratory data between treated and untreated HIV-1 infected patients and healthy control subjects. The results are expressed as percentages of lymphocytes ± SEM[a].

Variable	Untreated patients *n*=13	HAART treated patients *n*=30	Healthy controls *n*=12	*p*-value
Viral load (mean ± SEM)	177,615± 25,448	<200	ND[b]	-
CD4 count (mean ± SEM)	10.1 ± 2.8	16.9 ± 1.4	41.4 ± 1.6	<0.001[c]
CD25 count (mean ± SEM)	4.8 ± 0.7	7.6 ± 0.8	15.0 ±1.4	<0.001[c]
CD4+/CD25+(mean ± SEM)	1.9 ± 0.5	4.1 ± 0.6	14.2 ± 0.9	<0.001[c]
CD4+/ZAP70 (mean ± SEM)	7.2 ± 2.7	10.3 ± 0.8	25.0 ± 2.3	<0.001[c]
CD25+/ZAP70 (mean± SEM)	2.0 ± 0.6	3.2 ± 0.3	4.35 ± 0.7	0.023[c]
ZAP-70 + cell (mean± SEM)	74.8 ± 2.7	69.6 ± 1.3	64.4 ± 2.7	0.03[c]
MFI of ZAP-70 expression on CD4 cells (geomean± SEM)	40.7 ± 1.2	35.2 ± 1.2	30.47 ± 1.2	0.001[c]

[a]Standard error mean; [b]not determined; [c]statistically significant

$r = - 0.5, p<0.001$ · $r = - 0.6, p <0.001$
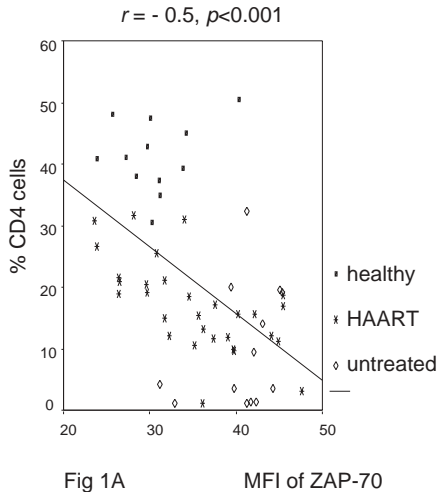
Fig 1A   MFI of ZAP-70

Fig 1B   MFI of ZAP-70

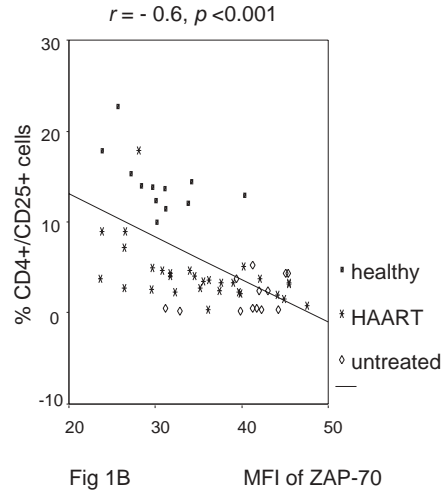r : correlation coefficient
HAART: highly active antiretroviral treatment

Fig 1–The MFI of ZAP-70+/CD4+ cells were correlated with percentages of CD4+cells (Fig 1A) and percentages of CD4+/CD25+ cells (Fig 1B).

CD4+, CD25+, CD4+/ZAP70+, and CD25+/ZAP70+ cells in healthy controls were significantly higher than those in the treated and untreated HIV-1 infected patients at $p<0.001$, $p<0.001$, $p<0.001$ and $p=0.023$, respectively. The percentages of ZAP-70 positive cells in the treated and untreated HIV-1 infected individuals were significantly higher than those of healthy controls ($p=0.03$). ZAP-70+/CD4+ cells in the treated and untreated HIV-1 infected individuals had a MFI of ZAP-70 greater than the healthy controls ($p<0.001$) (Table 1).

Relationships between MFI of ZAP-70 molecules on CD4+cells and immunological markers

By Pearson correlation analysis, a significant inverse correlation was seen between the percentages of CD4+cells and the MFI in the ZAP-70+/CD4+ cells ($r=-0.5$, $p<0.01$) (Fig 1A). A stronger correlation between the percentages of CD4+/CD25+ and the MFI in the ZAP-70+/CD4+

cells was observed ($r=-0.6$, $p<0.01$) (Fig 1B). No significant correlation was seen between the MFI in the ZAP-70+/CD4+cells and the viral load in the untreated HIV-1 infected individuals ($r=-0.4$, $p=0.16$). The correlation level was significant at $p <0.01$ (2 tailed). In HIV-1 treated patients, the viral load was too low to detect so it was not possible to calculate the correlation.

DISCUSSION

In this study, the significantly lower percentages of CD4+/ZAP-70+cells and CD25+/ZAP-70+cells in HIV-1 infected individuals were probably related to the small numbers of CD4+cells and lymphocytes in each case. Considering the ratio of CD4+/CD25+cells in infected patients, most CD4+cells were CD25+ as well. Our data indicate CD4+cells were activated continuously during the course of chronic HIV infection. According to previous reports, pathologic immune activation leads

to an increasing proportion of activated T-cells, which represents the preferential targets of the virus (Giorgi *et al,* 2002; Hazenberg *et al,* 2003; Deeks *et al,* 2004; Haase 2005). These results confirm the link between T-cell activation and HIV replication.

Both CD4+T-cell activation and depletion indicate immune dysfunction may occur at any steps during activation and/or proliferation. A large number of signaling molecules in immune activation, such as ZAP-70, p56lck, and Syk, have been postulated to be involved in immune dysfunction (Guntermann *et al,* 1997; Geertsma *et al,* 1999; Choe *et al,* 2002; Masci *et al,* 2003; Hung *et al,* 2007; Jerome 2008). In analyzing ZAP-70+ cells, the percentages of these cells in both the treated and untreated HIV-1 infected patients were higher than those in healthy controls (p<0.05). In general, ZAP-70 molecules are expressed in CD4, CD8 and NK cells (Catalfamo *et al,* 2008). Since the percentages of CD4+T-cells and NK cells were quite low, most of the ZAP-70+cells in these infected individuals were assumed to be CD8+T-cells. Unfortunately, CD8 surface staining was not performed. Our data indicate both CD4 and CD8+cells from HIV infected individuals had larger numbers of ZAP-70 molecules. Anti-ZAP-70 monoclonal antibody (clone 1E7.2) (www.e-bioscience.com) used in this study was generated against amino acid sequence residues 282-307, which corresponds to human ZAP-70. It may bind both phosphorylated and un-phosphorylated ZAP-70 molecules. In agreement with previous reports, the expressions of the zeta chain subunit (CD247), ZAP-70, Lck and Fyn were up-regulated in HIV-1 infected macaque peripheral mononuclear cells (mPBMCs). Up-regulation of these signals was confirmed by qRT-PCR (Hung *et al,* 2007). In another study, ZAP-70 was most evident in the CD4+ cells of HIV infected patients when compared with CD4+ cells from un-stimulated normal controls (Schweneker *et al,* 2008). The total amounts of protein tyrosine kinases (PTK) Lck, Fyn, ZAP-70 and the zeta chain of the TCR were found significantly decreased in T cells in both symptomatic and AIDS patients (Stefano *et al,* 1996). In both studies, cells were stimulated with anti CD3, and anti-CD4 *in vitro* before ZAP-70 analysis. In our study, cells were evaluated in the context of heterogeneous populations of T-cells in peripheral blood which contained a very small number of truly HIV-infected T-cells during the infection.

In HIV-1 infected patients, inverse correlations were observed between percentages of CD4+/CD25+ cells and the MFI of ZAP-70 protein (r =- 0.6, p<0.01). Low percentages of CD4+/CD25+ cells were the result of disease progression. Specific changes in the fraction of CD4+T-cells expressing CD25 and/or CD127 in HIV-1 patients with disease progression have been reported (Dunham *et al,* 2008). The greater intensity of ZAP-70 molecules in PBMCs with HIV-1 infection may be due to the activation state of T-cells. According to previous reports, T-cell activation may be enhanced in HIV-1 infection (Hung *et al,* 2007) and these cells represent the preferential targets of the virus (Haase, 2005). The effect of HIV infection on ZAP-70 expression has been previously reported using stimulated PBMC isolated from HIV-infected donors (Schweneker *et al,* 2008) and a stimulated T-cell line infected with HIV *in vitro* (Ottoson *et al,* 2001). Several HIV proteins have been shown to be associated directly with signaling molecules (Morio *et al,* 1997; Sol-Foulon *et al,* 2007) and T-cell activation (Sousa *et al,* 2002; Kinter *et al,* 2003).

Our study found no significant corre-

lation between the MFI of ZAP–70+/CD4+ cells and viral load in untreated individuals (r = -0.4, p = 0.16). The lack of correlation may be due to the small sample size or late stage of infection. The important role of ZAP-70 molecules in HIV-1 infection has been reported (Sol-Foulon *et al*, 2007). Schweneker *et al* (2008) found that basal levels of phosphorylation of ZAP-70 in T-cells in patients with progressive disease were higher than those in long-term nonprogressors and HAART responders. An up-regulated basal state of phosphorylation has been connected with disability of further activation. The results of our study suggest ZAP-70 is required to respond to HIV infection but the precise relationship between ZAP-70 molecules and disease progression is difficult to ascertain.

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