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Monocyte CD49e and 110–120 kDa fibronectin fragments: HIV prognostic indicators independent of viral load and CD4 T-cell counts

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Abstract

Objective—To investigate the prognostic impact of chronic inflammation associated with HIV infections. Previously, we had observed that proteases, released in the course of HIV infections, cause 110–120 kDa fibronectin fragments (FNf) to appear in the blood of many patients. *In vitro*, at concentrations within the range found in patients' plasma, FNf stimulate monocytes to release proteolytic enzymes that remove CD49e from the cell surface and produce cytokines that suppress proliferation of activated T cells when stimulated by agents that crosslink their antigen receptors.

Design—A long-term observational study of patients whose plasma FNf and monocyte CD49e had been measured at 90-day intervals for 1.4 ± 0.5 years.

Methods—Plasma FNf was measured by a quantitative western blot assay and monocyte CD49e expression by flow cytometry. Patients were monitored clinically for up to 5 years after enrollment.

Results—All-cause mortality was significantly higher in patients who had at least 5 µg/ml FNf in more than 50% of plasma samples and/or persistent depletion of monocyte CD49e. Persistence of FNf and depletion of monocyte CD49e were not associated with changes in viral load or CD4 T-cell counts.

Conclusion—Persistently reduced expression of blood monocyte CD49e and/or the persistent presence of FNf in plasma are adverse prognostic markers in HIV-infected patients.

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W.J.P. participated in the study design and the collection and analysis of the data.

J.T. was responsible for critical background studies concerning the biological effects of FNf and helped to design these studies and execute the experiments that provide the basis for this research. She participated in the writing of the manuscript.

F.M.O. participated in the design of the study, the analysis of the data and the writing of this manuscript.

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H.H.B. helped to design these studies, supervise the laboratory work on which it is based and helped to write the manuscript. The hypothesis that persistence of FNf in plasma may adversely affect prognosis is based on her in-vitro studies of the immunomodulatory roles of FNf.

Keywords

cell-binding 110; 120 kDa fibronectin fragments; inflammation; monocytes; patient survival; protease-antiprotease balance

Introduction

Inflammation is accompanied by proteolysis of tissue and plasma proteins. Even in HIV-infected patients receiving antiretroviral therapy, proteolytic enzymes of host and viral origin cause tissue breakdown and remodeling [1–4]. Catabolism of plasma proteins in HIV-infected patients results in the appearance of breakdown products of fibronectin [3]. One prominent fibronectin fragment, referred to as FNf in this study, has a molecular weight of 110–120 kDa, and contains a peptide motif that binds to leukocyte very late activation antigen 5 (VLA-5) (CD49e/CD29). Five microgram per millilitre of FNf, a concentration found in the plasma of many HIV-infected patients, induces leukocyte behaviors that could interfere with control of HIV and other infectious agents [3,5,6]. For example, *in vitro*, FNf stimulate transendothelial migration and subendothelial aggregation of leukocytes into clusters [5,7] that resemble those found around blood vessels of HIV-infected patients [8–10]. Colocalization of infected and uninfected cells in these clusters facilitates HIV-1 transmission [7]. In microenvironments depleted of α -1 antitrypsin, FNf also stimulate monocyte/macrophage cell surface display of proteinase 3 [3]; this hydrolyzes CD49e, thereby decreasing CD49e cell surface expression and VLA-5 function. FNf-induced loss of CD49e significantly reduces the ability of monocytes to migrate on fibronectin-rich collagen matrices [11]. Other fibronectin breakdown products, such as the III1-C fragment, stabilize HIV virions at the surface of infected cells, enhancing their ability to infect susceptible target cells [12].

In addition to promoting extravascular dissemination of HIV-1-infected cells, FNf inhibit host adaptive immune responses. FNf-stimulated monocytes secrete transforming growth factor-beta (TGF β), interleukin (IL)-10 and other immunomodulatory cytokines [6,13]. One effect of these cytokines is to suppress the proliferation of T cells in response to stimuli that crosslink their antigen receptors. We postulate that a similar suppressive effect of FNf *in vivo* would impair patients' ability to mount immune responses that control HIV and secondary infections. To evaluate this hypothesis, we monitored the occurrence of serious clinical events in patients whose plasma FNf levels had been measured repeatedly over a span of 1–2 years [3]. Here, we report the 5-year follow-up of this cohort and present data suggesting that all-cause mortality was associated with the persistent presence of more than 5 μ g/ml FNf in the blood during the early years of this study, particularly in those who also had a decreased fraction of monocytes expressing CD49e.

Materials and methods

Patients, clinical staging, treatment and follow-up

Fifty-one antiretroviral drug-experienced men participated in a longitudinal study [3] under a protocol approved by the Institutional Review Board of Baylor College of Medicine and the Michael E. DeBakey Veterans Affairs Medical Center. At enrollment, six patients were Centers for Disease Control and Prevention (CDC) stage A1, five were A2, seven each were B1 and B2, eight were C2 and 18 were stage C3 [14]. Patients were treated with three or more antiretroviral drugs, prescribed on the basis of sensitivity testing. These included protease inhibitors as well as nucleoside and nonnucleoside reverse transcriptase inhibitors. Plasma FNf levels, viral loads, CD4 T-cell counts and monocyte cell surface proteins, including CD49e, were measured at 90-day intervals [3]. After 1.4 ± 0.5 years (mean $\pm$ SD), the assays for FNf and monocyte CD49e were stopped, but patients were still followed at 90-day intervals until

5 years after the start of the study. Seventeen healthy age-matched and sex-matched controls were monitored concurrently.

Adherence to antiretroviral therapy

Adherence was measured by the timeliness with which patients refilled their prescriptions for antiretroviral drugs [15]. Patients were considered adherent if they picked up monthly refills within 14 days of the due date, 10 months of the year. We required timely refills for only 10 of 12 months because a previous analysis of 351 HIV-infected patients found that the frequency of secondary infections was no different in those who picked up 10 refills on time and those who picked up 12 refills on time over the course of a year [3].

Measurement of total plasma fibronectin, fibronectin fragments and monocyte CD49e

Whole blood was collected into heparinized polypropylene syringes. Leukocyte cell surface markers were identified by flow cytometry the same day [3]. Monocytes were defined as cells that expressed CD14. Plasma was stored at -120°C for later measurement of plasma fibronectin by rocket electrophoresis and FNf by a previously described quantitative western blot technique [3]. Complex formation of α -1 antitrypsin with itself and with various proteolytic enzymes in a subset of plasma samples was also evaluated by western blot analysis [3].

Statistical analyses and graphic representation of data were facilitated by STATISTICA 6 (StatSoft, Tulsa, Oklahoma, USA). Values in text and figures are reported as mean $\pm$ SEM unless otherwise noted.

Results

Circulating fibronectin fragments and long-term prognosis

Patients were stratified based on the frequency with which their plasma contained more than $5\text{ }\mu\text{g/ml}$ FNf during serial studies at 90-day intervals (Fig. 1). The 'infrequent' group ($n = 29$) included 19 patients whose plasma never contained FNf and 10 whose plasma contained FNf in less than 50% samples. The 'persistent' group ($n = 22$) included 17 patients who had more than $5\text{ }\mu\text{g/ml}$ FNf in all plasma samples and five who had this much FNf in more than 50% samples. Thirteen patients died: four from respiratory failure and pneumonia, four from hepatic failure, three from end-stage AIDS, one from sepsis and one from peritoneal tuberculosis and renal failure. Two died within the first year of the study, the remainder 2.4–4.9 years after enrollment. Deaths occurred significantly more often in those with persistent circulating FNf (Gehan–Wilcoxon test 2.36, $P = 0.018$, Fig. 1).

Prognosis correlated with persistence, not with concentration of fibronectin fragments

During the first 1.4 ± 0.5 years of the study, average FNf levels were no different in patients with undetectable viral RNA (<400 copies/ml) and those with measurable viral loads (13.6 ± 2.2 versus $13.7 \pm 2.3\text{ }\mu\text{g/ml}$, $P = 0.73$, t -test). Twelve patients were hospitalized 22 times for secondary infections during the initial 1.4 ± 0.5 years of the study. Those with secondary infections had significantly lower CD4 T-cell counts (196 ± 61 versus 405 ± 21 cells/ μl) and significantly higher viral loads ($73\,130 \pm 39\,499$ versus $18\,700 \pm 6454$ copies/ml) ($P < 0.02$, t -test for both measures). However, their average plasma FNf levels during this time were not significantly different from those in patients who remained free of secondary infections (20.0 ± 5.8 versus $12.8 \pm 1.6\text{ }\mu\text{g/ml}$, $P = 0.16$, t -test). Similarly, average plasma FNf levels were not significantly different in 30 nonadherent and 21 treatment-adherent patients (11.3 ± 1.9 versus $16.3 \pm 2.6\text{ }\mu\text{g/ml}$, $P = 0.12$, t -test), even though medication nonadherent patients had higher viral loads and lower CD4 T-cell counts (viral load: $43\,923 \pm 12\,993$ versus 4507 ± 2804 copies/ml; CD4 T-cell count: 292 ± 25 versus 485 ± 29 cells/ μl ; $P < 0.01$, t -test, for both).

Comparison of fibronectin fragments, CD4 cell count and viral load

During the initial 1.4 ± 0.5 years of the study, CD4 T-cell counts increased more than 10% in 24 patients and remained stable or fell 5 to 67% in 27; persistence of FNf did not differ in these two groups. Viral loads remained elevated in 26 patients and were persistently below the level of detection or became undetectable in 25. Similarly the presence or absence of viral copies in the plasma was not associated with persistence of circulating FNf (Table 1).

Relationship of monocyte CD49e expression and mortality

As previous work showed that incubation with FNf *in vitro* causes monocytes to lose CD49e [3,11], we postulated that measuring monocyte CD49e might provide a surrogate for circulating FNf. *In vitro*, FNf stimulation causes proteinase 3 to migrate from the cytoplasm to the monocyte cell surface [3,11], in which it rapidly degrades the extracellular component of CD49e [11]. *In vivo*, loss of monocyte CD49e was evident only when much of the α -1 antitrypsin in the blood sample was bound up in macromolecular complexes with elastase, proteinase-3 and cathepsin G [3]. As depletion of monocyte CD49e and circulating FNf has the same root cause, a chronic inflammatory response that generates proteolytic enzymes in excess, we postulated that enumerating the fraction of monocytes that express CD49e would also reflect this pathophysiologic process. Consistent with this hypothesis, monocyte CD49e was significantly reduced in patients whose blood persistently contained FNf (Fig. 2, Kruskal–Wallis test 8.59, $P = 0.003$).

To evaluate the prognostic utility of measuring monocyte CD49e, we stratified the patients into two groups: 31 whose monocyte CD49e expression was persistently within the normal range and 20 who persistently had less than 87% CD49e-positive monocytes (2 standard deviations below the mean for healthy donors). Persistent depletion of monocyte CD49e, as with persistence of FNf (Table 1), was not significantly related to changes in CD4 T-cell counts and viral load (Pearson chi-squared 3.36, DF = 3, $P = 0.34$, data not shown). Mortality was increased in patients with persistently reduced monocyte CD49e (Gehan–Wilcoxon 2.17, $P = 0.03$) (Fig. 3a) as was mortality of patients whose monocyte CD49e fluorescence intensity was below the 95th percentile for normal donors (data not shown).

Decreased monocyte CD49e, circulating fibronectin fragments and mortality

In 10 patients, monocyte CD49e was persistently decreased even though the plasma infrequently contained FNf. In an additional 12 patients, monocyte CD49e remained within normal limits despite persistence of FNf in the plasma. Neither decreased monocyte CD49e nor persistent FNf was found in blood from 19 patients. There were two deaths in each of these three groups. However, seven (54%) of the 13 deaths in this study occurred in the 10 patients whose blood persistently contained both decreased numbers of CD49e-positive monocytes and FNf (Fig. 3b).

Discussion

Both persistence of FNf and a low percentage of CD49e-positive monocytes in the blood of HIV-infected patients were associated with increased long-term mortality. In both cases, these associations were independent of viral load or CD4 cell count. All but two of the deaths in this study occurred for 1.3 or more years after we stopped testing the patients' blood, suggesting that once they commence, the mechanisms responsible for generating FNf and depleting monocytes' CD49e [1–3] continue for many years, despite therapy.

We emphasize that it is the fragments of fibronectin, not the native molecule, that are associated with adverse outcomes. Our previous measurements and those of others have demonstrated

that concentrations of native 450-kDa plasma fibronectin are not abnormal in HIV-infected patients [16].

Recent research has linked inflammation, exemplified by high levels of acute phase proteins, D-dimer and IL-6 with increased all-cause mortality in HIV infections [17]. Our studies help to explain how inflammation, normally considered a beneficial response to infection, could accelerate disease progression. A central feature of inflammation is leukocyte activation. Lysosomal proteases, released by activated neutrophils and monocyte/macrophages, readily degrade plasma and matrix fibronectin, producing FNf and other fragments that can stimulate leukocytes and modulate host–pathogen interactions. For example, FNf-stimulated monocytes produce tumor necrosis factor- α (TNF α) [18]. TNF α and other proinflammatory cytokines stimulate the synthesis of the lysosomal proteases that breakdown fibronectin and release FNf. FNf, acting together with TNF α , stimulate HIV-infected and uninfected leukocytes to cross endothelial tight junctions and cluster together in the perivascular matrix, creating an environment wherein transmission of virus from infected to uninfected leukocytes readily occurs [5]. In effect, a positive feedback loop involving TNF α and other proinflammatory cytokines, leukocyte proteases, fibronectin breakdown products [19], including the III1-C fibronectin fragments that can stabilize budding virus and promote cell-to-cell virus transmission [12], may help to produce the depots of infected cells, relatively inaccessible to antiretroviral drugs, that perpetuate this disease.

In addition to the effects of FNf on the propagation and dissemination of virus-infected cells, these fragments may also accelerate disease progression by interfering with adaptive immune responses to opportunistic infections. Exposure to FNf for as little as 15 min *in vitro* causes monocytes to produce sufficient IL-10 and TGF β to suppress proliferation of activated T cells [6]. Thus, FNf, produced as a result of the proteolysis of plasma or tissue fibronectin by leukocyte [3] or viral proteases [20,21], may cause host monocytes to become ‘suppressor’ cells that further impair the already compromised immune response system of HIV-infected hosts.

Although more than 60% of the HIV-infected patients had FNf in their plasma at least once, we never found FNf in serial samples collected concurrently from 17 normal controls [3]. As FNf are generated by the unopposed actions of diverse proteases, this comparison suggests that the generation of protease by HIV can outstrip the host’s ability to produce proteinase inhibitors. The concentrations of active, functional α -1 antitrypsin in patients with advanced HIV infections are reportedly low to normal [22]. As α -1 antitrypsin is an acute phase reactant, its concentration should rise with inflammation [22,23]. Low-to-normal α -1 antitrypsin levels in plasma from HIV-infected people, beset by a persistent inflammatory response engendered by this chronic virus infection, may indicate either that this proteinase inhibitor is produced in subnormal amounts or that it is being consumed and/or inactivated at an accelerated rate. Prior experience favors the latter explanation. In 15 plasma samples that contained between 38 and 100 μ g/ml FNf, we previously found that α -1 antitrypsin was often taken up in high molecular weight complexes with elastase, proteinase-3 or cathepsin G [3]. In these blood samples, the fraction of monocytes that expressed CD49e was reciprocally related to the quantity of α -1 antitrypsin that was incorporated in these complexes [3]. As α -1 antitrypsin in complexed form is not available to bind and inactivate proteinase 3, as it is expressed on the monocyte cell surface, it follows that CD49e on monocytes in blood containing FNf is at risk of being hydrolyzed.

Thus, deficiency of CD49e on patients’ circulating monocytes likely indicates that their microenvironment is deficient in functional proteinase inhibitors, most particularly α -1 antitrypsin, the inhibitor that opposes the protease responsible for FNf-induced loss of monocyte CD49e [3]. Increased mortality was as significantly associated with persistently

reduced monocyte CD49e as with the persistence of FNf in these patients' blood samples. Unfortunately, we were unable to systematically measure the relationship between monocyte CD49e display and α -1 antitrypsin function in the plasma samples of all patients; the potential importance of such an analysis became apparent only when we examined the 5-year survival results, by which time the antiprotease activity of the stored plasma could no longer be reliably measured.

α -1 antitrypsin, by itself, has antiretroviral activity. Physiological concentrations of this antiproteinase block HIV-1 infection of permissive target cells and inhibit viral replication in previously infected peripheral blood mononuclear cells [24]. Delivery of α -1 antitrypsin genes into cell lines and into primary human lymphocytes blocks HIV gp160 and p55 processing [25]. A carboxyterminal fragment of α -1 antitrypsin reportedly suppresses HIV transcription [26]. Thus, loss of functional α -1 antitrypsin in HIV-1-infected people may deprive the host of an element of the innate immune system that helps to control HIV replication. Increased viral replication, in turn, likely increases the inflammatory responses producing the proteases that generate FNf and inactivate α -1 antitrypsin.

Although HIV-specific aspartyl protease inhibitors have clearly had a major impact on the treatment of HIV-1 infections [27], the results of this study suggest that agents that suppress tissue injury mediated by other classes of proteolytic enzymes [28,29] or that amplify naturally occurring proteinase inhibitors may also contribute significantly to the control of this retroviral infection [24,30]. Indeed, if future studies show an association among FNf, depressed monocyte CD49e expression and depletion of functional α -1 antitrypsin, exploration of the effects of treatment with α -1 antitrypsin should be considered. In the interim, monitoring circulating FNf, or more easily, from a technical standpoint, the fraction of blood monocytes that express CD49e, may help to identify patients in whom the inflammatory response to this chronic infection may adversely affect prognosis.

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Fig. 1. Persistence of circulating fibronectin fragments and mortality

(a) Mortality was significantly greater in patients whose plasma frequently contained FNf, for example, in more than 50% of their samples (dotted line), compared with patients who had FNf in less than half of their samples (solid line) (Gehan–Wilcoxon test 2.36, $P = 0.018$). (b) Frequencies of patients with no FNf ($n = 19$) or with FNf in less than half of their serial samples ($n = 10$) (dark grey bars) versus those who had FNf in all ($n = 17$) or more than 50% ($n = 5$) of their plasma samples (slashed bars). FNf, fibronectin fragments.

Fig. 2. Patients' monocytes expressed CD49e less frequently than concurrently studied healthy controls

However, CD49e was significantly less frequently expressed on monocytes of patients whose serial blood samples persistently contained more than 5 µg/ml FNf (Kruskal–Wallis test 8.59, $P = 0.003$). Box shows the standard error, and the bars indicate the 95% confidence intervals of the mean. FNf, fibronectin fragments.

Fig. 3. Monocyte CD49e expression: relation to survival

Survival was significantly lower in patients who had reduced monocyte CD49e expression (Gehan–Wilcoxon test = 2.18, $P = 0.029$; panel a). The increased mortality associated with reduced monocyte CD49e expression was particularly evident in patients whose plasma persistently contained FNf (Gehan–Wilcoxon test 3.63, $P = 0.00028$; panel b). FNf, fibronectin fragments.

Table 1

Relationship of viral load and CD4 T-cell numbers with fibronectin fragments.

	FNf infrequent	FNf persistent
Viral load remained elevated	14*	12
Viral load became undetectable	15	10
Change in VL versus FNf frequency: $\chi^2 = 0.26$, DF = 1, $P = 0.87$		
Increasing CD4 T-cell numbers	18	8
Stable or decreasing CD4 T cells	11	14
Change in CD4 T cells versus FNf frequency: $\chi^2 = 2.36$, DF = 1, $P = 0.12$		

DF, degree of freedom; FNf, fibronectin fragments; VL, viral load.

* Numbers indicate patients in each category.