Specific Changes in the Posttranslational Regulation of Nucleolin in Lymphocytes from Patients Infected with Human Immunodeficiency Virus

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Lymphocytes isolated from human immunodeficiency virus (HIV)–infected patients have dysregulated cell-cycle control, consisting of increased activation of the cyclin B1/p34 cdc2 complex and abnormal nucleolar structure. To better characterize the molecular features of the HIV-associated cell-cycle perturbations, we performed a detailed analysis of the posttranslational regulation of nucleolin, a key structural protein in the nucleolus. We found that, in concanavalin A–stimulated lymphocytes from HIV-infected patients, the inappropriate activation of the cyclin B1/p34 cdc2 kinase complex is temporally associated with increased threonine phosphorylation, augmented fragmentation, and prominent extranuclear and cell-surface localization of nucleolin. Importantly, increased lymphocyte apoptosis is observed at the time of cell-surface localization of nucleolin. These results may delineate a direct molecular link between abnormal activation of cyclin B1/p34 cdc2 and the changes in the nucleolar structure, thus providing a better molecular definition of HIV-associated cell-cycle dysregulation.

The natural history of human immunodeficiency virus (HIV) infection is characterized by progressive CD4+ T cell depletion that occurs in the setting of chronic hyperactivation of the immune system [1, 2]. Although the "direct" cytopathic effect of HIV on infected CD4+ T cells is a well-established pathogenic phenomenon, the T cell depletion of HIV-infected patients is also related to the apoptotic cell death of large numbers of uninfected CD4+ and CD8+ T cells [3–5]. In previous studies, we found that peripheral blood lymphocytes (PBLs) from HIV-infected patients show complex perturbation of cell-cycle control, consisting mainly of increased intracellular levels of cyclin B1, with consequent inappropriate activation of the p34 cdc2 kinase, and of abnormal nucleolar structure, as shown by staining for the argyrophilic nucleolar organizing regions (AgNORs) and the subcellular localization of nucleolin by confocal microscopy [6–8]. Although these observations are compatible with the presence of a large fraction of lymphocytes committed to the cell cycle, both the DNA content and the metabolic pattern of the same cells indicate a resting G0 state [6, 7]. This discrepancy between the metabolic/biochemical profile and the expression of phase-dependent proteins led us to introduce the concept of HIV-associated cell-cycle dysregulation (CCD). Importantly, these abnormalities of CCD are exacerbated in vitro by mitogens and are associated with induction of apoptosis, but they can be ameliorated when lymphocytes are exposed to interleukin (IL)–2 in vitro [8]. On the basis of these findings,
we have proposed that, during HIV infection, CCD may represent a biological link between the increased activation and/or turnover and the high levels of apoptosis observed in uninfected T lymphocytes [4, 5].

To better understand the molecular pathogenesis of the nucleolar abnormalities involved in HIV-associated CCD and to define the basis of the apparently abnormal pattern of subcellular localization of nucleolin [7, 8], we have focused our attention on the posttranslational changes of nucleolin. Nucleolin is one of the key proteins of the nucleolar matrix and is involved in rDNA transcription, ribosomal RNA maturation, ribosome assembly, and chromatin condensation/decondensation [9, 10]. In addition, nucleolin plays a role in cell proliferation, nuclear/cytoplasmic shuttling, and apoptosis [11–15]. Posttranslational regulation of nucleolin is important in determining both the function and subcellular localization of this molecule [9, 10, 16]. One important posttranslational modification of nucleolin is phosphorylation of either threonine or serine residues, an event that is strictly controlled during the progression through cell cycle [9, 10, 16]. Because nucleolin is phosphorylated on threonine residues by the cyclin B1–dependent p34 cdc2 kinase during the G2-M phase of the cell cycle [17], we hypothesized that, in lymphocytes of HIV-infected patients, increased levels of cyclin B1 may translate into increased levels of threonine-phosphorylated nucleolin, which, in turn, would make this molecule more prone to fragmentation and membrane localization [17, 18].

Here, we report data from a series of experiments performed on in vitro–activated T lymphocytes of HIV-infected patients and control subjects, in which we show the following: (1) the existence of a series of temporally related changes in the posttranslational regulation of nucleolin; (2) how these changes are involved in determining the observed abnormalities in the nucleolar structure; and (3) how their appearance is associated with the presence of activation-induced apoptosis.

**MATERIALS AND METHODS**

**Patient population.** The present study included 20 asymptomatic, untreated HIV-infected patients with a mean (± SD) HIV RNA load of 41,050 ± 34,465 copies/mL (range, 7,000–101,000 copies/mL) and a mean (± SD) CD4+ cell count of 334 ± 128 cells/μL (range, 208–625 cells/μL). Twenty uninfected individuals served as control subjects. Informed consent was obtained from patients, and human experimentation guidelines of the authors’ institutions were followed in the conduct of clinical research. Blood samples were obtained, and HIV loads were measured by use of a bDNA technique (Quantiplex; Chiron).

**Lymphocytes studies.** For the in vitro activation studies, PBLs were isolated by gradient purification, according to standard procedures, and were cultured in 10% fetal calf serum and RPMI 1640 medium, at an initial density of 10^6 cells/mL. To induce lymphocyte activation, recombinant IL-2 (rIL-2; 50 UI/mL) or concanavalin A (ConA; 5 μg/mL) or both was added. After activation, cells were monitored for ornithine decarboxylase activity, proline uptake, production of IL-2, and activity of cell machinery for protein and DNA synthesis (data not shown). All cell cycle–related metabolic parameters were measured as described elsewhere [7, 8]. Studies of purified CD4+ and CD8+ T cell subpopulations were performed with cells isolated from total peripheral blood mononuclear cells with a FACS Vantage cell sorter (Becton Dickinson). Purity of the sorted subpopulations was always >97%.

**Western-blot analysis.** Nucleolin expression was measured by Western-blot analysis (monoclonal antibodies from Santa Cruz Biotechnology; 1:1000 dilution), and the bands were analyzed with SigmaGel (Handel Scientific); 2–3 replicas were performed for each sample. Internal controls always were performed, including lysis of equal cell numbers and loading equal volumes of protein.

**Immunoprecipitation of nucleolin.** Lymphocytes were lysed in 10 mmol/L Tris (pH 7.4), 1% SDS, 1 mol/L NaVO₃, and 50 mol/L NaF. After separation of the soluble fraction by centrifugation, the cell lysate was precleared by adding 50 μL of protein G agarose beads/1 mL of lysate, followed by incubation at 4°C for 30 min on an orbital shaker. The protein G agarose beads were removed by centrifugation, and a portion of the lystate corresponding to 300–500 μg of total protein was immunoprecipitated with 3–5 μg of anti-nucleolin antibody (Santa-Cruz Biotechnology) in 1 mL of lysis buffer. After 2 h of incubation at 4°C, protein G agarose was added for an additional 60 min. The immune complexes were harvested by centrifugation, washed once with 1× lysis buffer, washed twice with PBS, resuspended in an equal volume of 2× sample buffer, and boiled. The samples were separated on SDS-PAGE and subjected to immunoblot analysis. The membranes were incubated with a 1:1000 dilution of a phospho-threonine polyclonal antibody (Cell Signaling Technology) and with a 1:1000 dilution of a nucleolin monoclonal antibody.

**p34 cdc2 kinase assay.** After 24 h of activation with ConA, p34 cdc2 kinase activity in crude cell lysates was measured as the degree of phosphorylation of a specific substrate (histone-1), by use of the transfer of the γ-phosphate of adenosine-5’-[³²P] triphosphate ([³²P]ATP). The assay kit (Upstate Biotechnology) includes 3 inhibitors of other kinases (protein kinase A, protein kinase C, and calmodulin-dependent kinase) that could be responsible for histone-1 phosphorylation. The phosphorylated substrate was then separated from the residual [³²P]ATP by use of p81 phosphocellulose paper and was quantified by use of a scintillation counter (Packard Camberra).

**AgNOR staining.** AgNOR staining was performed as de-
permeabilization, propidium iodide (5 μM) was added. After cell washes, fluorescein isothiocyanate–conjugated goat anti–mouse immunoglobulin (1:200 dilution; Sigma) was added. After cell permeabilization, propidium iodide (5 μg/mL) plus RNAse (200 μg/mL) were added for 30 min. In addition, costaining with phycoerythrin-labeled annexin V (10 μL) also was performed. The Confocal microscope was from Leica, with a 63× zoom 1.6 objective.

RESULTS
In activated PBLs from HIV-infected patients, increased p34 cdc2 activation is associated with increased threonine phosphorylation and fragmentation of nucleolin. A main feature of HIV-associated CCD is the increased intracellular level of cyclin B1, a phenomenon observed in freshly isolated T cells and enhanced by activation with ConA [6]. Consistent with the presence of increased intracellular levels of cyclin B1, stimulation of PBLs isolated from HIV-infected patients, with ConA for 24 h, resulted in high levels of p34 cdc2 activity, as measured by histone-1 phosphorylation (figure 1A). Because the presence of high p34 cdc2 activity may result in nucleolin phosphorylation on threonine residues [16, 17], we then measured the level of threonine phosphorylation of nucleolin after stimulation with ConA. To this end, we first immunoprecipitated the total cell lysate with an anti–nucleolin antibody and then performed immunoblotting with an anti–phosphothreonine antibody. We found that, in T cells from both HIV-infected patients and control subjects, most of the threonine-phosphorylation signal was detected in 2 fragments of nucleolin, p33 and p29 (figure 1B), a finding consistent with the fact that threonine phosphorylation of nucleolin is known to promote its fragmentation [9, 18]. Interestingly, in ConA-activated PBLs from HIV-infected patients, the level of threonine phosphorylation of these nucleolin fragments was increased >2-fold, compared with the level in PBLs isolated from uninfected control subjects (figure 1C; P < .05).

To determine whether the increased threonine phosphorylation of nucleolin that follows ConA activation in PBLs from HIV-infected patients is associated with increased rates of nucleolin fragmentation, we then measured the ratio between fragmented and nonfragmented nucleolin by Western-blot analysis. As shown in figure 1D, the fragmented to nonfragmented ratio of nucleolin was ~3-fold higher in ConA-stimulated PBLs from HIV-infected patients, compared with those from control subjects (P < .01). The fact that the bands shown in figure 1D are indeed nucleolin fragments is indicated not only by their reaction with the anti–nucleolin monoclonal antibody, but also by their apparent molecular weights of 72 and 58 kDa, which is consistent with the described size of the most commonly represented nucleolin fragments found in Western-blot experiments [20]. It should be noted that, because of the exquisite sensitivity of nucleolin to proteolysis, smaller (33 and 29 kDa) fragments tend to predominate when more-complex procedures (i.e., immunoprecipitation) are performed on cell lysates (figure 1B). These results indicate that, in PBLs from HIV-infected patients, the increased activity of p34 cdc2 kinase after activation with ConA is temporally associated with 2 specific changes in the posttranslational regulation of nucleolin: increased levels of threonine phosphorylation and higher rates of fragmentation.

HIV-associated CCD is present in both CD4+ and CD8+ T cell subsets. Our previous observations suggest that HIV-associated cell-cycle abnormalities are not limited to CD4+ T cells that are directly infected with HIV but, in fact, involve a large number of uninfected T lymphocytes of both CD4+ and CD8+ lineage [6–8]. To confirm this earlier observation and to determine whether the HIV-associated changes in the posttranslational regulation of nucleolin also involve both CD4+ and CD8+ T lymphocytes, we analyzed the levels of cyclin B1 expression, nucleolar structure, and levels of nucleolin fragmentation in sorted CD4+ and CD8+ T cell subpopulations. As shown in figure 2A, we found similar levels of cyclin B1 expression in both CD4+ and CD8+ T cells, as measured by multiparametric flow cytometric analysis.

Consistent with this observation is the finding that both CD4+ and CD8+ T lymphocytes isolated from HIV-infected patients included a large proportion of cells showing abnormal nucleolar structure, as assessed by AgNOR staining (figure 2B). In addition, the levels of activation-induced, phosphorylation-dependent nucleolin fragmentation (figure 2C) were also similar in sorted CD4+ and CD8+ T cells that were isolated from HIV-infected individuals. These findings are consistent with the hypothesis that HIV-associated CCD is not caused directly by the virus, which would likely result in predominant abnormalities in CD4+ T cells, but rather is caused by HIV-related hyperimmune activation, which is known to involve both CD4+ and CD8+ T cells [21–23].

In vitro pretreatment with rIL-2 reestablishes the posttranslational regulation of nucleolin in ConA-activated lymphocytes from HIV-infected patients. In an earlier study, we...
observed that in vitro treatment with rIL-2 reverts the CCD of lymphocytes from HIV-infected patients [8]. This finding is consistent with the potential role of IL-2 as a survival factor for activated T lymphocytes and led us to propose that the beneficial effect of IL-2 on HIV-associated CCD may provide a new rationale for IL-2 therapy in patients with AIDS [8]. To analyze the effect of IL-2 on the HIV-associated changes in the posttranslational regulation of nucleolin, we measured the levels of threonine phosphorylation–induced fragmentation of nucleolin in lymphocytes that were activated with ConA for 24–48 h and were either pretreated with rIL-2 or left untreated. We found that the abnormally increased levels of nucleolin fragmentation present after 24 h (figure 1D) and 48 h (figure 3A, lane 2) of ConA activation in lymphocytes from HIV-infected patients were not observed when the same lymphocytes were pretreated with rIL-2 (figure 3A, lane 1, and figure 3B).

These results indicate that pretreatment with rIL-2 has a protective effect on the abnormal posttranslational regulation of nucleolin that we have observed in mitogen-activated lymphocytes from HIV-infected patients. Importantly, this finding is consistent with the previous observation that rIL-2 treatment reverts the HIV-associated CCD in vitro [8] and supports the hypothesis of a potential in vivo antiapoptotic role of IL-2 when administered to HIV-infected patients.
Figure 2. Cell-cycle dysregulation in both CD4+ and CD8+ T cells. A, Percentage of CD4+ and CD8+ T cells expressing cyclin B, as measured by multiparametric flow cytometry, on cells from human immunodeficiency virus (HIV)-infected patients (n = 8), with active viral replication (i.e., virus load >5000 copies/mL) and CD4+ T cell counts of 200–500 cells/mm³, and from uninfected healthy control subjects (n = 8). B, Pattern of argyrophilic nucleolar organizing region (AgNOR) staining in representative samples of sorted CD4+ and CD8+ T cells from the same group of HIV-infected patients (left) and average no. of AgNOR dots in HIV-infected patients and control subjects (right). Nos. in the box indicate the average total area of AgNOR staining per cell. At least 500 cells/sample were examined. C, Detection of the p72 and p58 fragments of threonine-phosphorylated nucleolin by Western blot test in sorted CD4+ and CD8+ T cells from a representative HIV-infected patient belonging to the same cohort.
In ConA-activated lymphocytes from HIV-infected patients, threonine phosphorylation and fragmentation of nucleolin are associated with its cell surface localization. Extracellular and cell-surface localization of nucleolin are enhanced by posttranslational covalent modifications that include threonine phosphorylation and fragmentation [9, 16]. In unstimulated lymphocytes from healthy control subjects, nucleolin staining is mainly confined to the nucleolus, whereas unstimulated PBLs from HIV-infected individuals show an enlarged area of nucleolin distribution with multiple spots [7].

To determine whether 24 h of stimulation with ConA induces further changes in the subcellular localization of nucleolin in PBLs from HIV-infected patients, we performed a series of analyses with confocal microscopy. As shown in figure 4A, activation with ConA, for 24 h, induced only a minimal level of membrane localization of nucleolin in lymphocytes from control subjects. In contrast, ConA-activated lymphocytes from HIV-infected patients consistently showed a markedly increased level of cell-surface nucleolin staining (figure 4B). Quantitative analysis of the surface expression of nucleolin indicated that the number of lymphocytes with positive membrane staining for nucleolin was nearly 3 times higher in HIV-infected patients than in control subjects (figure 4C). Because threonine phosphorylation of nucleolin is known to enhance its localization outside the nucleus and on the cell surface [9, 16], it is conceivable that, in our system, the increased level of p34 cdc2-mediated, threonine phosphorylation of nucleolin we observed is involved in the membrane localization of nucleolin and/or its fragments.

Cell-surface localization of nucleolin is associated with increased apoptosis, as detected by annexin V staining. In previous studies, we showed that the CCD of lymphocytes from HIV-infected patients is correlated with an increased susceptibility to activation-induced apoptosis [7, 8]. To determine whether changes in the posttranslational regulation of nucleolin are associated with increased levels of apoptosis among in vitro-activated lymphocytes from HIV-infected patients and control subjects, we visualized the costaining of these cells with an anti-nucleolin antibody and annexin V, by use of confocal microscopy. Annexin V positivity is a marker of apoptosis because it binds apoptotic cells that express phosphatidylycerine on their outer membrane layer [24]. We observed that colocalization of nucleolin and annexin V is commonly seen in ConA-activated lymphocytes from HIV-infected patients (figure 4E) but not in cells from uninfected control subjects (figure 4D). Of importance, in ConA-activated PBLs from HIV-infected patients, the majority of cells that were positive for surface localization of nucleolin also stained positive for annexin V (figure 4F). These results indicate that ConA-activated PBLs from HIV-infected patients show a 3-fold increase in the number of cells expressing nucleolin on their surface and that this increased cell-membrane localization of nucleolin is temporally associated with the biochemical changes typical of apoptotic cell death.

DISCUSSION

A well-known paradox of HIV disease is that a state of immunodeficiency is associated with high levels of lymphocyte activation and turnover [4, 5, 22]. It has been recently proposed that the perturbations of T cell dynamics that are associated with this chronic hyperimmune activation play a major pathogenic role in inducing the CD4+ T cell depletion seen in patients with AIDS [25]. This model is also supported by our studies of the nonpathogenic simian immunodeficiency virus infection in a natural host monkey species, the sooty mangabeys, indicating that, in the absence of the bystander effects of immune activation, the animals maintain near-normal CD4+ T cell counts and avoid disease despite high levels of viral replication [26]. In previous studies aimed at defining the potential biological links between hyperimmune activation and lymphocyte apoptosis [6–8], we observed that PBLs from HIV-infected pa-
Regulation of Nucleolin in HIV Infection

Figure 4. Membrane localization of nucleolin and costaining with annexin V in peripheral blood lymphocytes obtained from human immunodeficiency virus (HIV)–infected patients (n = 20) and control subjects (n = 20). Assessment of the subcellular localization of nucleolin was performed by confocal microscopy after 24 h of stimulation with concanavalin A (ConA). A–C, Detection of membrane-bound nucleolin (green) was performed before cell permeabilization and was followed by propidium iodide staining (red) after cell permeabilization. D–F, Membrane colocalization of nucleolin (red) and annexin V (green) was performed in nonpermeabilized lymphocytes. Images are derived from selected experiments on lymphocytes isolated from healthy control subjects (A and D) and HIV-infected patients (B and E). To quantify the no. of cells showing surface nucleolin staining (C) or showing costaining for nucleolin and annexin V (F), we took the average of the percentage of positive cells (500 cells/sample) for each sample.

Patients consistently show a discordance between different biochemical indicators of the cell cycle. Specifically, we found both an increase in the expression of phase-dependent proteins, suggesting an advanced cell cycle phase, and a DNA content and biochemical/metabolic profile that indicate a resting phase. These latter findings are consistent with the results of several studies, directed at assessing lymphocyte turnover during HIV infection and performed by using various techniques (i.e., Ki67 staining, telomer length analysis, BrdU staining, and 2H-glucose labeling), that have indicated that only a minority of circulating lymphocytes are actively proliferating during HIV disease [27–30]. We thus concluded that the discrepancy observed between the expression of phase-dependent proteins and the biochemical/metabolic profile represents a major perturbation of cell cycle regulations (i.e., CCD).

To expand our analysis of the molecular features of HIV-associated CCD, we investigated a possible relationship between the abnormal level and/or activation of the cyclin B1/p34 cdc2 kinase complex and the abnormalities of the nucleolar structure. Among the nucleolar proteins, a prominent role is played by nucleolin, a 77-kDa multifunctional protein whose posttranslational modifications exert a profound influence on both its function and subcellular localization [9, 10, 16]. We hypothesized that the abnormal nucleolar structure observed in PBLs from HIV-infected patients is related to changes in the posttranslational regulation of nucleolin, which, in turn, may be related to the inappropriate p34 cdc2 activation that results from the increased level of cyclin B1. Our results show that, after activation with ConA for 24 h, PBLs from HIV-infected patients manifest a consistent increase in the threonine phos-
 phosphorylation of nucleolin. This event is temporally associated with an increase of p34 cdc2 kinase activation, thus supporting the hypothesis that the inappropriate activation of the kinase p34 cdc2 may be responsible for the increased threonine phosphorylation of nucleolin observed in ConA-activated PBLs from HIV-infected patients. Of importance, lymphocytes from HIV-infected patients also show an increase in nucleolin fragmentation and nucleolin membrane localization, 2 events facilitated by increased levels of threonine phosphorylation of the molecule [9, 17, 18]. Finally, the surface localization of nucleolin was often observed in annexin V-positive lymphocytes undergoing apoptosis, indicating a strict temporal correlation between changes in the posttranslational regulation of nucleolin and the activation of apoptotic pathways in lymphocytes from HIV-infected patients.

The fact that several cell-cycle abnormalities—including cyclin B1 overexpression, abnormal AgNOR staining, and increased nucleolin fragmentation—are observed in both CD4+ and CD8+ T cells is consistent with previous observations indicating that HIV-associated CCD involves both T cell subsets [6–8]. These new data further support the idea that CCD in cells from HIV-infected patients is not caused directly by HIV or its gene products. Of importance, we found that, in ex vivo-activated lymphocytes from HIV-infected individuals, the observed changes in the posttranslational regulation of nucleolin are corrected by pretreatment with rIL-2. This finding is consistent with the previous observation that IL-2 reverts HIV-associated CCD [8] and provides a novel rationale for the therapeutic use of IL-2 during HIV infection.

Taken as a whole, the results of this study indicate that, in activated T cells from HIV-infected patients, the increased level of cyclin B1 and the resultant inappropriate activation of its catalytic partner, p34 cdc2 kinase, are temporally associated with increased levels of threonine-phosphorylated nucleolin, an increased ratio of fragmented to nonfragmented nucleolin, increased cell-surface localization of nucleolin, and increased levels of apoptosis. These data may provide a better understanding of the molecular mechanisms underlying HIV-associated CCD by delineating a direct biological link between increased levels of cyclin B1 and abnormalities in the nucleolar structure. The exact relationship between the presence of CCD and the increased sensitivity to apoptosis remains to be elucidated. Although it has been suggested that nucleolin may have proapoptotic potential [9, 14, 15], at present, we do not have data proving a direct causal relationship between nucleolin fragmentation and induction of apoptosis.

In conclusion, we have shown that specific changes in the posttranslational regulation of nucleolin are consistently found in lymphocytes from HIV-infected patients and that their presence may represent a link between inappropriate cyclin B1/p34 cdc2 activation and increased susceptibility to lymphocyte activation–induced apoptosis during HIV infection.

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References