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Letter to the Editor

ScopeMed

Preventive effect of ascorbic acid against biological function of human immunodeficiency virus trans-activator of transcription

To elucidate the inhibition mechanism of human immunodeficiency virus type 1 (HIV-1) replication by ascorbic acid (AA), we have investigated and compared the effect of noncytoxic concentrations of AA on HIV-1 replication. Using trans-activator of transcription (TAT) expressing cells or non-expressing cells transfected HIV-1-long terminal repeat (LTR) chloramphenicol acetyl transferase (CAT) plasmid, we examined the action of AA on TAT dependent transcriptional activation of provirus *HIV-1* gene through enhancer/promoter of HIV-1-LTR. In TAT expressing cells, AA strongly reduced the levels of intracellular CAT activity in a dose-dependent manner. Alternatively in TAT non-expressing cells, CAT activity was reduced somewhat. Using other *in-vivo* and *in-vitro* experiments, AA inhibited the activity of TAT dependent *HIV-1* genome RNA elongation but did not inhibit the activity of basal transcriptional activation of provirus *HIV-1* gene. The intracellular *HIV-1* genome RNA pattern in AA treated cells infected with HIV-1 showed significant differences in the synthesis and the processing of individual viral RNA compared to the patterns of untreated controls. HIV-1 transcription was specifically reduced because in contrast to HIV-1 transcription, transcriptional activities through other viral promoters were not reduced by treatment of AA. Furthermore, the activation of transcription factors was not affected by treatment of AA. These results show that AA specifically inhibits the replication of HIV-1 on down-regulation of TAT dependent *HIV-1* genome RNA elongation.

Previous reports demonstrated the antiviral activity of AA against a broad spectrum of RNA and DNA viruses including polio virus, herpes virus, HIV-1 *in-vivo* and *in-vitro* [1-5]. Already, it has reported that the suppression of virus production and cell fusion in HIV-1 infected $CD4^+$ T-cells grown in the presence of non-toxic concentration of AA. Among the earliest studies on viral replication, it is reported that the growth of HIV-1, after the first replication cycle, was suppressed by the addition of AA, glutathione-SH (GSH), N-Acetyl L-Cysteine (NAC), butylated hydroxyanisole (BHA) or α-tocopherol/vitamin E to human diploid-cell culture [6-11]. There is increasing evidence that reactive oxygen intermediates (ROIs) play an important role in cellular processes such as signal transduction and the controlling gene expression. As actions of GSH and NAC such as thiol-containing antioxidants on the replication of HIV-1 is previously reported, GSH and NAC reduce the target DNA binding activities of nuclear factor κ-B (NF-κB), AP1 (Jun/Fos) or upstream stimulatory factor (USF), by oxidation-reduction (redox) regulation system [6,9,12-17]. It is reported that when these antioxidants such as GSH, NAC, and BHA are added into culture medium, they play such as radical scavenger in the cytsole of cells stimulated by tumor necrosis factor-alpha (TNF-α) or H_2O_2 and then the induction of NF-κB activity by these stimuli is blocked [9]. The suppression of the HIV-1 replication by GSH or NAC is caused by the inactivation of these transcriptional factors by redox system [9,12,14,18]. Furthermore, activation of NF-κB induced by TNF-α is reduced by treatment of vitamin E [9,11]. AA may be considered to play as antioxidant free radical scavenger such like GSH or NAC [19]. Thus, it is possible to regulate DNA binding activity of NF-κB by AA. However, the previous report shows that the life cycle of HIV-1 is suppressed by treatment of 100 μg of AA per ml (0.57 mM) [8], which is more low concentration than NAC as 30 mM (4.9 mg/ml) [18]. Furthermore, it was not established whether AA exerted a virus-specific effect or interacted directly with the activating substances. In several earliest reports, it is demonstrated that the inhibitory effect of AA is not directed inactivation of transcriptional factors such as NF-κB, USF [8,20]. The several research groups already reported that when HIV-1 infected $CD4^+$ T-cells was treated by AA with NAC, the release of HIV-1 particles was reduced about 2^{nd} or 3^{rd} fold than with AA alone or NAC alone [7]. It is speculated from these observations that AA inhibits the replication of HIV-1 by other system except redox system. Furthermore, the virus particle production and the cell fusion are reportedly suppressed in HIV-1 infected $CD4^+$ T-cells grown in the presence of non-toxic concentrations of AA, this report shows that the metabolism in cells is not affected by treatment of these concentrations of AA. AA may specifically block one or several points on the steps of the HIV-1 replication cycle. TAT, REV, VIF, VPR are reported to function as viral regulating proteins which specifically play on HIV-1 replication. In addition, the complex nature of the genome and the action of the two best-characterized viral trans-acting regulatory gene products, TAT and REV, indicate that HIV-1 has an efficient way of regulating its own gene expression during its infection cycle [21-29]. Actions of TAT and REV are so much important for regulation of HIV-1 life cycle [16-23,28,30]. The regulation mechanisms of HIV-1

replication by these viral proteins are not clearly revealed, but if regulation activities of TAT or REV are reduced by AA, this riddle of AA is solved. We have investigated the action of AA on HIV-1 life cycle under the controlled conditions *in-vivo* and *in-vitro*. AA specifically is demonstrated to inhibit TAT dependent *HIV-1* genome RNA elongation system using *in-vitro* and *in-vivo* experiments.

It was reported by several research groups that continuous exposure of HIV-1 infected $CD4^+$ T-cells to non-cytotoxic AA concentrations resulted in significant inhibition of both virus replication in chronically HIV-1 infected cells and multinucleated giant-cell formation in acutely HIV-1 infected CD4+ T-cells [4,7,8,20]. However, the molecular mechanism by which AA suppresses HIV-1 replication was not fully understood yet. There is increasing evidence that ROIs play an important role in cellular processes such as signal transduction and the control of gene expression [13,14]. The suppression of HIV-1 replication is caused by NF-κB, AP1 and USF, which are down-regulated by the redox system of antioxidants such as NAC, GSH, and BHA [7,9,18]. When AA was added together with NAC into the culture medium, extra viral reverse transcriptase (RT) was reduced to 20.0% of the control, compared with values of 30.0% and 50.0% seen, respectively, with AA alone and NAC alone [7]. This result indicates that there are the different target point between AA and NAC. Several data indicate that HIV-1 suppression by AA was not due to secondary effects resulting from inhibition of cellular growth or metabolic activity. In recent report and this study, it is demonstrated that activities of transcription factors are not reduced by AA treatment [20]. The experimental evidence presented in this study has demonstrated that AA could inhibit the HIV-1 replication by blocking the regulation on the step of TAT dependent *HIV-1* genome RNA elongation [Figure 1]. AA dose not inhibit the basal transcriptional activation of HIV-1, however as shown in *in-vitro* and *in-vivo* experiments, the TAT dependent transcriptional activation are strongly reduced by AA treatment [Figure 1]. The several experiments demonstrate that this suppression of *HIV-1* genome RNA expression was not caused by reducing activities of basal transcriptional factors containing RNA polymerase II and transcriptional factors, NF-κB, SP1, USF. Further, the earliest report shows that HIV-LTR-directed β-galactosidase expression in transiently transfected Jurkat cells is not inhibited by AA [20]. The *in-vivo* experimental evidence presented in this study has revealed that the inhibition of HIV-1 replication by treatment with AA is caused by inhibition of TAT dependent RNA elongation, but the basal transcriptional activation through HIV-1-LTR is not affected by treatment with AA. Furthermore, comparison of intracellular *HIV-1* genome RNA patterns in AA treated cells with corresponding patterns of untreated controls showed significant differences in the synthesis of viral genome RNAs. Importantly, the smallest *HIV-1* genome RNAs 2.0 kb were detected in cells treated by AA, TAT protein translated from smallest RNAs possibly was exists in cells, but other length RNAs were not detected by RNA blot. Thus, the results indicated in *in-vitro* expreriments show that TAT dependent *HIV-1* genome RNA elongation system was strongly inhibited by AA. It is demonstrated in several reports that TAT could activate transcriptional activation and *HIV-1* genome RNA elongation after forming initiation complex with cellular cofactors [29,31-37]. Furthermore, the known species tropism of TAT protein appears to arise from the fact that not only TAT but also the cellular cofactor can markedly influence the RNA sequence specificity of the resultant protein complex [29,31]. In earlier studies, P-TEFb expressing in $CD4^+$ T-cells recognizes the loop structure in HIV-1 trans-activation response (TAR) and forms proteins/TAR complex, negative elongation factor, which directly interacts TAT protein [29,31,33,38], then the activation of transcription and *HIV-1* genome RNA elongation is activated by these proteins/TAT/TAR complexes [2,30]. In other result, Mammalian Suppresser of Sgv1 (MSS1), which strongly expresses in $CD4^+$ T-cells, activates with TAT the transcription through the promoter/enhancer of HIV-1-LTR, but activation mechanism by MSS1 is unclearly revealed [34,39]. It was demonstrated by RT-PCR with specific *MSS1* gene primer sets that expression of *MSS1* mRNA gene was not suppressed in cells treated by AA, expression of other cellular transcriptional cofactors have not been examined yet. TAT is demonstrated to recognize directory RNA polymerase II holoenzyme, which constructs with transcriptinal factor IID, transcriptinal factor IIB, then activates the transcription of *HIV-1* genome RNA as a mediator between TAR and basal transcriptional factors [40-44] [Figure 1]. There are possible two reasons why *HIV-1* genome RNA expression is downregulated by AA treatment. First, the expression of these cellular cofactors may be downregulated in cells treated by AA [Figure 1]. The second, the stereomatic conformation of TAT protein may be changed by the treatment of AA and be not able to play as the trans-activating mediator [Figure 1]. The previous report demonstrates that HIV-infected individuals have low levels of AA; however, this deficiency is not related to eating habits, since the intake of this nutrient was higher in this group than in the control group. HIV-infected individuals have specific characteristics that increase their oxidative stress, which is evidenced by increased C-reactive protein [45]. It is necessary to examine whether the TAT activity is downregulated in the cells treated by AA or not. Already, AA is used for the treatment of AIDS and AA at 90 μg/ml was attained in plasma in patients consuming oral AA to achieve urinary levels about 1 mg/ml [46]. Clinical facilities examined how AA interacts with antiretroviral therapy in individuals with HIV-1. AA usage appears to be associated with improved highly active antiretroviral therapy (HAART) adherence and HAART effectiveness as adjudicated by HIV viral loads and $CD4^+$ T-cell counts [2]. These findings are consistent with a high bowel tolerance reported for AIDS patients. Future AA studies should target specific HAART drugs and prospective clinical outcomes.

CONCLUSION

We conducted an analysis of HIV-1 life cycle examining the impact of AA usage. Significantly increased HAART adherence is demonstrated during periods of AA usage compared to when the patients were not consuming AA. Due to the potential impact this simple, inexpensive intervention may have on

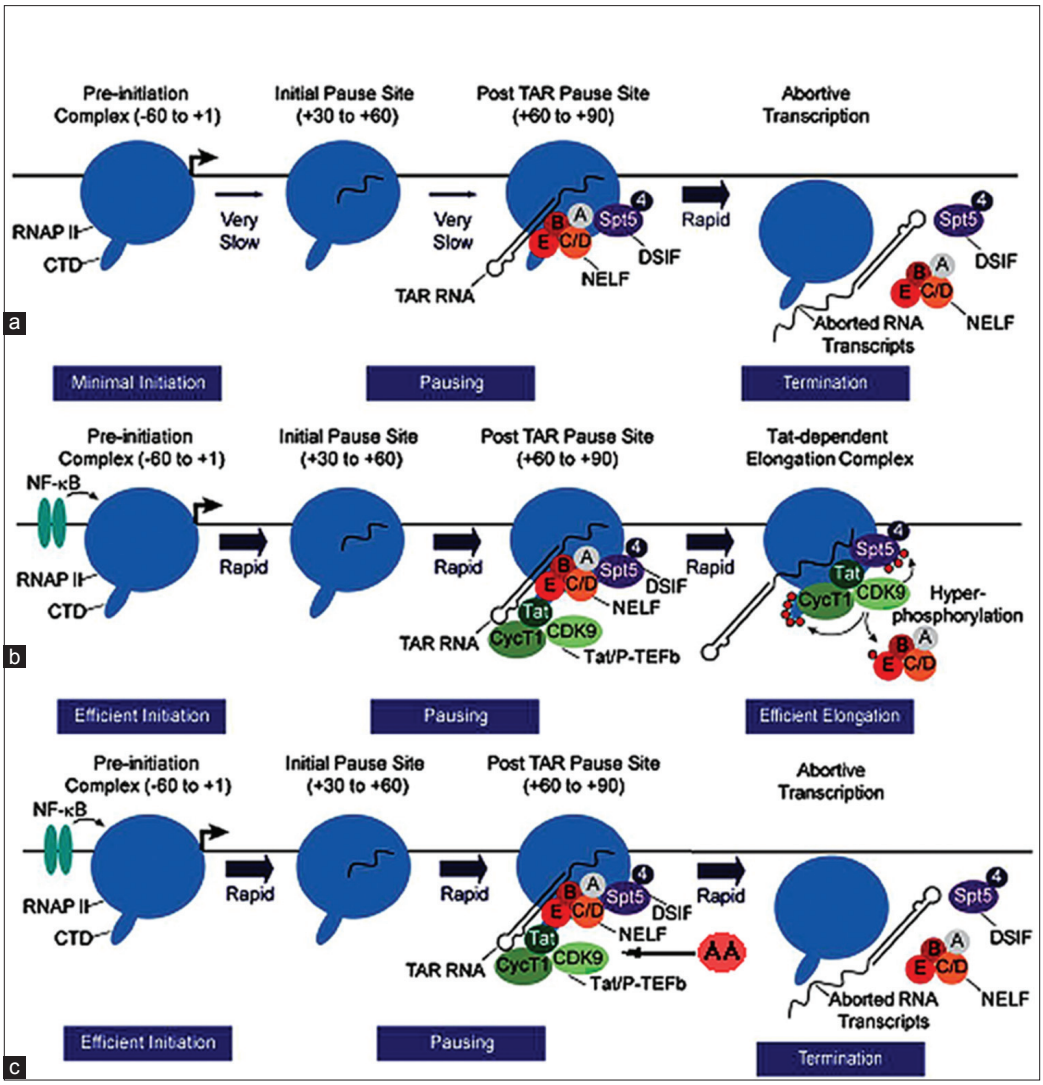

Figure 1: Preventive effect of ascorbic acid (AA) against trans-activator of transcription (TAT)-dependent *HIV-1* gene expression. (a) Latent human immunodeficiency virus type (HIV) provirus. In latent proviruses transcription elongation is very inefficient due to absence of the transcription elongation factor nuclear factor κ-B (NF-κB) as well as chromatin restrictions (not shown for simplicity). However, a significant number of proviruses carry RNAP II paused in the promoter-proximal region. The small number of transcription complexes that are able initiate and elongate through trans-activation response (TAR) are subject to additional elongation restrictions by negative elongation factor (NELF) which forces premature termination. (b) NF-κB and TAT-activated transcription. Initiation is strongly induced by NF-κB, which removes chromatin restrictions near the promoter through recruitment of histone acetyltransferases. Under these circumstances promoter clearance is also much more efficient, and there is an enhanced accumulation of elongation complexes in the promoter-proximal region. After the transcription through the TAR element, both NELF and the TAT/P-TEFb complex (the super elongation complex factors are not shown for simplicity) are recruited to the elongation complex via binding interactions with TAR RNA. This activates the CDK9 kinase and leads to hyperphosphorylation of the C-terminal domain of RNA polymerase II, Spt5, and NELF-E. The phosphorylation of NELF-E leads to its release. Although the promoter is transcribing more rapidly than in the latent condition, there is relatively little change in the amount of RNAP II that accumulates in the promoter-proximal region due to its rapid replacement by newly initiated transcription complexes. (c) There are possible two reasons why *HIV*-1 genome RNA elongation is downregulated by treatment with AA. First, the expression of these cellular cofactors may be downregulated in cells treated by AA. The second, the stereomatic conformation of TAT/cellular cofactors complex may be changed by the treatment of AA and be not able to play as the trans-activating mediator

HIV-positive patients, we believe a large community-based clinical trial is indicated.

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