Molecular Mechanism of Decreased Glutathione Content in Human Immunodeficiency Virus Type 1 Tat-transgenic Mice*

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Human immunodeficiency virus (HIV) infection is associated with a systemic decrease in the GSH content in humans (1, 2). Such biochemical alteration appears to be a direct consequence of retroviral infection rather than a late and indirect consequence of advanced disease state, is progressive in nature, and often occurs in the absence of any symptoms associated with AIDS (2–5). This decrease is significant in patients with AIDS-related Kaposi's sarcoma and B cell lymphoma. In this study, we report a decrease in GSH biosynthesis with Tat, using HIV-1 Tat transgenic (Tat+) mice. A significant decline in the intracellular GSH content in liver and erythrocytes of Tat+ mice was accompanied by decreased γ-glutamylcysteine synthetase regulatory subunit mRNA and protein content, which resulted in an increased sensitivity of γ-glutamylcysteine synthetase to feedback inhibition by GSH. Further study revealed a significant reduction in the activity of GSH synthetase in liver of Tat+ mice, which was linearly associated with their GSH content. Therefore, Tat appears to decrease GSH in vivo at least partially, through modulation of GSH biosynthetic enzymes.

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because of the reduced availability of cysteine (1, 35, 36), a limiting substrate for GSH biosynthesis, and cysteine supplementation may restore the GSH content in these individuals (19, 34). However, it has been debated whether the restoration of cysteine content truly restores intracellular GSH content in HIV+ patients (33, 36), suggesting some defect in the utilization of cysteine for GSH biosynthesis. Therefore, the molecular basis of decreased GSH synthesis in HIV infection has been obscure.

HIV type 1 (HIV-1) Tat is a transactivator protein encoded by HIV that is essential for efficient viral replication (37). Tat is associated with AIDS-related dementia (38) as well as two of the most frequent cancers associated with AIDS: Kaposi’s sarcoma (39) and B cell lymphoma (40). Tat has been shown to lower total and reduced GSH concentration in vitro in various cell lines and also the total sulfhydryl content in vivo in a transgenic animal model (27, 41, 42). In addition, Tat is sufficient to cause both the enhanced activation of NF-κB and the increased susceptibility to apoptosis, observed with HIV (42–44). Tat also enhances drug toxicity in vivo (27). Therefore, Tat may be a viral agent that participates in many of the pathological changes that occur upon HIV infection. Consequently, determining the mechanism whereby Tat decreases GSH content may serve as an important step toward understanding how HIV suppresses the GSH content in humans.

The aim of the present investigation, therefore, was to test whether GSH synthesis is decreased in Tat-transgenic (Tat+) mice and, in the process, determine the mechanism underlying such reduced GSH synthesis with Tat. A significant decrease in the GSH content in liver and erythrocytes of Tat+ mice was associated with specific modulation of GCS and GS, the enzymes involved in GSH de novo synthesis.

**EXPERIMENTAL PROCEDURES**

**Tat+ Mice and Reagents—**C57Bl mice (Jackson Laboratory) had been previously engineered to express HIV-1 Tat constitutively and ubiquitously under the control of the chicken β-actin promoter (40). All of the Tat+ mice used were homozygous for Tat and were derived from a single line. Food and water were provided ad lib. On the day of the experiment, 14-month-old Tat+ mice and age-matched control mice (NIA, National Institutes of Health, Bethesda, MD) were sacrificed in CO2 chambers. Immediately, blood was drawn directly from the heart and collected in EDTA-containing test tubes (VWR Scientific, San Diego, CA).

All high performance liquid chromatography (HPLC) solvents were Baker Analyzed HPLC-grade reagents from VWR Scientific. All other chemicals were at least analytical grade and obtained from Sigma, unless indicated otherwise.

**Intracellular GSH and Cysteine Measurements—**Total intracellular GSH ([GSH] + 2 × [GS-S]) and total cysteine ([cysteine] + 2 × [cystine]) concentrations were measured by HPLC according to the well-established method of Parissi and Beed (45). Immediately after obtaining samples, both erythrocytes and liver samples were washed twice in 1× phosphate-buffered saline and acidified to 10% perchloric acid, 2 mM EDTA that contained the internal standard, γ-glutamylglycine acid (Bachem, Torrance, CA). Liver samples were precipitated and sonicated twice in this solution to ensure complete GSH recovery. Samples were further processed and analyzed for their GSH and cysteine contents as described previously (46).

**Northern Analysis of GCS mRNA Content—**Liver samples were homogenized in Trizol reagent (Life Technologies, Grand Island, NY), and total RNA was isolated, following the manufacturer’s protocol. The amount of GCS light subunit (GCS-LS), GCS heavy subunit (GCS-HS), and glyceraldehyde-3-phosphate dehydrogenase mRNAs was measured by Northern analysis and quantitated with an InstantImager (Packard Instrument Co., Meriden, CT), as described previously (46).

**Western Analysis of GCS Proteins—**Mouse liver samples were sonicated briefly in 0.25 M sucrose, 1 mM EDTA, 20 mM Tris-HCl (pH 7.4) solution that contained a 2 μg/ml concentration each of leupeptin and aprotinin and 50 μg/ml phenylmethanesulphonyl fluoride. Erythrocytes were lysed by adding a 10× volume of distilled water. Following 20 min of centrifugation at 12,000 rpm and 1 h centrifugation at 100,000 rpm at 4 °C, supernatants were concentrated in Microcon-10 tubes (Millipore Corp., Bedford, MA). Then 10 μg of liver and 40 μg of erythrocyte cytosolic protein samples were denatured in 2% SDS, 6 M urea, and 300 mM dithiothreitol. Subsequently, GCS-LS and GCS-HS protein content was determined by Western analysis, using rabbit anti-rat GCS-HS and GCS-LS antibodies, as described previously (46).

**GCS and GS Activity Assays—**GCS activity was determined in vitro, according to the method described by Yan and Huxtable (47) with slight modifications. After concentrating liver and erythrocyte proteins in Microcon-10 tubes as described under “Western Analysis of GCS Proteins,” samples were washed further by repeatedly adding GCS lysis solution (0.1 M Tris-HCl, pH 8.2, 150 mM KCl, 20 mM MgCl2, and 2 mM EDTA) to remove endogenous inhibitors, acceptors, and substrates. The reaction mixture consisted of GCS lysis solution that was supplemented with 25 mM sodium glutamate, 5 mM cysteine, 5 mM dithiothreitol, 10 mM ATP, and 0.04 mg/ml acivicin. Reactions were allowed to proceed for 30 min at 37°C upon adding protein at a final concentration of 0.1–1 mg/ml. Reactions were then terminated with 5% (v/v) 5-sulfosalicylic acid. After adjusting pH to 8–9 with 40 mM KOH/40 mM N-ethylmorpholine, samples were derivatized for 20 min in the dark with 3 mM monobromobimane (Calbiochem). Next, samples were acified again with 5-sulfosalicylic acid and, after removing precipitated protein, were analyzed by HPLC, as described previously (46).

For the GS activity assay, glutamate was substituted with 30 mM glycine, and cysteine was substituted with 3 mM γ-glutamylcysteine (48). Dithiothreitol concentration was 6 mM. Protein was added to the reactions at a final concentration of 0–0.25 mg/ml.

Base-line levels of γ-glutamylcysteine and GSH were negligible, if present, in both GCS and GS activity assays; nevertheless, the base-line absorbance at the points where these compounds eluted was subtracted from each reaction. GCS and GS activities were both linear for at least 30 min at the protein concentrations used.

**Statistical Analysis—**Data were expressed as means ± S.E. unless indicated otherwise and evaluated by Student’s t test or one-way analysis of variance of balanced folowed by the Tukey test. Pearson product moment correlation and linear regression analysis were used to identify statistically significant correlation or association between variables. For all analyses, p < 0.05 was considered statistically significant. The number of samples used in each experiment is indicated under “Results” or in the figure legends.

**RESULTS**

**Intracellular GSH Content Is Decreased in Liver and Erythrocytes of Tat+ Mice Compared with Age-Matched Controls—**HIV-1 Tat+ mice used in this investigation had been previously reported to develop B-cell lymphomas, but their livers did not display any histological abnormalities (40). However, total intracellular GSH content was significantly decreased to 57 ± 3% (Fig. 1A) and 80 ± 5% (Fig. 1B) of the age-matched control level in liver and erythrocytes of these mice, respectively. There was no significant difference in total GSH content between genders within each group (data not shown). A significant, positive linear correlation existed between liver and erythrocyte GSH content (r = 0.56; p < 0.05).

The samples were also examined for their intracellular cysteine content. No significant change in the total cysteine content could be detected in liver (0.36 ± 0.07 and 0.23 ± 0.04 nmol/mg protein in control and Tat+ mice, respectively, p > 0.05) or erythrocytes (0.13 ± 0.02 and 0.12 ± 0.05 nmol/mg protein in control and Tat+ mice, respectively, p > 0.05) of control (n = 6) versus Tat+ mice (n = 6) with the statistical tests employed. There was no significant correlation between liver and erythrocyte total cysteine content (r = 0.38; p > 0.05). Furthermore, comparing the data with those in Fig. 1, no significant correlation was found between total GSH and cysteine concentrations in liver and erythrocytes (r = 0.31, p > 0.05) or erythrocytes (r = 0.12, p > 0.05) of control and Tat+ mice.

**GCS-LS mRNA Content Is Decreased in Liver from Tat+ Mice—**To test whether Tat modulated GCS, the first enzyme in GSH de novo synthesis, we determined the level of GCS heavy (catalytic, GCS-HS) and light (regulatory) subunit mRNA levels in liver from control and Tat+ mice. Northern analysis detected one GCS-HS and two GCS-LS mRNA species in mouse liver, as
in rat liver (Fig. 2A). GCS-HS mRNA content was not different between two groups of mice. There was also no significant change in the higher molecular weight GCS-LS mRNA band. However, the lower molecular weight GCS-LS mRNA content was significantly decreased to 56 ± 4% of the control level in Tat+ mice (Fig. 2B).

**GCS-LS Protein Content Is Decreased in Liver and Erythrocytes of Tat+ Mice**—Next, we examined whether the GCS-LS protein content was likewise reduced in Tat+ mice relative to controls by Western analysis. GCS-HS and -LS proteins were identified, based on the size of rat kidney GCS-HS and -LS proteins (data not shown). GCS-LS protein content was significantly decreased to 41 ± 16% of the control level in livers from Tat+ mice (Fig. 3A), consistent with the decrease in the lower molecular weight GCS-LS mRNA band (Fig. 2). Erythrocytes from the same mice exhibited a similar decline in the GCS-LS protein content, to 48 ± 20% of the control level (Fig. 3B). There was no statistically significant decline in GCS-HS protein content.

**Decreased GCS-LS Protein Content Was Accompanied by Increased Sensitivity of GCS to Inhibition by GSH**—GCS-HS exhibits all of the catalytic activity of the holoenzyme and is feedback-inhibited by GSH (49). GCS-LS, on the other hand, is believed to decrease the $K_m$ of GCS-HS for glutamate and increase its $K_i$ for GSH (50). Consistent with the lack of any change in the GCS-HS protein content, GCS activity was not decreased in liver or erythrocytes of Tat+ mice measured with saturating substrate concentration (5.2 ± 0.4 nmol/min/mg in control versus 6.2 ± 0.4 nmol/min/mg in Tat+ mouse liver; 0.81 ± .05 in control versus 0.81 ± .03 nmol/min/mg/min in Tat+ mouse erythrocytes).

To determine the effect of decreased GCS-LS protein content in Tat+ mice, we examined the effect of GSH on GCS activity in liver and erythrocytes from the two groups of mice. GCS activity from control and Tat mouse liver and erythrocytes were measured at low (0.5 mM) and high (25 mM) glutamate concentrations in the absence and presence of 10 mM GSH, and the ratios of the GCS activity in the absence and presence of GSH at low and high glutamate within the same sample were compared between the Tat and control groups. The results suggest that the major difference between the GCS activity in control and Tat is that the latter is inhibited to a greater extent by GSH (Fig. 4). This greater GSH feedback inhibition would be expected to contribute to a lowering of the GSH steady state that was observed.

**GS Activity Is Also Decreased in Livers from Tat+ Mice**—We also examined the possibility of a change in the activity of GS, the next enzyme in GSH biosynthesis. GS activity was significantly decreased to 73 ± 4% of the control level in Tat+ mouse liver (Fig. 5) but not in the erythrocytes (0.53 ± 0.03 in control versus 0.54 ± 0.01 nmol/min/mg/min in Tat+ mice, $p > 0.05$). Interestingly, GS activity was linearly associated with total intracellular GSH content of liver ($r = 0.72; p < 0.01; r^2 = 0.51$).

**DISCUSSION**

Acute infection of C57Bl mice with murine AIDS virus can diminish their liver GSH content 2 weeks postinfection (51). In this report, we show that HIV-1 Tat expression is sufficient to decrease GSH content in the same strain of mice and, furthermore, present a potential mechanism for this decreased GSH content: a specific modulation of GCS and GS. Specifically, down-regulation of GCS-LS in Tat+ mice was associated with an increased sensitivity of GCS to inhibition by GSH. No change in cysteine concentration could be demonstrated with the tests employed, perhaps due to the limitations in the instrument and the sample size. Regardless of whether cysteine content is altered in Tat+ mice, GCS is believed to be the rate-limiting enzyme in GSH biosynthesis (52), and therefore, such a decline in GCS-LS protein content is likely to result in a decreased rate of GSH biosynthesis. This, together with the down-regulation of manganese superoxide dismutase by Tat, may allow the observed reduction in GSH content. It is presently unknown whether the down-regulation of GCS-LS by Tat is sufficient to cause GSH depletion.

The role of GS modulation in decreased GSH concentration is less obvious. Although the high correlation between GS activity and GSH content ($r = 0.72; p < 0.05$) suggests a possible role of GS in determining GSH status, at least in mouse liver, GS is generally not believed to govern the rate of GSH synthesis. It is perplexing, therefore, how GS activity could be related to the GSH status. However, similar association between GS activity and GSH content has been reported by others (53, 54). In addition, while GS is usually regarded as the rate-limiting enzyme in GSH synthesis (52), increasing evidence also suggests that GS activity can be regulated (55–62). Obviously, if GS activity falls significantly, it could become the rate-limiting step. γ-Glutamylcysteine, produced by GCS, has been suggested to be channeled efficiently to GS (63). It is known that physical interaction or close spatial arrangement of proteins that participate in a given signal transduction or biochemical pathway enables an efficient relay of signals to effector molecules as well as the channeling of intermediates between interacting metabolic enzymes (64). Thus, it is not at all surprising that the enzymes in GSH biosynthesis may be regulated coor-
dinately and perhaps even interdependently, through protein-protein interaction. Nevertheless, the observed decline in the GCS-LS protein content may be sufficient to explain the decreased GSH content in Tat mice, and therefore, the physiological significance of a 30% decline in the GS activity in Tat mice remains unclear at the moment.

Our findings on Tat mice apparently lead to the next question, which is whether the modulation of GSH biosynthetic enzymes also occurs in HIV infection. A similar decline in GSH content has been found in the liver and erythrocytes of HIV individuals (34, 65). Hepatic GSH depletion is particularly interesting, as liver serves as a reservoir for HIV, and various cells found in liver, such as Kupffer cells, lymphocytes, sinusoidal endothelial cells, and even the hepatocytes themselves, can be infected by the virus (66). Thus, Tat may directly decrease GSH synthesis in the liver of HIV+ patients. This may explain the systemic decrease in the GSH content found in these individuals, because liver is the organ primarily responsible for GSH in circulation (21, 22). In addition, although HIV is not found ubiquitously in infected individuals, Tat is secreted by HIV-infected cells and also taken up rapidly by various cells (37). Thus, any cell that Tat enters can potentially suffer from decreased GSH biosynthesis. Moreover, Tat may affect GSH biosynthesis through an indirect mechanism, such as by production of diverse cytokines, which may also exert a systemic effect on GSH synthesis. Therefore, Tat is a potential viral agent that causes the systemic GSH depletion observed in humans infected with HIV. Whether GSH synthetic enzymes are indeed suppressed in HIV+ individuals in a Tat-dependent manner should be tested in the future.

It is uncertain whether a partial depletion in the GSH content will have significant effects on metabolism, and the answer is likely to depend on the GSH concentration and metabolic demands of each tissue. However, any effect of decreased GSH concentration will certainly be amplified in situations of increased oxidative burden. Due to the primary and secondary infections, HIV+ individuals tend to have activated respiratory burst of the immune cells, elevated level of pro-oxidative cytokine (24, 25), and increased intake of pharmaceutical compounds that either cause oxidative damage (e.g. zidovudine (27)) or require GSH for detoxification (13). These, along with a decrease in overall antioxidant capacity (14, 28), will undoubtedly create a higher demand for GSH and, in turn, result in an altered redox poise of cells. This may worsen various aspects of viral pathogenesis such as enhanced viral replication, potentiation of apoptotic cell death (44), and increased susceptibility to...
inhibition by GSH was calculated as follows: sensitivity = (1/\(v_{\text{soh}}\) – 1/\(v_{\text{sih}}\))/((1/\(v_{\text{soh}}\) – 1/\(v_{\text{sih}}\)) – (1/\(v_{\text{soh}}\) – 1/\(v_{\text{sih}}\)))^1, where \(v\) represents GCS activity; \(s\) represents glutamate concentration; and the subscripts \(v\), \(i\), and \(h\) represent uninhibited (–GSH), inhibited (+ GSH), low glutamate and high glutamate, respectively. In the experiments here, \(s_{\text{v}} = s_{\text{i}} = s_{\text{h}} = s_{\text{v}}\); therefore, sensitivity = (1/\(v_{\text{soh}}\) – 1/\(v_{\text{sih}}\))^2, statistically significant difference from control (p < 0.05).

oxidative stress and drug toxicity. A 10–40% decrease in GSH content can completely inhibit lymphocyte activation in vitro (8). Indeed, the very fact that Tat modulates GSH biosynthesis in addition to suppressing the activity of manganese superoxide dismutase (27, 41, 42) may testify to the importance of protein redox regulation in HIV pathogenesis. Specifically, these pro-oxidative effects of Tat may enhance its NF-

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Down-regulation of γ-Glutamylcysteine Synthetase by Tat

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