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Minireview

The extracellular microenvironment plays a key role in regulating the redox status of cell surface proteins in HIV-infected subjects

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Abstract

There is an overwhelming interest in the study of the redox status of the cell surface affecting redox signaling in the cells and also predicting the total redox status of the cells. Measuring the total surface thiols (cell surface molecule thiols, csm-SH) we have shown that the overall level of surface thiols is tightly controlled. In vitro, the total concentration of intracellular glutathione (iGSH) seems to play a regulatory role in determination of the amounts of reduced proteins on cells. In addition, short term exposure of the cell surface to glutathione disulfide (GSSG, oxidized GSH) seems to reduce the overall levels of csm-SH suggesting that the function of some cysteine containing proteins on the cell surface may be regulated by the amount of GSSG secreted from the cells or the GSSG available in the extracellular environment. Examination of peripheral blood mononuclear cells (PBMCs) from healthy or HIV-infected subjects failed to reveal a similar correlation between the intra- and extracellular thiol status of cells. Although there is a relatively wide variation between individuals in both csm-SH and iGSH there is no correlation between the iGSH and csm-SH levels measured for healthy and HIV-infected individuals. There are many reports suggesting different redox active proteins on the cell surface to be the key players in the total cell surface redox regulation. However, we suggest that the redox status of the cells is regulated through a complex and tightly regulated mechanism that needs further investigation. In the mean time, overall surface thiol measurements together with case specific protein determinations may offer the most informative approach. In this review, we discuss our own results as well as results from other laboratories to argue that the overall levels of surface thiols on the exofacial membrane are regulated primarily by redox status of the cell surface microenvironment.

Keywords: Cell surface issue; Redox control (invited)

There are vitally important proteins with pivotal cysteine residues in cells as well as on exofacial cell membranes. These cysteine residues have free sulfhydryl groups (-SH)[1] that are available for redox regulation. As in the internal redox environment, the redox status of the cysteines on the exofacial membrane proteins is closely regulated and is critical to the functions of these proteins. In HIV disease, the progression of the disease is accompanied by increasing oxidative stress. We and others have shown that intracellular glutathione (iGSH) in peripheral blood T cells tends to be markedly decreased at advanced stages of the disease. This decrease impairs T cell function and is associated with decreased survival. In recent studies, we have developed a FACS method for measuring cell surface (exofacial) molecules that have -SH groups available for redox interactions (csm-SH) and have shown that T cells from some HIV-infected people have increased csm-SH. Importantly, these infected people have very low T cell iGSH and have advanced manifestations of the disease. This finding, which suggested that csm-SH increases as iGSH

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[1] *Abbreviations used:* ALM; Alexa maleimide, BSO; buthionine sulfoximine; csm-SH, cell surface molecule-SH, GSH; reduced glutathione, PSSG, glutathionylated proteins FACS; fluorescence activated cell sorter, Trx; thioredoxin, TrxR; thioredoxin reductase.

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decreases, led us to a further examination of this relationship. The FACS method we have developed for measuring T cell csm-SH requires initial reaction of a maleimide coupled fluorescent molecule (Alexa maleimide) with free -SH groups on csm-SH. Once this reaction is complete (in 15 min), cells are stained with fluorochrome-coupled antibodies that detect the T cell surface markers that distinguish T cell subsets. Hi-D (high-dimensional, 11-color 13-parameter) FACS analysis then reveals the amount of Alexa dye associated with individual T cell subsets. Extending this method by using two Alexa-maleimide dyes that have distinct emission spectra allows evaluation of csm-SH before and after cells are treated with various agents. Thus, we have been able to detect alterations in the overall surface levels of csm-SH following exposure of cells to agents that alter the redox status of cells. Findings from these studies indicate that the levels of surface thiols are modified in response to short-term exposure to oxidative or reductive conditions. Redox-active proteins on cell surface, including proteins with -SH, are known to be involved in sensing redox changes in the medium. Current studies focus mainly on protein disulfide isomerase (PDI) and γ-glutamyltransferase (GGT), which are viewed as major factors in modulation of csm-SH. The method we use ranges more broadly since it detects all csm-SH capable of reacting with maleimide. Thus, it provides a more comprehensive view of the impact of redox changes on the availability of -SH on the exofacial portion of the cell surface proteins on individual subsets of cells. Logically, one might expect that oxidative stress, which decreases intracellular -SH, would also decrease csm-SH. Indeed, several studies report that decreasing iGSH by treating cells in vitro with agents like buthionine sulfoximine (BSO) results in decreased csm-SH. Our in vitro findings are consistent with this earlier evidence. However, our studies with cells from HIV-infected people, in whom T cell iGSH tends to be markedly decreased, indicate that there is relatively little correlation between iGSH and csm-SH levels in T cells. In our discussion here, we consider explanations for this apparent paradox and discuss the idea that the extracellular microenvironment may also play a key role in regulating cell surface redox status.

Intracellular redox regulation

The main intracellular redox buffer is reduced glutathione (GSH, *N*,*N*-L-γ-glutamyl-L-cysteinylglycine), a tripeptide synthesized in cells from its precursor amino acids, two of which are also synthesized while the third, cysteine, must be obtained exogenously. GSH is present in mM range intracellularly [1]. It has been shown to control several functions in cells including calcium flux and nuclear factor-κB (NF-κB) activation [2,3]. Intracellular levels of GSH are lowered in advanced HIV disease [4], suggesting a role for oxidative stress in pathogenesis of HIV disease. The importance of this role is underscored by evidence demonstrating that T cell function is impaired in HIV disease when iGSH levels are low and is restored by treatments that replenish GSH. There are several possibilities for the molecular mechanisms through which iGSH regulates cell function. iGSH levels can directly alter the activity of antioxidative proteins and enzymes like glutathione peroxidases [5]. In addition, oxidations, which result in loss of iGSH in biological systems, has long been known to result in formation of mixed disulfides between protein-SH and GSH, i.e., formation of glutathionylated proteins (PSSG) [6]. Such "glutathionylation" is known to alter enzyme function, causing either inactivation or activation depending on the enzyme and the site that is glutathionylated.

Glutathionylation, a means for regulating enzyme activity in cells

Protein glutathionylation, the formation of a disulfide between a -SH in a protein and the -SH in GSH, occurs when cells are under oxidative stress and internal levels of glutathione disulfide (GSSG) are high. Glutathionylation occurs by (i) direct interaction between protein-SH and GSH under oxidizing conditions; (ii) thiol disulfide exchange between a protein -SH and GSSG; and, (iii) reaction of protein-SH with *S*-nitrosoglutathione (GSNO). Glutathionylation is a reversible process. A second redox enzyme, glutaredoxin (Grx), is involved in this deglutathionylation process. A modified Grx, in which one of the two cysteines in the active site is naturally modified to a serine, is able to form glutathionylated proteins [7]. Glutathionylation of protein on critical residues may either activate or deactivate enzymes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is perhaps the most well-known enzyme whose activity is destroyed by glutathionylation [8–10]. The HIV-1 protease contains conserved and critical Cys residues that, when glutathionylated, activate or deactivate the protease depending on the Cys involved [11–14]. As a measure of the importance of this activation, the HIV-1 genome also encodes a glutaredoxin-like molecule (EC 1.8.4.2), which has been implicated in protecting the protease function [15].

The thioredoxin system is also regulated by glutathionylation

Thioredoxin (Trx), one of the key redox enzymes in the cell, has been found to have both intracellular and extracellular activities. Human Trx is reported to enhance the activity and expression of cytokines (soluble proteins produced in response to and modulating the immune system) and has therefore been termed a

co-cytokine [16–18]. In addition, human Trx has been shown to be a chemotactic factor that induces migration of neutrophils, T cells, and monocytes to the site of inflammation [19]. Furthermore, when overexpressed in transformed cells [20], Trx has been shown to control the activity of the immunologically important transcription factors NF-κB and AP-1 [21]. Finally, Trx has been shown to directly regulate the induction and maintenance of high levels of hypoxia induced factor (HIF-1α), a factor responsible for tumor progression [22,23]. Trx is part of the 'Trx system,' which includes Trx, thioredoxin reductase (TrxR), and NADPH. It is present in all cells. Trx is a 12 kDa protein with two cysteines in its active site and a third, regulatory cysteine that is a target for glutathionylation. TrxR (EC 1.6.4.5) is a homodimeric flavoprotein that uses the reducing equivalent from NADPH to reduce disulfide bonds (e.g., in the active site of oxidized Trx). It contains a selenocysteine as its penultimate amino acid, encoded by what was earlier thought to be a stop codon [24,25]. The selenocysteine is essential to TrxR function. Reduced Trx is involved in the redox regulation of protein substrates. The redox regulation of protein substrates denotes the post-translational alteration of proteins through a thiol group (-SH) on the amino acid cysteine (Cys) with another thiol group giving rise to a disulfide bond (–S–S–) or direct binding to glutathione (GS-protein). The amino acid Cys is often located on the strategically important parts of enzymes and transcription factors. Therefore, alternation of this amino acid causes major changes in the folding of the whole protein, which originate from the redox status in the cell or in subcellular compartment such as nucleus and mitochondria [21,26].

PDI on the cell surface

Protein disulfide isomerase (PDI, EC 5.3.4.2) is a redox active protein that has reducing activity when on the exofacial membrane of the cell. It has been implicated in regulation of HIV entry into cells and, on B chronic lymphatic leukemia (B-CLL) cells, its overexpression has been shown to increase csm-SH mediated protection and drug resistance [27–29]. Recently, the redox active cysteine residues on domain 2 of the CD4 molecule were shown not to support HIV entry in oxidized state. Reduction of the cell surface CD4 with reduced Trx made the cells susceptible to HIV entry [30]. PDI has also been shown to be the epithelial cell receptor for *Chlamydia trachomatis Servoar E*, the leading bacterial agent responsible for sexually transmitted disease [31], and has been shown to be involved in the activation of L-selectin shedding from the cell surface of leukocytes [32]. In one study, PDI has been shown to account for 40% of total cell surface thiols on platelets [33] and has been shown to play a key role in platelet aggregation [34,35]. It has been implicated in the conversion of GPIIbIIIa to a fibrinogen-binding conformation and in the changes that occur after receptor binding [36]. Csm-SH are involved in sustained integrin ligation [37–39]. In a recent study, integrin α-4 was shown to have csm-SH critical for adhesion to fibronectin. The redox status of integrin α-4 is changed with brief treatment with NAC and other antioxidants, independent of intracellular redox changes. The treatment did not increase fibronectin but increased its ability to adhere by inducing conformational changes that are dependent on the redox status of integrin [40]. Importantly, inhibitors of PDI have been shown to inhibit the ligation [39], suggesting that the redox influence on the receptor may be mediated, at least in part, by PDI.

Measurement of total cell surface molecule thiols and mixed disulfides

The Hi-D FACS assay that we have developed to measure cell surface molecule thiols (csm-SH) on peripheral blood mononuclear cells (PBMCs) allows analysis of the redox status of the proteins on surface of cells that belong to distinct PBMC subsets. In this assay, the reaction between Alexa maleimide and csm-SH measures the total amount of cell surface thiol available for reaction with the maleimide moiety of the dye (Fig. 1). This staining to detect csm-SH does not interfere with subsequent monoclonal antibody staining to identify PBMC subsets. Thus, with a Hi-D FACS instrument capable of detecting up to 11 fluorescence "colors," PBMC subsets can readily be resolved and evaluated for csm-SH levels. Studies with this assay have shown that lymphocyte subsets in freshly isolated PBMC have different csm-SH levels. Hi-D FACS analysis of cell subset markers as well as the ALM showed that monocytes have very high csm-SH followed by $CD19^+$ B cells, $CD3^+CD8^+$cells, and $CD3^+CD4^+$ cells [41]. Several Alexa dyes coupled to maleimide are available. Typically, we use Alexa maleimide 594 in our staining protocols. However, we have also developed a 2-color, 2-stage Alexa maleimide (using Alexa maleimide 594 and 488, Fig. 2) that identifies changes in surface thiols after oxidative or reductive treatments. In this assay, the basal csm-SH level is established by staining cells with one of the Alexa dyes (emission at 630 nm) prior to treatment and the response csm-SH level is measured by staining with the second Alexa dye (emission at 594 nm) after the cells are treated. With this 2-stage assay, we have found that in vitro treatment of PBMC for 24 h with BSO, a GSH synthesis inhibitor, decreases csm-SH (Fig. 3) as does short term (15 min) treatment with GSSG. In contrast, csm-SH is increased following short-term treatment with *N*-acetylcysteine (NAC). The mechanisms responsible for these csm-SH changes are not clear.

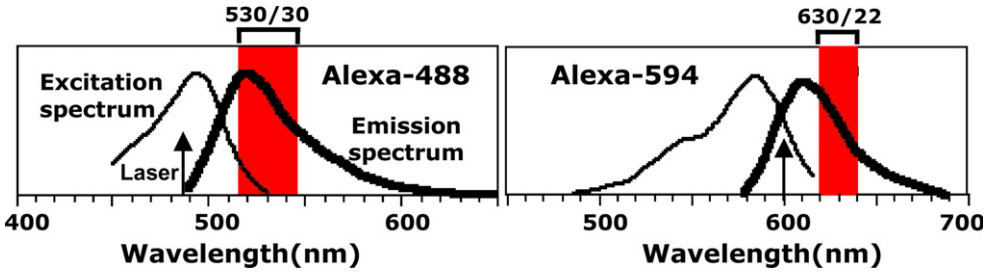

Fig. 1. FACS detection of cell staining with ALM-488 and ALM-594. ALM staining was detected with Hi-D 13-parameter FACS in the Stanford Shared FACS Facility. Alexa 488 is excited by an argon laser at 488 nm, whereas the Alexa 594 is excited by a dye laser at 600 nm. The Alexa 488 emission is detected by using a 530 ± 30 nm band pass filter, and the Alexa 594 is detected by a 630 ± 22 band pass filter.

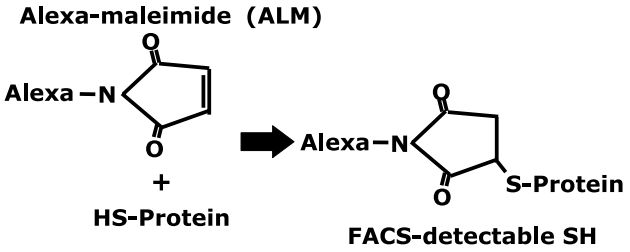

Fig. 2. Mechanism of ALM (Alexa maleimide) reaction. ALM reacts with csm-SH via the maleimide portion of the ALM molecule. The reaction results in a covalently bound fluorescent conjugate that is detectable by FACS. ALM is a nontoxic charged molecule that does not enter cells at the concentrations used for staining.

Csm-SH in chronic oxidative stress in HIV disease

By demonstrating that redox mechanisms regulate NF-κB activation, the Herzenberg laboratory and others opened a new window on the mechanisms that regulate gene expression. These findings were obtained in a series of in vitro reporter gene and HIV infection studies [2,42]. Later the Herzenberg laboratory turned to redox studies in HIV-infected people. They and others showed that HIV infection results in a progressive decrease in T cell iGSH [3,4,43]. Later, they showed that the lower intracellular levels of GSH, and the increased secretion of Trx, correlate with the curtailment of life expectancy [44,45]. In a double blind clinical trial, Breitkreuts and Dröge also revealed a massive cysteine loss that correlated with lower life expectancy and increased levels of secreted Trx and IL-6. Importantly, these investigators also showed that the HIV-induced redox imbalance is reversible and can be corrected by oral administration of *N*-acetylcysteine (NAC), which increases iGSH in HIV-infected individuals by delivering the cysteine for synthesis of GSH [46]. T cell function also improved in the group treated with NAC. In recent studies, we examined csm-SH in PBMC subsets in HIV-infected people who had CD4 T cell counts below 250/μl blood. Surprisingly, although iGSH levels were low in these individuals, we found that csm-SH levels on CD4 T cells were elevated in about 10% of the individuals being investigated [41]. Importantly, this group contained the sickest subjects in the

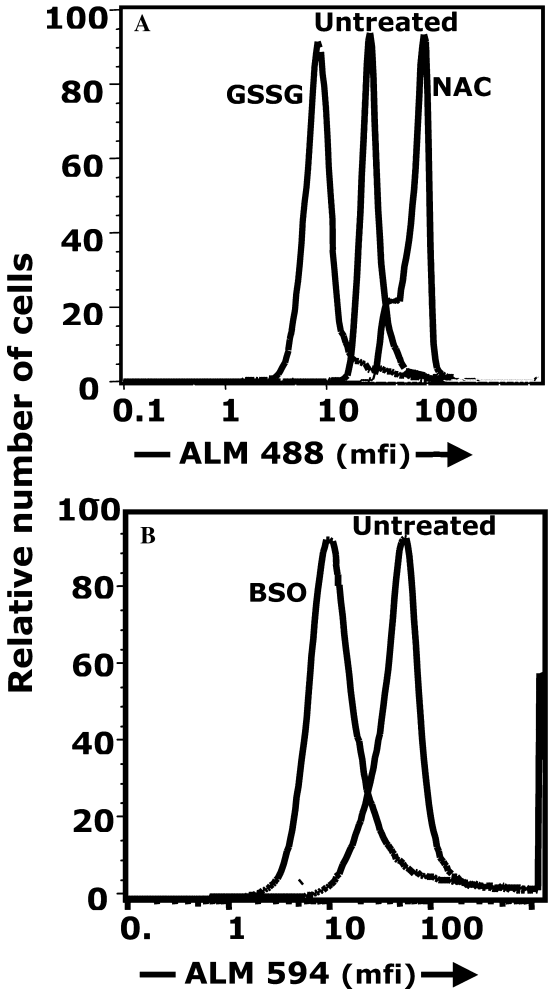

Fig. 3. Manipulation of cellular redox status alters csm-SH. (A) Lymphocytes isolated from PBMCs were stained with ALM-594 before treatment and then treated for 15 min with either an oxidizing agents (GSSG) or reducing agent (NAC) fro 15 min on ice. Treated cells were then stained with ALM-488 and reagents to detect lymphocyte surface markers and analyzed by Hi-D FACS. (B) Cells were treated with BSO, which inhibits GSH synthesis. After 24 h treatment in vivo, cells were stained with ALM-594 and subset detecting reagents and analyzed by Hi-D FACS.

study. Analysis of the median iGSH levels and csm-SH levels in individuals failed to reveal a correlation between these two variables, compared either across the

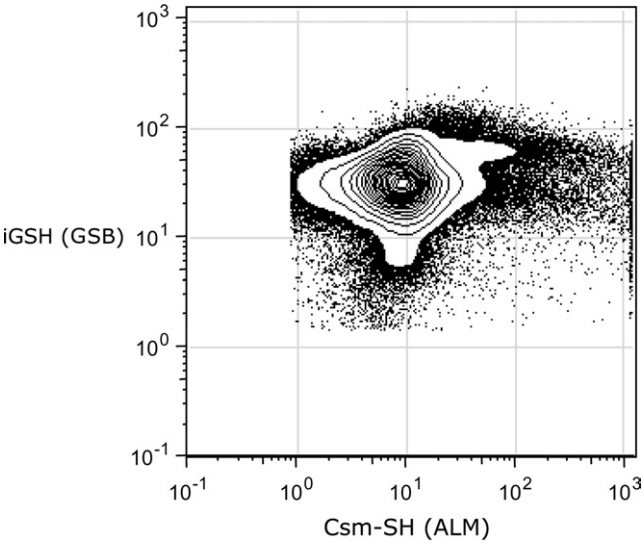

Fig. 4. Lack of correlation between iGSH and csm-SH levels in HIV-infected individuals at single cell level. PBMCs from HIV-infected individual were stained for iGSH using monochlorobimane (MCB) that is conjugated to GSH resulting in the fluorescent GS-bimane (GS-B). Cells were then stained for csm-SH and lymphocyte subset markers. Dot plot shows the lack of any correlation between the iGSH and csm-SH for the same HIV-infected representative individuals at single cell level. Each dot represents a $CD4^{+}$ cell.

subject population or across the cells in an individual subset. Further, we found no correlation between iGSH and csm-SH in CD4 T or other cells in individual samples at single cell level (Fig. 4). This lack of correlation between iGSH and csm-SH suggests that the extracellular microenvironment plays a pivotal role in regulation of csm-SH [41], and hence of the functional status of cell surface proteins like PDI and integrin α-4 [40].

Other redox active proteins on the cell surface

γ-Glutamyltransferase (GGT, EC 2.3.2.2.) is a key enzyme in the GSH economy of the cell. It is a plasma membrane enzyme whose catalytic site faces the extracellular environment. It binds extracellular GSH and acts to remove the γ-glutamyl group from the GSH molecule. The resulting cysteinyl–glycine dipeptide is then cleaved by a membrane dipeptidase and the cysteine that is released is transported into the cell for use as a substrate for de novo synthesis of GSH.

GGT often is upregulated on tumor cells. Several studies have suggested that its ability to increase cellular supply of cysteine increases internal GSH [47,48]. Other studies demonstrate that increasing GGT activity decreases csm-SH [49,50] and suggest that GGT may have pro-oxidant activity. Our findings, which suggest a complex relationship between iGSH and csm-SH that can vary depending on the cell treatment conditions, point to the difficulties in resolving the apparent conflict between the two types of GGT studies.

Taken together, the newly emerging regulatory role of the redox status of the cell surface proteins has proven to be important in the regulation of cell function. Using Hi-D FACS we have shown in vitro and also on freshly isolated PBMC that the cell surface redox status is regulated. Upon HIV infection, however, the proteins on the cell surface show a change that is more obvious in patients with clinically worse prognosis.

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