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Commentary

Liver Immunity and Glutathione

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ABSTRACT

Redox processes have been implicated in various biologic processes, including signal transduction, gene expression, and cell proliferation, and several molecules have been identified as redox regulators in cell activation. Glutathione is the oldest and most investigated molecule among them. Although details of the mechanisms by which glutathione regulates various aspects of cell biology remains to be characterized, the relationship between immunodeficiency and cellular glutathione status is well established. Redox dysregulation contributes to the pathogenesis of acquired immunodeficiency syndrome (AIDS). Human immunodeficiency virus (HIV)-infected patients and simian immunodeficiency virus (SIV)-infected rhesus macaques have, on the average, significantly decreased plasma cysteine and intracellular glutathione levels. Liver contains abundant levels of reducing factors. However, glutathione levels in serum and peripheral blood mononuclear cells of cirrhosis patients are lower compared to values detected in healthy individuals. In the present article, the significance of glutathione in regulating the functions of lymphocytes, especially those of liver-associated lymphocytes, has been described. A novel strategy for immune therapy of liver neoplasms with the use of redox-modulating agents has been proposed. Antiox. Redox Signal. 1, 245–253, 1999.

INTRODUCTION

REDOX PROCESSES have been implicated in various biologic processes, including signal transduction, gene expression, and cell proliferation, and several molecules have been identified as redox regulators in cell activation (Sen, 1998). Glutathione (GSH) is the oldest, and the most investigated molecule among them (Sen, 1997). Details of mechanisms by which GSH regulates various aspects of cell biology remain to be characterized. However, it is well established that GSH plays an important role in regulating human immunity, including antibody responses (Ohmori *et al.*, 1982), killing activity (Yamauchi and Bloom, 1993), and lymphocyte proliferation (Iwata *et al.*, 1994).

IMMUNODEFICIENCY

Human immunodeficiency virus (HIV)-infected patients have been observed to have decreased levels of plasma as well as lymphocyte GSH (Buhl *et al.*, 1989). Agents such as *N*-acetyl-L-cysteine (NAC) (Roederer *et al.*, 1992) or selenium (Hori *et al.*, 1997), which are known to

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strengthen the GSH-dependent antioxidant defense system, show beneficial effects in acquired immunodeficiency syndrome (AIDS) patients. Other immunodeficient diseases, including common variable immunodeficiency, have also been reported to be associated with low levels of GSH in lymphocytes (Aukrust *et al.*, 1995).

MALIGNANCY

We and others have reported that liver-associated mononuclear cells (liver MNC) of humans (Kanai *et al.*, 1993a; Winnock *et al.*, 1993), rats (Kanai *et al.*, 1993b), and mice (Johnkoski *et al.*, 1992) have higher cytotoxicity than peripheral blood mononuclear cells (PBMNC). The cytotoxic function of liver MNC was impaired in animals via various immunological conditions, such as malignancy (Johnkoski *et al.*, 1992) and surgical stress (Oka *et al.*, 1994; Vujanovic *et al.*, 1995). On the other hand, production of large amounts of reactive oxygen intermediates (ROS) is reported by human tumor cell lines and tumor-bearing macrophages. In addition, plasma and hepatic GSH levels are significantly decreased in patients with liver cirrhosis (Willems *et al.*, 1985; Otsuji *et al.*, 1996). These results suggest that the tumor-bearing cirrhotic liver may be exposed to oxidative stresses. To explore the merit of a novel strategy of cytokine therapy using liver MNC for liver cancers, we evaluated the cytotoxicity of liver MNC obtained from rats with liver cirrhosis (Tsuyuki *et al.*, 1998a) and from patients with hepatocellular carcinoma (Tsuyuki *et al.*, 1998b). The role of the redox state of liver MNC was studied by increasing the intracellular GSH level using NAC. NAC proved to be effective as adjunct of cytokine therapy for hepatocellular carcinomas (Tsuyuki *et al.*, 1998a,b).

CELL PROLIFERATION

Thiol compounds, including GSH, regulate the proliferation and several other functions of lymphocytes (Sen and Packer 1996; Sen 1998). Reducing agents such as β-mercaptoethanol (2-ME) or GSH potentiate the ability of interleukin-2 (IL-2) to induce cytotoxic activity (Kuppen *et al.*, 1991; Ting *et al.*, 1992) and proliferation (Sugama *et al.*, 1987; Ting *et al.*, 1992) of murine lymphocytes. Consistently, L-buthionine-(*S,R*)-sulfoximine (BSO), a selective inhibitor of GSH synthesis, decreases the cytotoxic activity of murine lymphocytes (Liang *et al.*, 1991). The role of thiol compounds and the relevance of thiol-sensitive pathways in cell activation have been reported in systems ranging from the age-related decline of immune function (Fong and Makinodan, 1989) to HIV infection (Buhl *et al.*, 1989; Eck *et al.*, 1989; Baruchel and Wainberg, 1992). Immunodeficiencies, other than AIDS, are also associated with diminished levels of GSH or cystine in serum and decreased natural killer (NK) cell function (Aoki *et al.*, 1993; Aukrust *et al.*, 1995). We investigated thiol-sensitive target molecules and observed that IL-2-induced expression and phosphorylation of the retinoblastoma (RB) protein was decreased in NK3.3 cells cultured in thiol-deficient medium, although CDK6 and CDK2 showed a more rapid and higher kinase activity and phosphorylation under conditions of thiol deprivation (Yamauchi and Bloom, 1997).

These findings suggest that inadequate kinase activities caused by excessive enhancement of phosphorylation may account for cell cycle arrest in thiol-deficient medium. These observations lead to the hypothesis that thiol deprivation may down-regulate the activity of phosphatase(s) such as CDC25a, which, in turn, may result in premature and prolonged phosphorylation of CDK6 and CDK2. On the basis of these findings, we speculate that decreased NK function associated with immunodeficiency may be due, in part, to disrupted cell cycling, and that thiols function, in part, to maintain normal cell division by regulating the function of cyclin-dependent kinases. Intracellular redox status has been shown to influence signal transduction in a variety of systems through reduction of key proteins such as protein kinase C (Fong *et al.*, 1990), nuclear factor-κB (NF-κB) (Matthews *et al.*, 1992), the *jun* and *fos* proto-oncogenes (Abate *et al.*, 1990), or the

iron-responsive element of human transferrin receptor gene (Hentze *et al.*, 1989). Iwata *et al.* observed that cystine incorporation by human mononuclear lymphocytes was enhanced by thioredoxin (Iwata *et al.*, 1994), a thiol-related reducing enzyme that is involved in the activation of transcriptional factors including NF-κB, AP-1, and p53 (Yamauchi *et al.*, 1998). Thus, an interplay between thioredoxin and GSH, two major redox regulators of cell activation, seems likely.

LIVER-ASSOCIATED LYMPHOCYTE FUNCTIONS IN RATS

Liver cirrhosis, which is associated with decreased plasma and hepatic GSH, has been reported to cause the suppression of NK activity in PBMNC (Hirofuji *et al.*, 1987; Vento and Eddleston, 1987). Previously, we have reported that liver MNC obtained from perfusates via the portal vein of normal rat and human liver have different characteristics and higher cytotoxicity than PBMNC (Kanai *et al.*, 1993a,b). Our works have also shown that hepatic MNC is sensitive to the redox environment in the liver (Kinoshita *et al.*, 1997). For example, hepatic MNC, isolated from normal human livers, underwent apoptosis, but this death pathway was inhibited by the addition of reducing agents such as 2-ME or reduced GSH. Because low GSH levels in lymphocytes are known to alter lymphocyte function, we have examined the correlation between intracellular GSH levels and the cytotoxic activity of liver-associated mononuclear cells (Tsuyuki *et al.*, 1998a). We observed that rat liver contains a highly active population of NK cells ($CD3^-$ NKR-$P1^+$ cells) that are dominantly responsible for the cytotoxicity in the liver MNC of rats and that the cytotoxic activity of this NK population is directly proportional to liver MNC GSH, despite no major alterations in the subpopulation (Fig. 1). This proportionality is independent of the methods used to alter GSH level. Thus, *in vitro* treatment of liver MNC with BSO to lower GSH levels lowers the cytotoxic activity. MNC from cirrhotic liver, in which implanted tumor cells grow faster (Fig. 2), have low GSH levels as well as low cytotoxicity. Supplementation of cirrhotic liver MNC with NAC raises GSH levels and increases cytotoxicity. NAC has been widely reported to be effective in various conditions. Administration of NAC has been shown to improve the survival of AIDS patients (Herzenberg *et al.*, 1997). NAC also prevented the decrease of the CD3ζ chain in tumor-bearing mice (Otsuji *et al.*, 1996). Enhanced cell proliferation and cytotoxicity of lymphokine-activated killer cells induced by IL-2 *in vitro* have been observed in NAC-treated, GSH-rich cells (Yim *et al.*, 1994). Conjugate formation between NK cells and target cells is promoted by NAC (Malorni *et al.*, 1994). We observed that the administration of NAC restored both suppressed NK activity and the intracellular GSH contents of liver MNC in the cirrhotic livers, while having no effect on NK activity mediated MNC from normal livers. This finding further supports the requirement of adequate intracellular GSH for optimal NK activity by liver MNC, and suggests a physiological mechanism, *i.e.*, decreased GSH, may be causally associated with the increased incidence of hepatoma in cirrhotic individuals and the increased growth of hepatoma cells in cirrhotic animals. Thus, GSH is important to the

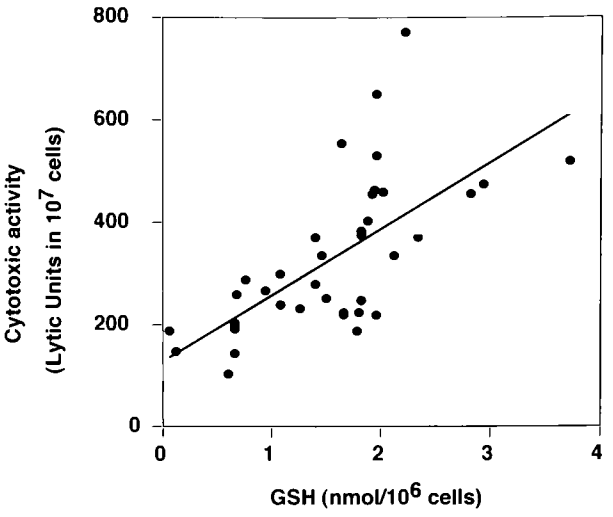


FIG. 1. Correlation between cytotoxic activity and intracellular GSH levels in liver MNC. All data from experiments to examine the relationship between GSH and cytotoxic levels are shown. $r^2 = 0.444158$, $p < 0.0001$ (Tsuyuki *et al.*, 1998a).

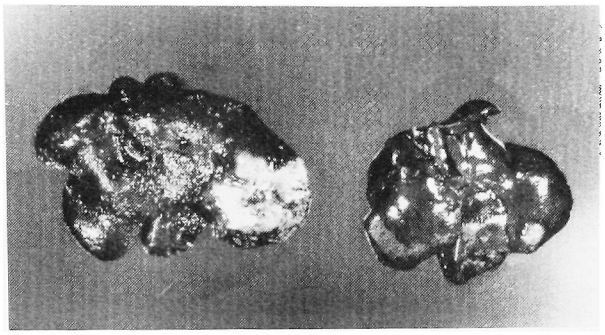


FIG. 2. Macroscopic appearance of intrahepatic tumor progression in the cirrhotic and normal liver. These are typical samples representative of seven independent experiments. The implanted syngeneic AH66F hepatoma cells in the cirrhotic liver **(left)** significantly formed more progressively than in the control **(right)** (see Tsuyuki *et al.*, 1998a).

optimal functioning of the hepatic immunity that protects against hepatoma development.

LIVER-ASSOCIATED LYMPHOCYTE FUNCTIONS IN HUMANS

Surgical resection is generally accepted as the first choice of treatment for hepatocellular carcinoma. However, most patients with hepatocellular carcinoma cannot be helped only by surgical therapy. Because of its multifocal nature, association with chronic liver diseases, and frequent post-resectional recurrence, non-resectional therapies are still important in the management of a significant proportion of patients with hepatocellular carcinoma. Systemic chemotherapy and immunotherapy, however, have not had satisfactory results, although some investigators report some beneficial effect using adjuvant immunotherapy [*e.g.*, tumor-specific cytotoxic T-lymphocyte (CTL) therapy, or locoregional IL-2 administration for patients with hepatocellular carcinoma] (Yamamoto and Fujii, 1995). It has also been reported that lymphokine-activated killer (LAK) activity by IL-2 activated NK cells is modulated by intracellular GSH levels (Yamauchi and Bloom, 1993). In addition, plasma and hepatic GSH levels are significantly decreased in patients with liver cirrhosis, a background of hepatocellular carcinoma (Altomare *et al.*, 1988; Shigesawa *et al.*, 1992), in patients with gastric cancer (Engin and Ferahköse, 1990), and in tumor-bearing rats (Blumberg *et al.*, 1995). These reports suggest the possibility that the tumor-bearing liver may be exposed to oxidative stress. To examine the possibility of immunotherapy for activating liver MNC in hepatocellular carcinoma, we evaluated the cytotoxicity of liver MNC and PBMNC in hepatocellular carcinoma patients. Strategies to activate these cells by cytokines and to regulate such activation by redox-dependent processes were examined (Tsuyuki *et al.*, 1998b). Cytotoxicity of liver MNC but not PBMNC in hepatocellular carcinoma patients was significantly decreased compared with that of controls, despite no alteration in the subpopulation of liver MNC between the two groups. We next measured intracellular GSH, which is thought to be required for the enhancement of the cytotoxicity by IL-2. Intracellular GSH levels of liver MNC in hepatocellular carcinoma were significantly lower than those of controls. *In vitro* administration of NAC not only restored intracellular GSH levels, but also enhanced the IL-2-stimulated cytotoxicity of liver MNC in hepatocellular carcinoma patients (Fig. 3). These observations indicate that intracellular GSH of liver MNC in hepatocellular carcinoma may modulate the cytotoxicity of liver MNC *in vitro*, and that NAC may be effective as an adjunct to immunotherapy for hepatocellular carcinoma.

NAC, a precursor of GSH (Sen, 1997), has been reported to induce changes in redox states that may improve immunosuppression or induce enhanced responses. We have observed that NAC significantly enhances IL-2-stimulated cytotoxicity of liver MNC from hepatocellular carcinoma patients, especially against NK-insensitive targets, Daudi cells, and also enhanced intracellular GSH levels. These findings were consistent with a previous finding in LAK cells, showing that LAK activity of IL-2-activated NK cells is modulated by intracellular GSH, and suggests a similarity between liver MNC and LAK cells. NAC could not, however, increase the IL-2-stimulated cytotoxicity of PBMNC, which have intracellular GSH levels similar to the control group, suggesting that NAC is not effective for enhancing IL-2-stimulated cytotoxicity of PBMNC, which have adequate concentrations of intracellular GSH.

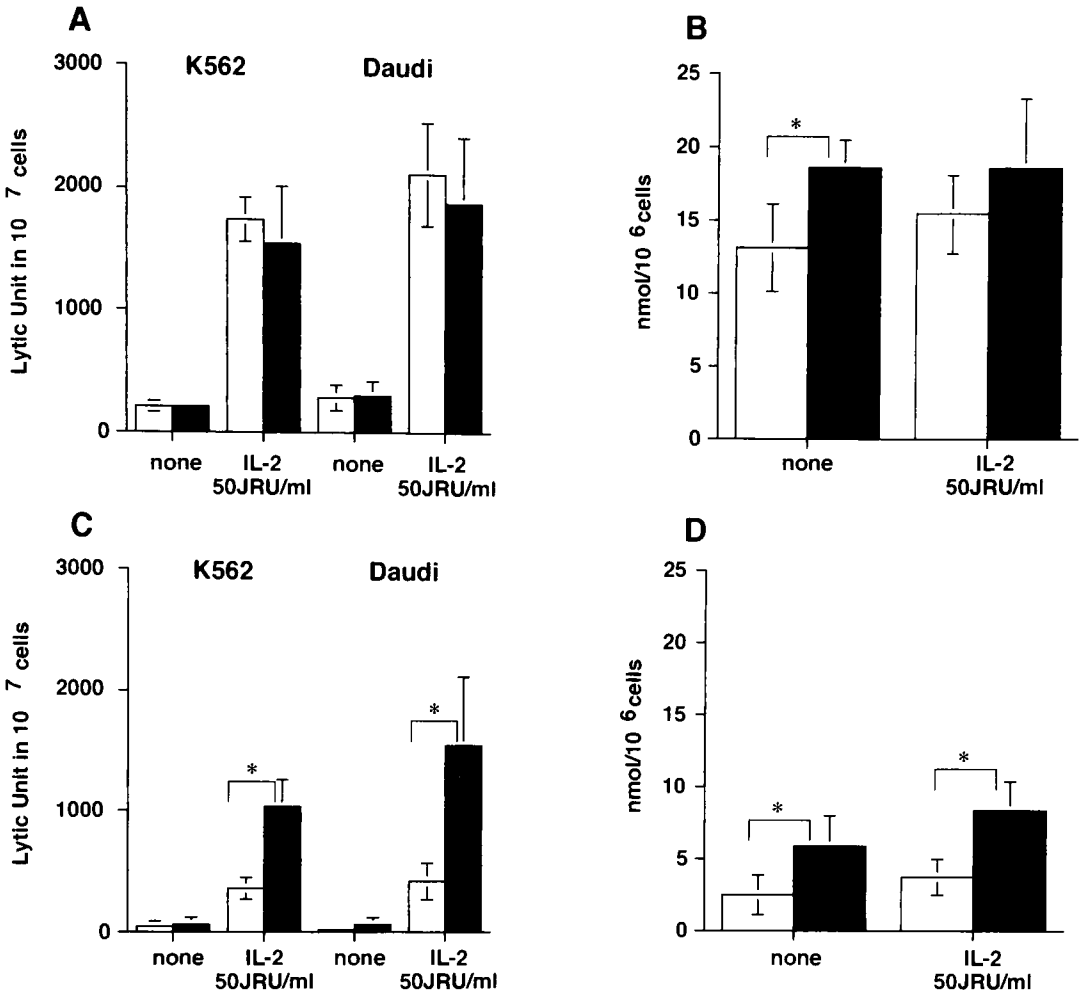


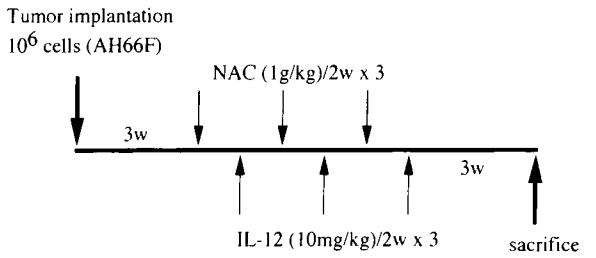
FIG. 3. Cytotoxicity against K562 and Daudi (A and C) and intracellular GSH concentration (B and D) of liver MNC obtained from donors (A,B) and patients bearing hepatocellular carcinoma with cirrhosis (C,D) 72 hr after incubation with NAC (1 m*M*). Open bars, Liver MNC without NAC; **closed bars,** liver MNC with NAC. Values are given as means ± SE of five independent experiments and analyzed by the Wilcoxon signed-rank test. (*) $p < 0.05$ (Tsuyuki *et al.*, 1998b).

Our finding that the cytotoxic activity of fresh normal liver MNC against an NK-insensitive target, *i.e.*, Daudi cells, was higher than that of PBMNC suggests that liver MNC are more activated than PBMNC and may be more LAK-like. In addition, our unpublished data show NK-T ($CD3^+$ $CD56^+$) cells in fresh liver MNC from normal donors had significantly higher expression of the activated marker CD69, compared to those in PBMNC, supporting the suggestion that liver MNC are preactivated. These data suggest that the restoration of intracellular GSH levels may be essential for IL-2 to enhance the cytotoxic activity of liver MNC from hepatocellular carcinoma patients, and that activation of liver MNC using NAC plus IL-2 may be effective in enhancing intrahepatic antitumor effects *in vivo*. Further investigation is, however, necessary to determine the precise mechanism of effect of GSH in cytotoxicity of liver MNC.

IMMUNE THERAPY FOR HEPATOMA AND BEYOND

Previous immune therapies, such as adoptive immunotherapy, or therapies using antigen peptides or biological response modifiers including cytotoxic cytokines have had a big black box in the detail of the mechanisms, although the important processes of immunological functions have recently been elucidated. Cancer patients suffer from cachexia, a condi-

tion mediated by reactive species. Cytokines of the tumor necrosis factor family are generally regarded as inducers of oxidative stresses in cancer-bearing hosts. Therefore, these cytokines may be expected to disrupt the intracellular reducing microenvironment. Redox condition in immune cells of the hosts is an important aspect of research. Previous therapies using immune cells have mostly aimed for cell activation by lymphokines or antigens without considering the intracellular redox status, a major determinant of cell activation. In view of the remarkable implications of cellular redox status, we trust that strategies involving the manipulation of intracellular redox status will prove to be beneficial in enhancing immune therapy. This contention is firmly supported by observations made in our laboratory showing that a combination of cytokine and NAC is much more effective than cytokine alone to control the progression of hepatoma cells implanted in cirrhotic livers (Fig. 4). Thus, transarterial or transportal administration of redox-active agents such as NAC should prove to be markedly beneficial to activate liver MNC.



Treatment	cirrhotic liver	normal rat
no	2.2g	0.3g
NAC	2.2g	
IL-12	0.7g	
NAC+IL12	0.3g	

FIG. 4. Effect of *in vivo* administration of IL-12 with NAC. NAC enhanced the effect of IL-12 on the progression of syngeneic AH66F hepatoma cells implanted in the cirrhotic livers.

Liver has a big subpopulation of macrophages called Kuppfer cells, which are on the upstream of T cells or NK cells in the view point of antigen presentation and Th1 cascade activation. It is known that processing of some antigens depends on reduction of disulfide bonds. The amount of reduced glutathione and cysteine markedly increase in response to stimulation of bone marrow-derived macrophages with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Frosch *et al.*, 1993). The high level of these thiols induced by GM-CSF correlates with a prominent capacity to present the antigen bovine insulin (Frosch *et al.*, 1993). Activation of $CD4^+$ T cells requires processing of exogenous protein antigens by antigen-presenting cells. Using macrophage hybridoma and B cells, it has been shown that the intracellular levels of cysteine and glutathione based on protein content were comparable in these cell lines. The intracellular location of cysteine transport activity determines whether an organelle is a productive site of processing antigens with disulfide bonds that is necessary for $CD4^+$ cell activation (Gainey *et al.*, 1996). Moreover, it has been shown that the hybrid poorly processed hen egg lysozyme and ovalbumin, which are rich in disulfide bonds such as under BSO-treated GSH-deficient conditions. These observations suggest that low intracellular GSH levels in antigen-presenting cells correlate with defective processing of antigens with disulfide bonds, indicating that GSH may be a critical factor in regulating productive antigen processing (Short *et al.*, 1996).

The balance between Th1 and Th2 determines host immunity. GSH levels in antigen-presenting cells determine whether Th1 or Th2 response patterns predominate, and show that intracellular GSH is essential for the production of IL-12 in macrophages (Peterson *et al.*, 1998). These findings indicate that macrophages as well as T or NK cells require an intracellular reducing microenvironment for their functions and provide us with crucial tips to develop a novel strategy against not only hepatocellular carcinoma, but also neoplasms in other organs.

ABBREVIATIONS

AIDS, acquired immunodeficiency syndrome; BSO, L-buthionine-(*S*,*R*)-sulfoximine;

CDK, cyclin-dependent kinase; CTL, cytotoxic T-lymphocyte; GM-CFS, granulocyte-macrophage derived colony stimulating factor; GSH, reduced-type glutathione; HIV, human immunodeficiency virus; IL, interleukin; LAK, lymphokine-activated killer; 2-ME, β-mercaptoethanol; MNC, mononuclear cells; NAC, N-acetyl-L-cysteine; NF-κB, nuclear factor-κB; NK, natural killer; PBMNC, peripheral blood mononuclear cells; RB, retinoblastoma gene products; ROS, reactive oxygen species; SIV, simian immunodeficiency virus; TNF, tumor necrosis factor.

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