Exogenous cysteine and cystine promote cell proliferation in CaCo-2 cells

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Received 18 June 2001; revision accepted 5 September 2001

Abstract. Previous studies have shown that intracellular glutathione, a ubiquitous intracellular thiol, is related to cell proliferation and that cysteine or its disulphide form, cystine, also induces cell proliferation. Cysteine is a thiol containing amino acid and a rate-limiting precursor of glutathione. Therefore, it is still unresolved as to whether the proliferative effect of cysteine or cystine is entirely mediated by a change in the intracellular glutathione status. The objective of this study was to delineate the relationship among cysteine/cystine (thereafter referred to as cyst(e)ine), intracellular glutathione and cell proliferation in the human colon cancer CaCo-2 cell line. CaCo-2 cells were cultured in cyst(e)ine-free Dulbecco's Modified Eagle Medium without serum, and treated with 200 µM cysteine and/or 200–400 µM cystine for 24 h. In the presence of DL-buthionine-[S, R]-sulfoximine (BSO), a glutathione synthesis inhibitor, exogenously administered cyst(e)ine did not change the intracellular glutathione content, but increased the intracellular cysteine as well as cystine level. Addition of exogenous cyst(e)ine following 5 mM BSO treatment significantly increased cell proliferation as measured by ³H-thymidine incorporation and protein content. Cell cycle analyses revealed that cyst(e)ine promoted cell progression from the G₁ phase to the S phase. Correspondingly, cyst(e)ine treatment induced expression of cyclin D1 and phosphorylation of retinoblastoma protein (Rb). In conclusion, these data indicate that both cysteine and cystine have proliferative effects in CaCo-2 cells independent of an increase in intracellular glutathione. Induction of cyclin D1, phosphorylation of Rb, and subsequent facilitation of G₁-to-S phase transition were involved in the proliferative effect of exogenous cyst(e)ine.

INTRODUCTION

In recent years, there is increasing recognition that intracellular redox status affects cellular and molecular events in various cells. Glutathione, L-γ-glutamyl-L-cysteinylglycine, is present in all tissues. Its reduced form (GSH) and oxidized form (glutathione disulphide, GSSG)

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constitute the major intracellular thiol-disulphide redox system in mammalian cells. Glutathione serves several vital functions, including: (1) scavenging peroxides; (2) modulating critical cellular processes such as cell proliferation, apoptosis, microtubular-related processes, and immunological function; (3) maintaining the essential thiol status of proteins by preventing oxidation of -SH groups or by reducing disulphide bonds induced by oxidative stress; (4) detoxifying electrophiles; and (5) providing a reservoir for cysteine (Meister 1988; Suthanthiran *et al.* 1990; DeLeve & Kaplowitz 1991; Hutter *et al.* 1997; Lu 1999; Wang *et al.* 2000). Among the many functions of glutathione, the relationship between intracellular glutathione status and cell proliferation is of particular interest. Several studies have demonstrated a close association between the two (Suthanthiran *et al.* 1990; Shaw & Chou 1986; Godwin *et al.* 1992; Kang *et al.* 1994; Obrador *et al.* 1997; Chang *et al.* 1999; Noda *et al.* 2001).

Cysteine is a thiol containing amino acid. Together with cystine, its disulphide form, they constitute an important extracellular thiol-disulphide system. However, cysteine and cystine can also be transported into cells through distinct membrane transporters (Bannai & Tateishi 1986). Within cells, cystine can participate in disulphide bond formation with intracellular thiols, such as GSH, or be reduced to cysteine. Intracellular cysteine can be incorporated into proteins or serve as a precursor for glutathione synthesis. Indeed, the availability of cysteine is rate limiting for glutathione biosynthesis (Bannai 1984; Lu 1999).

Previous studies have shown that cyst(e)ine stimulated cell proliferation in various cells (Eagle *et al.* 1966; Lazarus *et al.* 1974; Livingston *et al.* 1976; Iglehart *et al.* 1977; Uren & Lazarus 1979). However, as cysteine is a precursor of GSH synthesis and the administration of cysteine increases intracellular GSH under physiological conditions, it is still unclear as to whether the stimulatory effects of exogenous cysteine are entirely mediated by intracellular GSH synthesis or mediated, at least in part, by a glutathione-independent mechanism. Moreover, because cystine can be readily reduced to cysteine, the mechanism by which exogenous cystine mediates cell proliferation is similarly unresolved.

In the current study, we have used human colon cancer CaCo-2 cells to evaluate the role of exogenously administered cyst(e)ine in cell proliferation. The specific objectives of this study were: (1) to determine whether exogenously administered cyst(e)ine have stimulatory effects on cell proliferation that are independent of their role in promoting GSH synthesis, and (2) to examine the role of cyst(e)ine in cell cycle changes.

MATERIALS AND METHODS

Reagents

L-cystine and DL-buthionine-[S, R]-sulfoximine (BSO) were obtained from Sigma Chemical (St. Louis, MO). L-cysteine was purchased from Fluka chemical (Ronkonkoma, NY). [Methyl-³H]-thymidine was obtained from NEN Products (Boston, MA). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Dulbecco's Modified Eagle Medium (DMEM) and other cell culture supplies were from Gibco BRL (Grand Island NY). All other chemicals were of reagent grade and were obtained from local sources.

Cell culture and treatment

The human colon cancer cell line, CaCo-2, was obtained from the American Type Culture Collection (Rockville, MD) and the cells were seeded in 75 cm² culture flasks. The cells were grown in standard cystine (200 µM)-containing DMEM12800, supplemented with 10% fetal

Figure 1. High performance liquid chromatography separation of cystine, cysteine, GSH and GSSG in extracts of CaCo-2 cells. (a) Separation of standard mixtures. (b) Separation of cystine, cysteine, GSH and GSSG in cell extracts. The numbers 1–4 represent the cystine, cysteine, GSH, and GSSG peaks with retention times of 12, 19, 25 and 27 min, respectively.

bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and 10 mg/ml gentamicin (standard medium). The cell cultures were maintained in a humidified atmosphere with 5% CO₂/95% air at 37 °C. The culture medium was changed every 2 days.

For experimentation, CaCo-2 cells were seeded in 6- or 24-well plates and were cultured in standard medium. When cells reached 60% confluency, the standard medium was replaced with cysteine and cystine-free DMEM21013 that was supplemented with 1% nonessential amino acids, 100 U/ml penicillin, 10 mg/ml gentamicin, but not with serum. Just after medium change, cells were treated with cysteine and/or cystine and incubated for 24 h. In some experiments, 5 mM BSO was administered 1 h prior to the treatment with cyst(e)ine and was present throughout the incubation time to prevent synthesis of glutathione (Griffith & Meister 1979).

Determination of GSH, GSSG, cysteine, and cystine

GSH, GSSG, cysteine, and cystine levels were determined by the high-performance liquid chromatography (HPLC) method of Reed *et al.* (1980). At the end of timed incubations, the cells were washed once with PBS while still attached and then treated with ice-cold 5% trichloroacetic acid (TCA). The acid-treated cells were harvested by scraping, and the supernatants were separated from TCA-insoluble proteins by centrifugation. The acid supernatants were treated with 6 mM iodoacetic acid, pH 7 for 1 h. This derivatization step yielded S-carboxymethyl derivatives and thereby protected the thiol groups of GSH and cysteine. After 1 h, a 1% solution of 2, 4-dinitrofluorobenzene was added and the pH adjusted to 10. The amino groups were derivatized to yield the 2, 4-dinitrophenyl derivatives of GSH, GSSG, cysteine, and cystine which have a strong absorbance at 365 nm. Separation of GSH, GSSG, cysteine, and cystine derivatives was achieved on a 25-cm × 4.6 mm × 10 µm Lichrosorb NH₂ column using a Shimadzu chromatograph (Shimadzu Scientific Instruments Inc., Columbia, MD) with the operating conditions of 1 ml/min flow rate and an elution gradient of buffer A (80% methanol) to buffer B (4 M acetate in 64% methanol) over 55 min as follows: 10 min isocratic at 90% buffer A/10% buffer B, 30 min gradient to 100% buffer B, and 15 min isocratic at 100% buffer B. Representative chromatograms of HPLC separations of authentic standards (a) or cell samples (b) are presented in Fig. 1.

[³H]-thymidine incorporation

Three hours before collecting the cells, 1 μ Ci [³H]-thymidine was added to each well. At each indicated time point, the labelled cell monolayer was washed twice with phosphate-buffered saline (PBS), and the cells were detached with trypsin-EDTA. Cells were precipitated with 5% TCA at 4 °C. The precipitate was washed twice with 95% ethanol at 4 °C followed by drying at room temperature, and was solubilized with 1 N NaOH. Aliquots of dissolved precipitate were mixed with Poly fluor (Packard, Meriden, CT), and the cell-associated radioactivities were determined using a liquid scintillation counter.

Cell cycle analysis by flow cytometry

At designated times, the cells were trypsinized, washed with cold PBS, and fixed in 70% ethanol for 30 min at 4 °C. Fixed cells were washed with PBS and incubated in 1 mg/ml DNAase-free RNAase for 40 min at 37 °C. Then, the samples were incubated with 50 μ g/ml propidium iodide (PI) at 4 °C for 30 min in the dark. Flow cytometric analysis was performed using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). Minimums of 5×10^5 cells per sample were analysed. Data were analysed using Cell Quest software (Becton Dickinson, San Jose, CA), and was gated on pulse-processed PI signals to exclude doublets and large aggregates, using a multiparameter gate strategy. Cell viability was determined as 100% minus the percent of dead cells at subG0.

Western blot analysis

Cell monolayers were washed twice with PBS, detached with trypsin-EDTA, collected, and centrifuged at 500 *g* for 5 min. Cell pellets were lysed for 45 min at 4 °C in lysis buffer (pH 7.6) consisting of 50 mM Tris-HCl, 300 mM NaCl, 0.5% Triton X-100, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatine A, and 1 mM phenylmethylsulphonyl fluoride. Equal quantities (50 μ g) of lysates were resolved by 10% sodium dodecyl sulphate polyacrylamide gel and electroblotted to a nitrocellulose membrane (Trans-Blot, Bio-Rad, Hercules, CA). After blocking with PBS containing 0.1% polyoxyethylene sorbitan monolaurate (Tween-20; Sigma) and 5% dry milk at 4 °C overnight, the membrane was incubated with mouse monoclonal anticyclin D1 antibody (2 μ g/ml; Santa Cruz Biotech, Santa Cruz, CA) or anti-Rb antibody (1 μ g/ml; Oncogene, Cambridge, MA) for 2 h. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated antimouse IgG (1 : 1000; Amersham, Piscataway, NJ). Detection of chemoluminescence was performed with an ECL Western blot detection reagent kit (Amersham) according to the supplier's recommendation.

Protein assay

Proteins were measured using Bio-Rad Protein Assay kit (Bio-Rad) according to the manufacturer's protocol.

Statistical analysis

Results are expressed as mean $\pm$ SE. Data were analysed using a one-way ANOVA with Bonferroni corrections for multiple comparisons. *P*-values of < 0.05 were considered as statistically significant.

RESULTS

Effect of cyst(e)ine on intracellular glutathione status

Figure 2 illustrates the effects of cysteine and/or cystine on intracellular glutathione levels. Deprivation of cyst(e)ine in the medium caused marked decreases in the intracellular GSH and

Figure 2. Effect of cysteine and/or cystine on intracellular glutathione levels in GSH-sufficient CaCo-2 cells (without BSO treatment). Cells were incubated in standard medium or cyst(e)ine-free medium and treated with 200 μM cysteine and/or 200–400 μM cystine for 24 h. (a) GSH. (b) GSSG. Results are the mean $\pm$ SE of 6 separate experiments. a: $P < 0.05$ vs. standard medium, b: $P < 0.05$ vs. corresponding cyst(e)ine-free medium without cyst(e)ine treatment.

GSSG levels (open bars) as compared to those in cells incubated with standard medium (dotted bars). The addition of 200 μM cysteine and/or 200–400 μM cystine to the medium significantly elevated intracellular GSH to near control levels (Fig. 2a). Interestingly, significantly higher intracellular GSSG levels were also observed in cells exposed to exogenous cystine alone or cystine in combination with cysteine, but not in cells treated with cysteine alone (Fig. 2b).

Influences of cyst(e)ine on cell proliferation

A baseline proliferative activity in GSH-sufficient cells (i.e. without BSO treatment) cultured in cyst(e)ine-free media was maintained at a rate of ^3H -thymidine incorporation of 10 000 cpm/mg protein and a cellular protein content of 0.12 mg (Fig. 3a and b). The addition of cyst(e)ine at all concentrations did not affect ^3H -thymidine incorporation and total protein contents, despite the significant increases in intracellular GSH and GSSG levels induced by cyst(e)ine exposure (see Fig. 2). Pretreatment of cells with 5 mM BSO (which lowered cellular GSH) markedly decreased cellular proliferative activity as measured by ^3H -thymidine incorporation (685 ± 452.6 vs. 10385 ± 2265.5 ; $P < 0.05$) and total protein contents (0.046 ± 0.008 vs. 0.124 ± 0.027 ; $P < 0.05$). However, in these GSH-deficient cells, the addition of exogenous cyst(e)ine significantly increased both ^3H -thymidine incorporation and total protein (Fig. 4a and b). Notably, the increases in ^3H -thymidine incorporation and protein contents were similar regardless of whether cells were exposed to cysteine, or cystine alone, or to combinations of both amino acids (Fig. 4a and b). Collectively, the results from Figs 3 and 4 show that exogenous cyst(e)ine exerted proliferative effects on CaCo-2 cells under GSH-deficient (BSO-treated, Fig. 4), but not GSH

Figure 3. Effect of cysteine and/or cystine on cell proliferation in GSH-sufficient CaCo-2 cells (without BSO treatment). Cells were incubated in cyst(e)ine-free medium and treated with 200 μM cysteine and/or 200–400 μM cystine for 24 h. (a) ³H-thymidine incorporation. (b) Total protein. Results are the mean ± SE of 4 separate experiments.

sufficient (Fig. 3) conditions, suggesting that the mitogenic effect of exogenous cyst(e)ine in the GSH deficient state may be related to its restoration of cellular GSH levels.

Effect of cyst(e)ine on intracellular GSH, GSSG, cysteine, and cystine in the presence of DL-buthionine-[S, R]-sulfoximine (BSO)

To test this suggestion, intracellular glutathione levels were measured. Figure 5 shows that the baseline GSH and GSSG levels in BSO-treated cells grown in cyst(e)ine-free media were low compared to the levels in cells without BSO treatment (see Fig. 2a and b). Moreover, the administration of cyst(e)ine at all doses did not increase GSH levels in BSO-treated cells except at the highest concentration of cyst(e)ine tested, namely 200 μM cysteine plus 400 μM cystine (Fig. 5a). GSSG levels were unchanged in all groups (Fig. 5b). These results are largely consistent with an inhibitory effect of BSO on glutathione synthesis. In comparison, the administration of exogenous cyst(e)ine significantly elevated the intracellular cysteine and cystine levels, and the magnitude of elevation was dependent on the concentrations of the administered cysteine and cystine (Fig. 6a and b). Taken together, these results indicate that the stimulatory effect of cyst(e)ine on cell proliferation occurred independently of intracellular glutathione synthesis.

Comparison of the data in Figs 4 and 6 further indicates that the stimulation of cell proliferative activity by exogenous cyst(e)ine is not linearly correlated with the intracellular cysteine or cystine concentrations. For instance, maximal stimulation of cell proliferative activity occurred with 200 μM cysteine or cystine alone (Fig. 4), while the highest intracellular levels of the amino acids were only achieved with the combined treatment with 200/400 μM cysteine and cystine (Fig. 6). The results in Fig. 6 also show that exogenously administered cystine increased

Figure 4. Effect of cysteine and/or cystine on cell proliferation in GSH-depleted CaCo-2 cells (with BSO treatment). Cells were incubated in cyst(e)ine-free medium and treated with 200 μM cysteine and/or 200–400 μM cystine for 24 h. Cells were pretreated with 5 mM BSO 1 h prior to administration of cyst(e)ine. BSO was present throughout the incubation time to prevent synthesis of glutathione. (a) ^3H -thymidine incorporation. (b) Total protein. Results are the mean $\pm$ SE of 4 separate experiments. a: $P < 0.05$ vs. the respective condition without cysteine or cystine addition.

intracellular cysteine and cystine levels more effectively than exogenous cysteine administration (compare 2nd and 3rd bars, Fig. 6a and b), which suggest that the cellular uptake of cystine is highly efficient and that the transported cystine is readily reduced to cysteine intracellularly.

Flow cytometric analysis

Because the greatest effects on cell proliferation mediated by cyst(e)ine were observed in GSH deficient cells (i.e. BSO treated) cultured in cyst(e)ine free media, this experimental model was used to further examine the role of cyst(e)ine on cell survival and cycle changes by cytometric analysis. Addition of 200 μM cysteine and 400 μM cystine did not affect the viability of cells, indicating that the stimulatory effect of cyst(e)ine on cell proliferation was not due to the preservation and/or the improvement of cell survival (Fig. 7a). In contrast, cyst(e)ine elicited significant changes in cell cycle progression. Notably, exposure of cells to exogenous cyst(e)ine decreased the percentage of cells in the G_0/G_1 phase and increased the percentage of cells in the S phase (Fig. 7b and c), which directly correlated with enhanced thymidine incorporation and DNA synthesis. Interestingly cyst(e)ine treatment did not affect the percentage of cells in the G_2/M phase (Fig. 6d).

Western blot analysis

To determine whether the mitogenic effect of cyst(e)ine is mediated by modulating cell cycle activity, we examined the status of two key proteins of the G_1 checkpoint, namely the expression

Figure 5. Effect of cysteine and/or cystine on intracellular glutathione levels in BSO-treated CaCo-2 cells. Cells were incubated in cyst(e)ine-free medium and treated with 200 μM cysteine and/or 200–400 μM cystine for 24 h. Cells were pretreated with 5 mM BSO 1 h prior to administration of cyst(e)ine. BSO was present throughout the incubation time to prevent synthesis of glutathione. (a) GSH. (b) GSSG. Results are the mean ± SE of 6 separate experiments. a: $P < 0.05$ vs. the respective condition without cysteine or cystine addition as well as vs. addition of 200 μM cysteine/0 cystine.

of cyclin D1 and the phosphorylation state of Rb. The western analysis in Fig. 8a shows that treatment with cyst(e)ine significantly increased cyclin D1 expression. Moreover, cyst(e)ine treatment significantly increased the phosphorylation of Rb, such that the ratio of phosphorylated Rb to hypophosphorylated Rb is markedly elevated (Fig. 8b). Taken together with the results of flow cytometric analyses, these data indicate that the stimulatory effect of cyst(e)ine on cell proliferation is mediated by increased cyclin D1 expression and Rb phosphorylation which facilitated the G₁-to-S phase transition and cell cycle progression.

DISCUSSION

Cysteine is an important extracellular thiol (Bannai 1984; Lu 1999) that has been demonstrated to play an important role in cell proliferative activity (Eagle *et al.* 1966; Lazarus *et al.* 1974; Livingston *et al.* 1976; Iglehart *et al.* 1977; Uren & Lazarus 1979). In addition, cysteine is a precursor for GSH synthesis whose rate of synthesis is limited by the availability of cysteine (Bannai 1984; Lu 1999). In the current study, exogenously administered cysteine increased intracellular GSH level, consistent with its role as a GSH precursor. Moreover, cellular GSH was also increased by exogenous cystine, indicating that cystine can be used for GSH production after its reduction to cysteine intracellularly. The blockade of GSH increases with BSO

Figure 6. Effect of cysteine and/or cystine on intracellular cysteine and cystine levels in BSO-treated CaCo-2 cells. Cells were incubated in cyst(e)ine-free medium and treated with 200 μM cysteine and/or 200–400 μM cystine for 24 h. Cells were pretreated with 5 mM BSO 1 h prior to administration of cyst(e)ine. BSO was present throughout the incubation time to prevent synthesis of glutathione. (a) Cysteine. (b) Cystine. Results are the mean ± SE of 6 separate experiments. a: $P < 0.05$ vs. no cysteine/cystine.

pretreatment followed by exogenous cyst(e)ine administration is consistent with this interpretation. Interestingly, cystine, but not cysteine, treatment also increased cellular GSSG. The reason for this increase is not clear. One possibility may be that elevated cellular cystine levels can induce a more oxidizing intracellular environment that favour GSH oxidation.

Previous studies reported that administration of cyst(e)ine elevated intracellular glutathione levels and stimulated cell proliferation, suggesting that the effect of cyst(e)ine is mediated by GSH increases (Yamauchi & Bloom 1993, 1997). However, our current results show that cyst(e)ine stimulated cell proliferation independently of GSH synthesis. For example, the administration of cyst(e)ine did not elicit stimulatory effects on cell proliferation as measured by ^3H -thymidine incorporation and protein content (Fig. 3a and b) in spite of an increase in intracellular glutathione levels (Fig. 2). The reason for the differences observed in previous studies and our current study is unclear. One possible explanation may be that we cultured cells in serum-free medium. As serum is a potent proliferative stimulus, the lack of serum suppresses cell proliferation. Thus, in the absence of BSO wherein cellular glutathione was preserved, cell proliferation was restricted by serum deprivation rather than by intracellular glutathione status. Another possible reason is that neither intracellular GSH levels nor GSSG levels *per se* were important for cell proliferation. This suggestion is supported by our recent finding that it is the decrease in GSH to GSSG ratio rather than the decrease in GSH level *per se* that was important in mediating cell proliferation (Noda *et al.* 2001). Therefore, while exogenous cyst(e)ine increased both GSH and GSSG, the small changes in GSH to GSSG ratio might be insufficient

Figure 7. Flow cytometric analysis of GSH-depleted cells. Cells were incubated in cyst(e)ine-free medium and treated with 200 μ M cysteine and 400 μ M cystine for 24 h. Cells were pre-treated with 5 mM BSO 1 h prior to administration of cyst(e)ine. BSO was present throughout the incubation time to prevent synthesis of glutathione. (a) Percentage of viable to total cells. (b) Percentage of cells in G₀/G₁ phase to total viable cells. (c) Percentage of cells in S phase to total viable cells. (d) Percentage of cells in G₂/M phase to total viable cells. Results are the mean $\pm$ SE of 3 separate experiments. a: $P < 0.05$ vs. BSO alone.

to modify cell proliferation in GSH-sufficient cells. In contrast, BSO significantly depleted intracellular glutathione pools and prevented the utilization of exogenous cyst(e)ine for *de novo* glutathione synthesis. In these GSH compromised cells, exogenous cyst(e)ine was found to stimulate cell proliferation, indicating that exogenous cyst(e)ine can directly exert mitogenic effects independently of GSH synthesis. These results are in agreement with the study by Gout *et al.* (1997) who demonstrated that the proliferative effect of exogenous cystine in Nb-2 lymphoma cells was not primarily based on its maintenance of normal GSH levels.

The mechanism by which cyst(e)ine promoted CaCo-2 cell proliferation is not known. The possibility that the proliferative effects of exogenous cyst(e)ine were related to their roles as substrates for protein synthesis is not supported by the current results. For example, BSO itself did not affect protein synthesis, yet, the proliferative effects of cyst(e)ine was dependent on the presence of BSO to induce a GSH-deficient state. Support for another mechanism comes from previous studies by Jonas *et al.* (2000) who indicated that the effect of cyst(e)ine might be mediated by the extracellular redox potential. These investigators found that extracellular thiol-disulphide redox status (cysteine-cystine balance in the medium) regulated proliferation in CaCo-2 cells. In our study, the administered cysteine was minimally converted to cystine in the medium throughout the incubation period (data not shown), which meant that the extracellular cysteine-cystine balance in various treatment groups was as defined by the ratios of the added reduced and oxidized amino acids in the incubation media. The results in Fig. 4 show that exogenous cysteine or cystine alone was as effective in stimulating CaCo-2 cell proliferation as the combination of the two amino acids. Hence, it is unlikely that extracellular cysteine-cystine balance was a critical determinant in modulating the stimulatory effect. However, we cannot rule out the possibility of a direct effect of cysteine or cystine on extracellular membrane receptors.

Figure 8. Western analyses on cyclin D1 expression and Rb phosphorylation in GSH-depleted CaCo-2 cells (with BSO treatment). Cells were incubated in cyst(e)ine-free medium and treated with 200 μ M cysteine and 400 μ M cystine for 24 h. Cells were pre-treated with 5 mM BSO 1 h prior to administration of cyst(e)ine. BSO was present throughout the incubation time to prevent synthesis of glutathione. (a) Cyclin D1 expression (adjusted by actin). (b) Rb expression and ratio of phosphorylated Rb (ppRb) to hypophosphorylated Rb (pRb). Results are the mean $\pm$ SE of 4 separate experiments. a: $P < 0.05$ vs. BSO alone.

Previous studies showed that cells that exhibited low cystathionase activity (limited generation of cysteine from methionine) required cysteine for cell proliferation, while cells with high cystathionase activity were able to proliferate when cultured in methionine-supplemented cyst(e)ine-free media (Eagle *et al.* 1966; Livingston *et al.* 1976). These findings support our current suggestion that the proliferative effect of exogenous cysteine was mediated by its intracellular conversion to cysteine. We recently demonstrated that the depletion of GSH *per se* had no effect on cell proliferation, but intracellular GSH depletion exacerbated the suppression of cell proliferation induced by cellular redox imbalance (Noda *et al.* 2001). Interestingly, our current results show that the mitogenic effect of exogenous cyst(e)ine was maximally manifested following BSO depletion of cellular GSH, but was unrelated to GSH restoration. One possible explanation of this interesting finding is that the increase in intracellular cysteine (resulting from uptake of exogenous cyst(e)ine) functions to stabilize the intracellular thiol pool that was compromised by BSO in order maintain cell proliferation, without necessarily restoring the GSH content itself. This contention is supported by previous studies which showed that 2-mercaptoethanol was able to stimulate cell proliferation in human peripheral blood mononuclear cells cultured in cystine-free medium and depleted of their cellular GSH by BSO (Messina & Lawrence 1992). Based on these considerations, we speculated that the glutathione redox system is an important intracellular thiol redox system governing cell proliferation, and that in GSH-depleted cells, other thiols (such as cysteine) can substitute for the function of GSH. Interestingly, the intracellular

thiol levels achieved by 200 μ M exogenous cysteine alone in GSH-depleted cells (i.e. with BSO treatment) (0.307 ± 0.038 nmol/mg protein, Fig. 6a) did not reach the equivalent intracellular concentrations of GSH in control cells (i.e. GSH-sufficient cells without BSO treatment) (9.810 ± 1.653 nmol/mg protein, Fig. 2a). Yet, this exogenous cysteine concentration was sufficient to stimulate cell proliferation. This suggests that the thiol threshold for initiating a proliferative response was lower for cysteine than GSH. However, it should be noted that cell proliferative activity (3 H-thymidine incorporation) in GSH-sufficient cells (with or without cysteine) was threefold higher (11125 ± 2461 cpm/mg protein, Fig. 3a) than that elicited by exogenous cysteine in GSH-deficient cells (3796 ± 853 cpm/mg protein, Fig. 4a). Notably, the combined addition of cysteine and cystine did not further promote cell proliferation, indicating that a ceiling threshold for cysteine-induced proliferation was reached at 200 μ M exogenous cysteine.

Cyclin D1 and Rb act in the late G_1 phase to regulate the G_1 -to-S phase transition. The phosphorylation of Rb is mediated by the cyclin D1/CDK 4 or CDK6 complexes which results in the release of the E2F transcriptional factor that recognizes the binding sequences in the genes that control cell cycle progression (Chellappan *et al.* 1991; Chittenden *et al.* 1991; Israels & Israels 2000). Previous studies have shown that the lack of cyst(e)ine and glutathione in the medium decreased intracellular GSH levels and inhibited the G_1 -to-S phase transition (Iwata *et al.* 1994; Yamauchi & Bloom 1997). In interleukin-2 stimulated natural killer cells, this G_1 arrest was mediated by a reduced expression and phosphorylation of the Rb protein. Addition of cystine restored the intracellular GSH levels and promoted cell proliferation (Yamauchi & Bloom 1997). In our current study, the combined manipulation of culturing cells in cyst(e)ine-free media and pre-treating cells with BSO exhausted the intracellular glutathione and cyst(e)ine levels. Under these thiol compromised conditions, exogenous cyst(e)ine caused an increase in intracellular cysteine (but not glutathione) (Figs 5 and 6) that mediated the transition of cells from G_1 -to-S phase (Fig. 7) in parallel with the induction of cyclin D1 and the phosphorylation of Rb (Fig. 8). These results suggest that the intracellular thiol status is an important factor in the control of cell cycle checkpoint.

In summary, the current study shows that exogenous cyst(e)ine administration stimulated proliferative activity in GSH-deficient CaCo-2 cells in the absence of GSH synthesis. This mitogenic effect of exogenous cyst(e)ine was associated with enhanced expression of cyclin D1, phosphorylation of Rb protein, and facilitation of cell transition from G_1 -to-S phase.

ACKNOWLEDGEMENTS

This study is supported by a grant from the National Institutes of Health DK 44510.

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