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Zinc and Selenium Requirements for Glutathione Peroxidase Activity and Cell Survival in Chinese Hamster Ovary Cells Overexpressing Metallothionein

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Abstract

Many defined cell culture media were formulated over 30 years ago and may be deficient in certain micronutrients whose essentiality has only subsequently been recognised. The objective of this study was to evaluate whether alpha-minimal essential medium (MEM) supplemented with 10% foetal bovine serum contained sufficient selenium for optimal activity of the selenium containing enzymes cytosolic glutathione peroxidase (cGPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) in cultured Chinese hamster ovary (CHO) cells. Additionally, the effect of zinc deficiency and metallothionein (MT) overexpression on cGPx and PHGPx activity was studied. The addition of 100 nM of selenous acid to the culture medium increased cGPx expression by 10-fold and PHGPx by about 2-fold in both wild-type CHO-K1 cells and CHO-K1 cells overexpressing mouse MT-1. Zinc deficiency had no significant effect on enzyme activity, but cells overexpressing mouse MT-1 had higher levels of cGPx activity. Zinc deficiency decreased cell survival but overexpression of MT-1 was partially protective, probably because its presence in quantity favoured the uptake, sequestration and cellular retention of any remaining zinc. This study demonstrates that selenium in complete alpha-MEM is insufficient for optimal cGPx and PHGPx activity and may compromise the cellular response to oxidative stress.

Key words: Zn, Se, cytosolic glutathione peroxidase (cGPX), phospholipid hydroperoxide glutathione peroxidase (PHGPx), Chinese hamster ovary (CHO) cells

INTRODUCTION

Defined cell culture media have been formulated for specific nutritional requirements of particular cell types. These media vary in complexity from simple compositions, such as Eagles minimum essential medium (MEM) (1) to complex media such as 199 (2) and RPMI 1640 (3). While the composition of many media has undergone minor modification in order to optimise conditions for new or established cell lines, the basic formulae for these media has not changed in over 30 years. This is in spite of improvement in the understanding of cellular nutrient requirements and the essentiality of a range of important micronutrients. For example, zinc is an essential trace metal, and yet it is not included in the formulation of most cell culture media. Therefore, the only supply of zinc to cultured cells comes from serum supplements, and it is likely that cells will become marginally zinc deficient during culture. The level of deficiency may not be sufficient to affect normal metabolism and cell division, but may influence metabolic or signalling pathways under stress conditions. Without any serum addition to culture medium, cells tend to become quiescent and eventually apoptotic, and partial prevention of apoptosis by addition of zinc shows that zinc deficiency contributes to this phenomenon (4). The essentiality of selenium was relatively unrecognized when original media formulations were being designed, and so there are few commercially available cell culture media containing this element. Supply of selenium is dependent on supplementation of the media with serum, but as in the case of zinc, the level of these elements in the complete medium may be insufficient for optimal cell function under stress conditions. The activity of selenium requiring enzymes, such as the antioxidant glutathione peroxidases, is known to be correlated to selenium status and assay of plasma glutathione peroxidase is used to assess selenium deficiency (5). Therefore, a principal objective of this work was to evaluate the degree of selenium deficiency in cell culture medium by measuring the cellular activity of cytosolic glutathione peroxidase (cGPx) and phosopholipid hydroperoxide glutathione peroxidase (PHGPx). Since zinc is known to have antioxidant properties (6), we also investigated whether zinc deficiency could affect cGPx and PHGPx activities. Acute zinc deficiency was induced by addition of the metal-chelating compound diethylenetriaminepentaacetic acid (DTPA) and simultaneous iron defi-

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ciency was prevented by supplementation with iron sulphate (7).

The cellular status for a particular nutrient may be influenced by metabolic state or expression of proteins that influence micronutrient metabolism. For example, the zinc-binding protein metallothionein (MT) can significantly influence zinc status (8). MT is a low molecular weight protein (about 6,500 Da) which binds 7 gram atoms of zinc per mole protein in a 2-domain secondary structure (9). Its expression is normally induced by zinc and a diverse range of stress factors (10). We therefore investigated the influence of MT overexpression on the effect of zinc and selenium deficiency. This was achieved by transfecting Chinese Hamster Ovary cells, which are known to have low endogenous levels of MT, with a mouse MT-1 expression vector.

MATERIALS AND METHODS

Generation of CHO cells overexpressing metallothionein

A modified cell line overexpressing metallothionein was formed by stable transfection of parental CHO-K1 cells with a gene construct containing the mouse metallothionein-1 gene (mMT-1), using the lipofectin method (11). The mMT-1 gene was inserted into the pBPV expression vector downstream of a non-inducible promoter derived from the mMT-1 gene and an enhancer from the long terminal repeat of the Moloney murine sarcoma virus (12). The pBPV plasmid also contains the bovine papilloma virus genome, which in several rodent cell lines, causes the plasmid to be maintained as an episome, replicating autonomously, with 20 ~ 150 copies per cell. Following transfection, cells were selected by addition of 5 ~ 30 μM cadmium chloride to the culture medium. Zinc chloride was added at equimolar concentrations to ensure that cells did not become zinc deficient due to the high expression of MT. Since only those cells expressing higher levels of MT could survive exposure to normally toxic levels of cadmium, clones overexpressing this protein could be selected following cadmium treatment. Several of these clones were characterised for their growth rate, MT levels and endogenous antioxidants to obtain a single clone that was similar to the parental (wild-type) cells in all respects except for MT expression. The level of MT in this clone was measured by radioimmunoassay (13) and found to be >350 times higher than in the wild-type cells. Stocks of the cells overexpressing mMT-1 (hereafter referred to as OEX cells) were preserved in liquid nitrogen and when revived for experimental use, the MT levels were periodically analysed after several passages.

Cell treatment conditions

OEX and wild-type (WT) CHO-K1 cells were grown to confluence in 10 cm culture dishes using alpha-minimal essential medium (α-MEM, Sigma, Poole, UK) supplemented with 10% fetal calf serum, 1% L-glutamine and 0.5% penicillin/streptomycin. The growth rate of both cell types was not significantly different and confluence was attained 4 ~ 5 days after seeding (seeding density 2×10^8 cells/mL). Zinc deficiency was induced by addition of 600 μM diethylenetriaminepentaacetic acid (DTPA) and since this zinc chelator also binds iron, 200 μM $FeSO_4$ was added to the medium to prevent cells also becoming iron deficient (7). As a control for zinc deficient conditions, $ZnSO_4$ was added back to the medium to a final concentration of 400 μM. To investigate the effect of Se supplementation, 100 nM selenous acid was added to the Zn-supplemented medium. The cells were exposed to the treatment media for 3 days and they were then washed and harvested by scraping into Hanks balanced salt solution (HBSS) using a cell scraper. They were then centrifuged at 3000 rpm at 4°C for 5 min to pellet the cells. The HBSS supernatant was discarded and the pellets were maintained at 4°C prior to analysis.

Selenium-dependent enzyme and protein analysis

The level of cytosolic glutathione peroxidase (cGPx) in cell cytosol samples was measured using the enzymatic method of Bermano et al. (14). This method follows the enzymatic oxidation of NADPH by cGPx spectrophotometrically at 340 nm. For phospholipid hydroperoxide glutathione peroxidase (PHGPx) analysis, cell samples were suspended in 0.1% triton X-100 with 0.125 M phosphate/1 mM EDTA buffer, and freeze-thawed 3 times. After centrifugation at 12,000 × g for 20 min at 4°C, enzyme activity was measured in the supernatant by a method modified from that of Weitzel et al. (15). Briefly describing here, cGPx and PHGPx enzyme activities were measured in cells which had been washed and resuspended in HBSS and then incubated with 0.1% peroxide-free Triton X-100 in 0.125 M KPO_4/1 mM EDTA solution for cell lysis. Cell cytosol sample was then incubated again with 0.3 mM NADPH, 5 mM reduced glutathione and 0.7 units/mL glutathione reductase. Oxdation of NADPH was followed spectrophotometrically at 340 nm after addition of either 2.2 mM H_2O_2 (cGPx activity) or phospholipid hydroperoxide PC-OOH (PHGPx activity) to the samples (14,15).

Total cell protein was measured using the bicinchoninic acid (BCA) method (16).

RESULTS

Effect of zinc deficiency on cell survival

The viability of WT cells exposed to 600 μM DTPA

and 200 μM $FeSO_4$ without addition of zinc was markedly reduced compared to all other treatments. Few cells remained attached to the culture dishes after induction of zinc deficiency but these cells were nevertheless used for assay of cGPx and PHGPx. Although OEX cells also showed a loss of viability, the concentration of cell protein recovered from the culture dishes (6.86 ± 1.48 mg/mL) was significantly greater than that recovered from the WT culture dishes (3.85 ± 1.20 mg/mL).

Effect of zinc deficiency and selenium supplementation on GPx activity

In both WT and OEX cells, the activity of GPx was found to be unaffected by Zn status (Table 1). Zn-deficient and Zn-supplemented cells showed the same GPx activity as cells in medium containing no added DTPA, $FeSO_4$ or $ZnSO_4$. However, OEX cells demonstrated significantly higher levels of GPx than WT cells, regardless of their zinc status. In contrast to the lack of effect of zinc deficiency, Se supplementation of the medium resulted in a >10-fold increase in GPx activity in both cell types (Table 1).

Effect of zinc deficiency and selenium supplementation on PHGPx activity

As found for cGPx activity, PHGPx activity was unaffected by zinc status (Table 2).

However, Se supplementation of the cell medium increased WT PHGPx acitivity 2-fold and also significantly in the OEX cells.

DISCUSSION

Cell culture media are designed to give a complete nutrient supply for the growth and maintenance of particular cell types. Superficially, nutrient requirements of a particular cell line may appear to be met by several types of medium and, for example cell growth, may appear to be unaffected by changing the type of medium. However, selection of cell culture medium requires careful consideration of specific cell requirements, and results presented here clearly demonstrate that CHO-K1 cell antioxidant capacity may be compromised by using a medium that is effectively selenium deficient. Supplementation of α-MEM with 100 nM selenous acid increased cGPx activity by 10-fold, suggesting that the degree to which the antioxidant capacity is compromised in the absence of selenium supply is considerable. The effect of selenous acid supplementation on PHGPx activity was less marked than observed with cGPx, but was nevertheless significant. This observation further supports the evidence that α-MEM requires supplementation with selenium to enable full antioxidant function in this cell line. It also supports previous observations with a variety of cell lines indicating that cell culture medium, including addition of fetal calf serum, does not meet cellular selenium requirements, as evidenced by compromised Se-dependent enzyme activity (17,18). It is important to note that no commercial media are supplied supplemented with selenium in any form, and that the effects of selenium deficiency may have significant influences on cellular function in many cell model systems, particularly the resistance to oxidative stress.

Zinc deficiency had a profound effect on cell survival, as indicated by attachment of cells to the culture dishes. This is consistent with the evidence showing that severe zinc deficiency induces apoptosis (19), since zinc inhibits caspase 3 activity (20,21) and downstream endonuclease activity (22). However, cGPx and PHGPx activities in the remaining cells were not different to those in the Control or zinc-supplemented cells. Clearly, if detached cells had been analysed along with those cells remaining on the dishes, the results for Se-dependent enzyme activity would

Table 1. Level of cytosolic glutathione peroxidase (cGPX) activity in wild-type (WT) and mMT-1 overexpressing (OEX) CHO-K1 cells under different conditions of zinc and selenium status

	Control	+Zn	-Zn	+Zn/+Se
WT	0.016 ± 0.002	0.014 ± 0.006	0.012 ± 0.006	0.136 ± 0.032*
OEX	0.030 ± 0.004	0.026 ± 0.005	0.030 ± 0.004	0.152 ± 0.019*

Values are the mean ± SD of 6 replicate dishes expressed as units of cGPx activity/mg protein.
Asterisks indicates significant difference ($p < 0.05$) from the Control using a one way ANOVA and Duncan's multiple comparison test.

Table 2. Level of phospholipid hydroperoxide glutathione peroxidase (PHGPx) activity in wild-type (WT) and mMT-1 overexpressing (OEX) CHO-K1 cells under different conditions of zinc and selenium status

	Control	+Zn	-Zn	+Zn/+Se
WT	2.49 ± 0.69	1.95 ± 0.95	2.23 ± 2.02	4.72 ± 0.54*
OEX	1.63 ± 0.64	2.26 ± 1.40	2.52 ± 0.58	3.79 ± 0.56*

Values are the mean ± SD of 6 replicate dishes expressed as mUnits of PHGPx activity/mg protein.
Asterisks indicates significant difference ($p < 0.05$) from the Control using a one way ANOVA and Duncan's multiple comparison test.

likely have been considerably lower than recorded for attached cells only.

cGPx activity but not PHGPx activity was significantly elevated in OEX cells as compared to WT cells. This was in contrast to data for cGPx obtained during characterisation of this clone, which showed no difference in activity between the parental cells and the OEX cells. It is possible that the difference in cGPx developed after repeated subculturing of either or both cell lines. The concentration of cell protein recovered from the culture dishes was however significantly higher in zinc deficient OEX cells compared to that in zinc deficient WT cells. This may indicate that overexpression of MT helped to resist zinc deficiency by binding and retaining zinc within the OEX cells. It has previously been noted that mice deficient in MT are more susceptible to the effects of zinc deficiency as regards abnormal development of embryos (23). It was therefore suggested that the retention of zinc was improved in the presence of MT.

In conclusion, the Se-stimulated increase in cGPx and PHGPx activity in both strains of CHO-K1 cells indicates that α-MEM supplemented with 10% fetal calf serum contained insufficient Se to ensure optimal endogenous antioxidant status. It is concluded that Se supplementation for optimal enzyme activity is determined for different cell types and medium prior to their use as models in oxidative stress studies. While severe zinc deficiency resulted in cell detachment from the culture dishes and probably cell death, lack of zinc apparently had no effect on cGPx and PHGPx activity in the remaining attached cells. This suggests that zinc does not regulate their enzyme activity, but it remains a possibility that zinc may have an effect at the transcriptional level. OEX cells showed higher cGPx activity under all treatment conditions and MT may partially protect against deficiency-induced cell death.

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