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Evaluation of insulin-mimetic trace metals as insulin replacements in mammalian cell cultures

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Abstract

Insulin is involved in a number of cellular functions, including the stimulation of cell growth, cell cycle progression and glucose uptake and is a common protein supplement in serum-free mammalian cell culture media. However, several trace metals have previously been reported to exhibit insulin-like effects on specific cell types. As a step towards developing chemically-defined, protein-free media for mammalian cells, we tested the effectiveness of five trace metals (cadmium, nickel, lithium, vanadium and zinc) as a replacement for insulin. Four cell lines of biotechnological relevance were used, including the hybridoma CRL1606, the myeloma NS0, and the Chinese hamster ovary cell lines CHO-IFN and CHO-K1. Zinc was found to be an effective insulin replacement for the hybridoma, myeloma and CHO-K1 cells. Cell growth, cell cycle progression and antibody production was not affected by the substitution. Furthermore, no adaptation procedure was required.

Introduction

Insulin is a common protein supplement found in most serum-free media formulations for the *in vitro* culture of mammalian cells (Barnes and Sato 1980; Jayme and Smith 2000). It is known to exert a number of physiologic effects, including the stimulation of cell growth and cell cycle progression and the regulation of glucose and lipid metabolism (Saltiel and Kahn 2001). Traditionally, insulin for mammalian cell culture is usually derived from bovine pancreases. However, there are regulatory concerns over potential contamination of the therapeutic product from animal-sourced media components (Jayme and Smith 2000). Some researchers have overcome the use of animal derived insulin by substituting with recombinant insulin produced from *Escherichia coli*, although this is a relatively costly option. Thus, identifying a non-protein substitute for insulin with equivalent bioactivity is an important step towards developing a chemically-defined protein-free media.

Several trace metals had previously been reported to exhibit insulin-like effects. For example, Tang and Shay (2001) reported that zinc exerted an insulin-like stimulation of glucose transport in mouse fibroblasts and adipocytes. Similarly, the elevated blood glucose levels of diabetic rats were normalized by oral administration of lithium and vanadium (Srivastava et al. 1993). Other insulin-mimetic trace metals include nickel (Saggerson et al. 1976) and cadmium (Ezaki 1989).

This study evaluates the potential ability of five trace metals, previously reported to have insulin-like effects, to replace insulin in mammalian cell culture media. To test the general applicability, four mammalian cell lines (a hybridoma, a myeloma and 2 CHO) were used.

Materials and methods

Cell lines and culture media

The four mammalian cell lines used in this study were the hybridoma CRL1606, the mouse myeloma NS0 and two Chinese hamster ovary cell lines, CHO-IFN and CHO-K1. The CRL1606 and CHO-K1 cell lines were obtained from the American Type Tissue Collection (ATCC), while NS0 was obtained from the European Collection of Animal Cell Cultures (ECACC). The CHO-IFN cell line, expressing human interferon-gamma, was a gift from Professor Daniel Wang (Massachusetts Institute of Technology) and was derived from a dihydrofolate reductase-deficient ($dhfr^-$) CHO cell line. CRL1606, a fusion of antigen-primed BALB/c mouse spleen cells and the BALB/c mouse myeloma P3-X63-Ag8, produces a monoclonal antibody against human fibronectin (Schoen et al. 1982).

CRL1606 and NS0 were routinely maintained in an in-house-developed serum-free formulation based on Iscove's modified Dulbecco's medium (IMDM) supplemented with 10 mg l^{-1} of bovine insulin (Sigma, MO). In addition, the medium for the sterol-dependent NS0 cell line was further supplemented with a cholesterol lipid concentrate (Cat. 12531, Invitrogen, CA). The CHO cell lines were maintained in an in-house-developed serum-free formulation based on a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12) supplemented with 10 mg l^{-1} insulin. In addition, 0.1% (w/v) Pluronic F-68 was added to all media as a shear protectant. The basal IMDM formulation (Invitrogen, CA) did not include any of the trace metal supplements evaluated in this study. The classical basal DMEM/F12 formulation (Invitrogen, CA) contains 0.432 mg/l^{-1} of $ZnSO_4{\cdot}7H_2O$, but none of the other trace metal supplements evaluated in this study. Unless specified otherwise, the amount of $ZnSO_4{\cdot}7H_2O$ indicated in the results presented refers to the total concentration, including any $ZnSO_4{\cdot}7H_2O$ that may already be present as part of the basal media (e.g. in classical DMEM/F12 medium). An exception is the DMEM/F12 basal medium for the CHO-K1 cultures, which was a custom-made (Hyclone, UT), zinc-free version of the classical DMEM/F12 medium. Stock solutions of the trace metal supplements were prepared in MilliQ water and added to the media at the following concentrations: $NiCl_2{\cdot}6H_2O$ (1.4×10^{-4} mg l^{-1}), $CdCl_2{\cdot}2.5H_2O$ (4.5×10^{-3} mg l^{-1}), LiCl (8.47 mg l^{-1}), Na_3VO_4(9.4×10^{-4} mg l^{-1}) and $ZnSO_4{\cdot}7H_2O$ (1.5 mg l^{-1}). All media and stock solutions were filter-sterilized through a 0.22 μm filter (Nalgene, NY).

Culture conditions

Cells were cultivated as suspension cultures in either multiwell plates (5 ml/well) or 250 ml shake flasks (40 ml/flask) placed in a humidified incubator (95% relative humidity) at 37 °C, 8% CO_2. CRL1606 and NS0 cultures were seeded at 3×10^5 cells ml^{-1} while CHO-IFN and CHO-K1 were seeded at 4×10^5 cells ml^{-1} . In each of the experiments, the inoculum was prepared by centrifuging the seed cultures grown in insulin-supplemented media at 950 rpm for 5 min, removing the supernatant before washing and resuspending in the test media. During the course of the batch culture, samples were taken at regular intervals for cell density and viability measurements. In addition, cells were harvested by centrifugation for cell cycle analysis while culture broth was collected for metabolite and product quantification. The results presented are the mean of duplicate or triplicate runs.

Analytical methods

The cell density was determined either by manual cell counting with an Improved Neubauer hemocytometer (Weber, UK) or a Cedex automated cell counter (Innovatis, Germany). Viability was estimated by trypan blue exclusion.

The concentration of anti-fibronectin in CRL1606 culture broth was determined by the ELISA technique previously described in Follstad et al. (2002).

Determination of the integral of viable cell density

The integral of viable cell density (IVCD) was calculated using the method described in Sauer et al. (2000). Briefly, the integral of viable cells for each discrete time interval was calculated using Eq. (1) and (2), where $(X_vV)_1$ and $(X_vV)_2$ are the total number of viable cells at time points t_1 and t_2 respectively, and $(X_vV)_{\text{ave}}$ is the average total number of viable cells for the time interval t_2-t_1.

$$\int_{t_1}^{t_2} X_v V \mathrm{d}t = (X_v V)_{ave}(t_2 - t_1) \tag{1}$$

$$(X_v V)_{ave} = \frac{(X_v V)_1 + (X_v V)_2}{2} \tag{2}$$

The IVCD was determined by summing all the discrete time intervals and dividing by the culture volume (V), as presented in Eq. (3):

$$\text{IVCD} = \frac{\sum_t (X_v V)_{\text{ave}}(t_2 - t_1)}{V} \tag{3}$$

Cell cycle analysis

Cell cycle analysis was carried out using the CycleTEST Plus DNA Reagent kit (Becton Dickenson, CA), following the manufacturer's protocol. Briefly, 5×10^5 cells were harvested by centrifugation at 8000 rpm for 5 min in a microcentrifuge. Two-hundred and fifty μl of trypsin buffer (Solution A in the manufacturer's protocol) was added and the cells incubated for 10 min at room temperature. Two-hundred μl of trypsin inhibitor and RNase buffer (Solution B) was then added and the suspension incubated for a further 10 min at room temperature. Finally, 200 μl of propidium iodide stain solution (Solution C) was added at 4 °C and the suspension incubated on ice for 10 min in the dark. At this stage, the cells were either analyzed immediately by flow cytometry or stored at 4 °C until further analysis.

Flow cytometric analysis was carried out on a FACScan (Becton Dickenson, CA). Thirty-five-thousand events per sample was analyzed in each measurement. The resolution of G_0/G_1, S and G_2/M phases was performed using the ModFit Lt analysis program (Verity Software House, ME).

Results

Evaluation of trace metals as insulin replacements

To determine the potential for different trace metals to replace insulin for various cell lines of biotechnological importance, a series of batch experiments were conducted in multiwell plates. Exponentially growing cells from insulin-supplemented media were inoculated into insulin-free media containing each of these trace metals. For this initial screening study, the concentration of trace metals used was the average of the range of concentrations previously reported in the literature. As a negative control, cells were also inoculated into insulin-free media without trace metal supplements. The cultures were sampled daily to determine the cell density and viability. To compare the results of the various media, the integrated viable cell density (IVCD) was calculated from the growth profiles. Figure 1a–d show the IVCD (up to day 3) and viability (at maximum cell density) for the four cell lines, CRL1606, NS0, CHO-IFN and CHO-K1 respectively. For all four cell lines, inoculation into insulin-free media without any trace metal supplements resulted in poor cell growth and low viability (compare bars for supplement F and G). Of the five trace metals tested, zinc was observed to be the most effective in replacing insulin. Addition of $ZnSO_4{\cdot}7H_2O$ into the insulin-free media resulted in cell growth and viabilities that were comparable to insulin-supplemented cultures for CRL1606 and NS0 (Supplement E in Figure 1a and b respectively). Although addition of $ZnSO_4{\cdot}7H_2O$ to the insulin-free media did not significantly increase the day 3 IVCD of CHO-K1 cultures in the multiwell plates, the viability stayed above 97%, which is significantly higher than the viability of CHO-K1 without insulin or trace metal supplementation (Figure 1d). In contrast to the other cell lines, $ZnSO_4{\cdot}7H_2O$ was not an effective replacement for insulin in CHO-IFN cultures (Figure 1c). Apart from zinc, the other trace metals were unable to replace insulin for the four cell lines. Preliminary testing of these trace metals in insulin-supplemented media showed that the concentrations of trace metals used were non-lethal (data not shown).

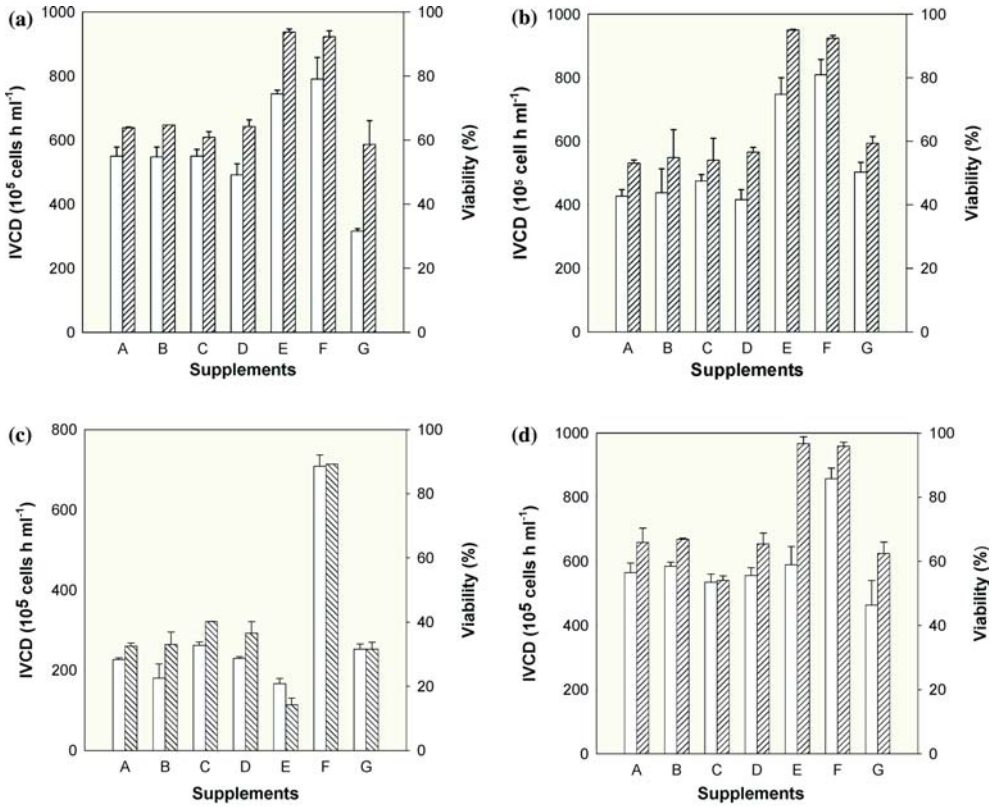

Figure 1. Integrated viable cell density up to day 3 (IVCD, unshaded bars) and viability (shaded bars) at maximum cell density of (a) CRL1606; (b) NS0; (c) CHO-IFN; (d) CHOK1 in 6-well plate cultures. A: $NiCl_2{\cdot}6H_2O$ (1.4×10^{-4} mg l^{-1}), B: $CdCl_2{\cdot}2.5H_2O$ (4.5×10^{-3} mg l^{-1}), C: LiCl (8.47 mg l^{-1}), D: Na_3VO_4 (9.4×10^{-4} mg l^{-1}), E: $ZnSO_4{\cdot}7H_2O$ (1.5 mg l^{-1}), F: Insulin (10 mg l^{-1}), G: No insulin or trace metal supplement.

Growth in zinc-supplemented, insulin-free cultures

The multiwell plate screening experiments identified zinc as a potential insulin replacement. To verify this, shake flask cultures of the four cell lines were then conducted. As shown in Figure 2a and b, supplementation with 1.5 mg l^{-1} of $ZnSO_4{\cdot}7H_2O$ was able to stimulate cell growth of CRL1606 and NS0 in insulin-free media to cell densities which were similar to that of insulin-supplemented media. Viable cell densities reached a maximum of about 2.9×10^6 cells ml^{-1} and 2.5×10^6 cells ml^{-1} for CRL1606 and NS0 respectively in both insulin and zinc-supplemented cultures. Similarly, the viability for both insulin and zinc-supplemented cultures remained over 90% up to days 2 and 3 for CRL1606 and NS0 respectively (Figure 3 and data not shown). On the other hand, CRL1606 and NS0 cells in insulin-free media with no zinc supplementation barely reached a viable cell density of 5×10^5 cells ml^{-1} (Figure 2a and b) and viability dropped below 80% by day 2 (Figure 3). It is worth noting that the cells were able to grow in the zinc-supplemented, insulin-free media without adaptation.

In contrast, zinc supplementation was not sufficient to support the growth of CHO-IFN in insulin-free media (Figure 2c). As described in the Materials and Methods section, the basal medium used for both CHO cell lines were based on DMEM/F12. The classical basal DMEM/F12 formulation already contains 0.432 mg l^{-1} of $ZnSO_4{\cdot}7H_2O$. However, this level of zinc was not sufficient to sustain growth of CHO-IFN cells without insulin supplementation. Increasing the concentration of $ZnSO_4{\cdot}7H_2O$ to 1.5 mg l^{-1} could not rescue cell growth in insulin-free media. On the other hand, CHO-K1 cells were able to grow without insulin supplementation when $ZnSO_4{\cdot}7H_2O$ was present at the level found in classical

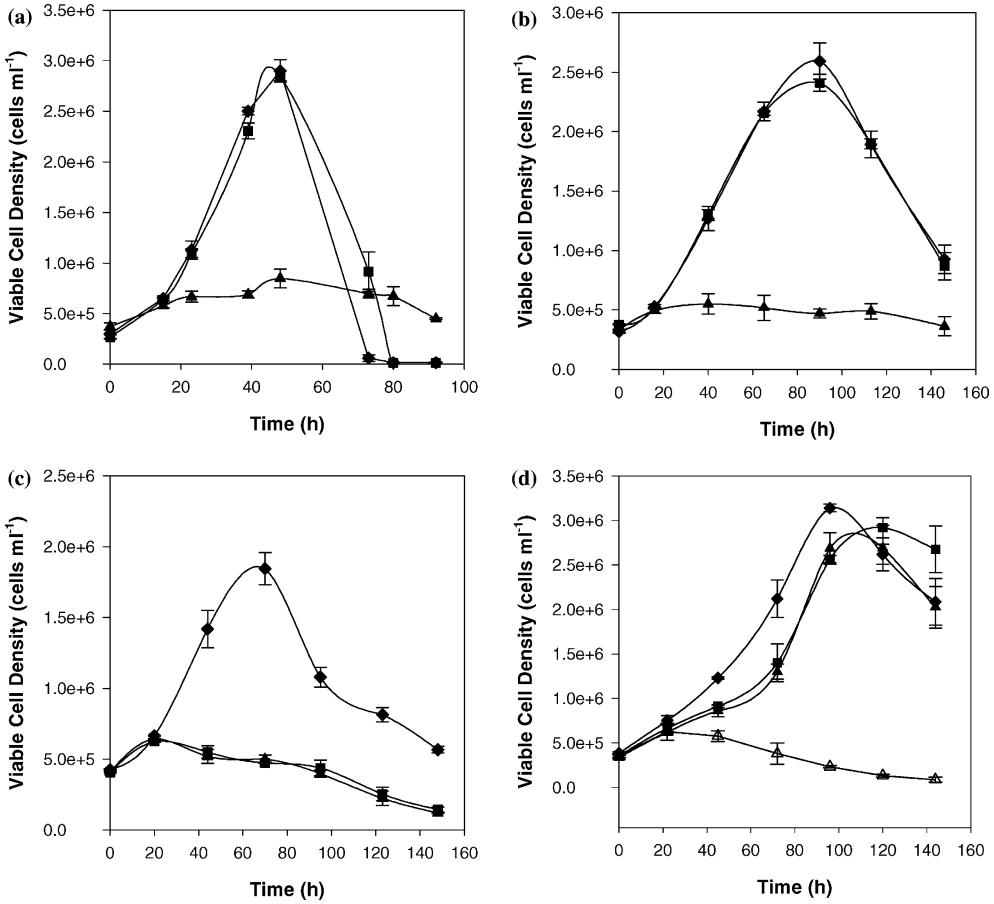

Figure 2. Viable cell density of (a) CRL1606; (b) NS0; (c) CHO-IFN; (d) CHO-K1 in shake-flask cultures. (◆) Insulin (10 mg l^{-1}), (■) $ZnSO_4{\cdot}7H_2O$ (1.5 mg l^{-1}), (▲) No insulin or zinc supplement (IMDM or zinc-containing DMEM/F12), (△) No insulin supplement (zinc-free DMEM/F12, CHO-K1 only).

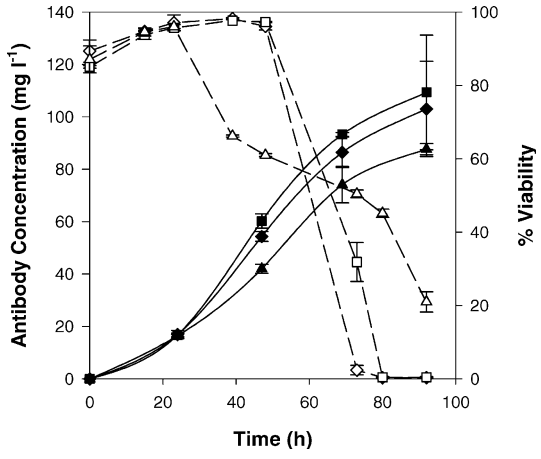

Figure 3. Antibody production (filled symbols, solid lines) and viability (open symbols, broken lines) of CRL1606 in shake-flask cultures. (◆, ◇) Insulin (10 mg l^{-1}), (■, □) $ZnSO_4{\cdot}7H_2O$ (1.5 mg l^{-1}), (▲, △) No insulin or Zn supplement.

DMEM/F12 media (Figure 2d), albeit at a slower rate than insulin-supplemented media. Increasing the concentration of $ZnSO_4{\cdot}7H_2O$ to 1.5 mg l^{-1} did not lead to further increases in cell growth. To investigate if the ability of CHO-K1 cells to grow in insulin-free DMEM/F12 media may be related to zinc, cells were inoculated into an insulin and zinc-free version of the media. As seen in Figure 2d, CHO-K1 cells were not able to grow in insulin-free media in the absence of zinc.

Figure 3 shows the antibody production and viability of the CRL1606 cultures. Substitution of insulin with $ZnSO_4{\cdot}7H_2O$ did not have any adverse effect on antibody production. Interestingly, the antibody titre from CRL1606 cultures grown in insulin-free media managed to reach 80 mg l^{-1} despite the lower viable cell density, suggesting that the remaining viable cells in the population

continued to produce antibody at a high specific productivity. However, the low viability of the insulin-free cultures may be detrimental to the quality of the product. Moreover, extended passaging of CRL1606 in insulin-free media showed very unstable growth profiles compared to insulin or zinc-supplemented cultures (data not shown). For both CRL1606 and NS0 cultures, the profiles of glucose uptake and lactate production between the insulin and zinc-supplemented cultures were highly similar (data not shown).

Cell cycle analysis

Insulin is known to be essential for stimulating cell cycle progression and withdrawal of insulin can lead to cell cycle arrest (Barnes and Sato 1980; Sanfeliu et al. 2000). The multiwell and shake flask experiments had demonstrated that for two of the four cell lines tested, zinc or insulin-supplemented cultures exhibited very similar growth (i.e. cell density and viability) and metabolism (e.g. glucose, lactate and antibody production profiles). We were, thus, also interested to study the effects of zinc and insulin on cell cycle progression.

Measurement of the cell distributions at different phases of the cell cycle are illustrated in Figure 4. The percentage of cells in the G_0/G_1 phase was around 30% for both insulin and zinc-supplemented CRL1606 cultures for the first 48 h before increasing to 60% by 80 h (Figure 4a). Similarly, the percentage of cells in the G_0/G_1 phase ranged below 55% for both insulin and zinc-supplemented NS0 cultures within the first 65 hours (Figure 4b). The increase in G_0/G_1 residence at the late stages of the batch culture corresponds with the death phase. Similarly, the cell distributions in the S phase and G_2/M phase were very comparable between the insulin and zinc-supplemented cultures for both CRL1606 (Figure 4d and g) and NS0 (Figure 4e and h) cells. Thus, for both cell lines where zinc was found to be an effective insulin-replacement (i.e. CRL1606 and NS0), the cell cycle distribution of the insulin and zinc-supplemented cultures were very similar.

CHO-K1 cultures in insulin-supplemented medium showed an initial decrease followed by a gradual increase in G_0/G_1 phase as the cells progressed from mid to late exponential growth (Figure 4c). Correspondingly, the S phase proportion gradually decreased from 22 to 96 h (Figure 4f). In zinc-supplemented medium, the decrease in G_0/G_1 proportion, and the corresponding increase in S phase residence, was delayed compared to the insulin-supplemented cultures (Figure 4c and f). This agrees with the slower growth of the zinc-supplemented cultures relative to the insulin-supplemented cultures. There was no apparent difference in the G_2/M phase distribution between the insulin and zinc-supplemented cultures (Figure 4i).

Cell cycle arrest in the G_0/G_1 phase with a corresponding decrease in S phase residence is typical for most cell lines upon insulin or serum withdrawal. For the hybridoma CRL1606, the percentage of cells in the G_0/G_1 phase for all three media remained within the range of 25–35% for the first 48 h (Figure 4a), even though the viable cell density and viability of the insulin and zinc-supplemented cultures were significantly higher than the non-supplemented cultures during that period. Thus, there was an anomalous lack of G_0/G_1 arrest in CRL1606 cultures upon insulin withdrawal. In contrast, for the myeloma NS0, the percentage of cells in the G_0/G_1 phase increased to 75% by 40 h for the non-supplemented cultures (Figure 4b), while the insulin and zinc-supplemented cultures remained at around 50%. Similarly, CHO-K1 cultures in zinc-free medium without insulin supplementation exhibited a decreased S phase residence (Figure 4f) and increased G_0/G_1 residence (Figure 4c) relative to insulin-supplemented cultures.

Discussion

In this study, we evaluated the potential of various insulin-mimetic trace metals to replace insulin in cell culture media for cell lines of biotechnological importance (e.g. hybridoma, myeloma, CHO). Previous groups have reported insulin-mimetic properties for cadmium (Ezaki 1989), nickel (Saggerson et al. 1976), lithium (Srivastava et al. 1993), vanadium (Thompson et al. 1999) and zinc (Tang and Shay 2001), albeit through *in vivo* studies or using primary cultures. From the multiwell plate and shake flask experiments, zinc (in the form of $ZnSO_4{\cdot}7H_2O$) was found to stimulate growth of CRL1606 hybridomas and NS0

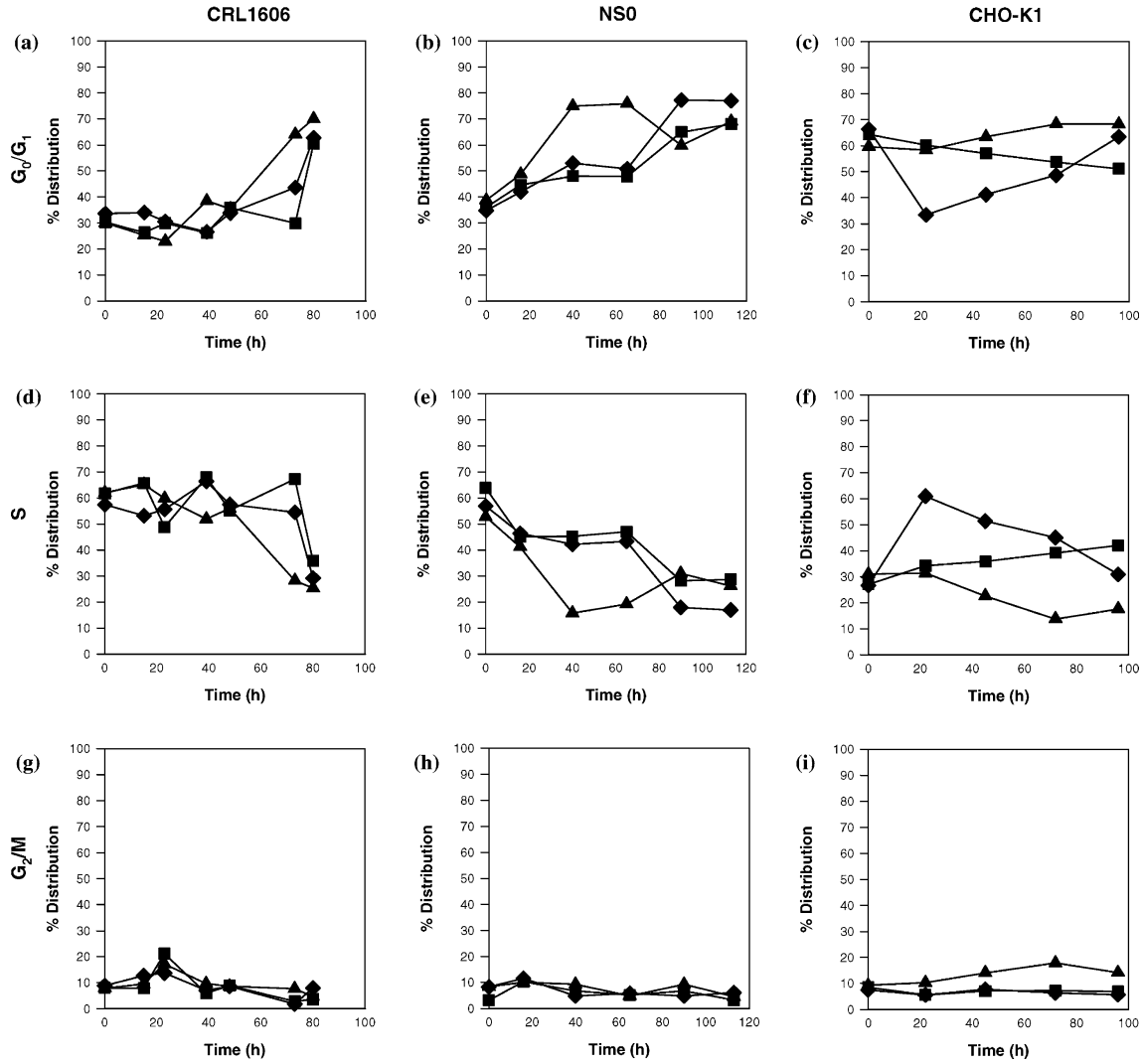

Figure 4. Cell cycle distribution of CRL1606 (a, d and g), NS0 (b, e and h) and CHO-K1 (c, f and i) in shake flask cultures. Top panel (a–c) for distribution in G_0/G_1 phase, middle panel (d–f) for S phase and bottom panel (g–i) for G_2/M phase. (◆) Insulin (10 mg l^{-1}), (■) $ZnSO_4 \cdot 7H_2O$ (1.5 mg l^{-1}), (▲) No insulin or zinc supplement (for CHO-K1, zinc-free DMEM/F12 was used).

myelomas in insulin-free media without the need for adaptation (Figures 1 and 2). Both cell growth and antibody production was maintained at the same levels as the insulin-supplemented cultures (Figure 3). Zinc was also found to stimulate growth of CHO-K1 cultures in insulin-free media, although cell growth was marginally slower than in insulin-supplemented cultures. The slower growth rate of zinc-supplemented cultures may have contributed towards the low IVCD results in the multiwell experiments. In contrast, growth of CHO-IFN in insulin-free medium could not be restored by any of the trace metals tested. The reason for the inability of zinc to replace insulin in CHO-IFN cells is presently unclear. In our experience, different cell lines, and even different clones of the same cell line, adapt to growth in protein-free media with varying levels of difficulty. Further studies would be required to determine why the insulin-mimetic effect of zinc is cell line specific. Although an extensive screen of various insulin-mimetic trace metals at different dosages and combinations was not the intent of this study, it does not preclude the possibility that an effective

insulin-mimetic trace metal may be identified for this CHO cell line.

It is interesting to note that zinc is a component found in certain classical basal media formulations (e.g. DMEM/F12) but not in others (e.g. IMDM). In light of the present work (e.g. Figure 2d), the apparent lack of insulin-dependence in certain cell lines previously reported may be confounded by the zinc present in the basal media.

Insulin has been implicated in a number of cellular functions, including promoting glucose and amino acid transport, regulating lipid metabolism and stimulating cell cycle progression (Saltiel and Kahn 2001). While further work is required to study the impact of the insulin-replacement on each of these functions, the present study demonstrated that, in addition to cell growth, the cell cycle progression (Figure 4) as well as the glucose and amino acid consumption profiles (data not shown) for CRL1606 and NS0 cultures were unaffected by substituting zinc for insulin. The anomalous lack of G_0/G_1 phase arrest for CRL1606 hybridomas following insulin withdrawal was similar to observations reported by Balcarcel and Stephanopoulos (2001) in batch cultures of this cell line. The ability of this cell line to enter the cell cycle in the absence of growth factors, such as insulin, leads to the phenomena where continuous proliferation occurs in the presence of continuous death, which has been termed 'abortive proliferation' (Chung et al. 1998). It has been hypothesized that this may be due to the over-expression of *c-myc* in this particular cell line, although this remains to be verified (Chung et al. 1998).

The mechanism for the insulin-mimetic effects of zinc is still largely unknown. In rat adipocytes, the insulin-like stimulation of glucose transport by zinc is likely to occur via a post-insulin receptor kinase mechanism (Ezaki 1989). Later studies showed that the effect of zinc is mediated by two key proteins in the insulin-signaling pathway, phosphoinositol-3-kinase and Akt (Tang and Shay 2001). To gain a better understanding of the mechanism of zinc's action, we have employed transcriptional analysis using DNA microarrays and Western blot techniques to analyze the gene and protein expression of the hybridoma CRL1606 cultured in insulin or zinc-supplemented media (manuscript in preparation).

Regulatory concerns over the potential contamination of therapeutic proteins by adventitious agents introduced via medium components has spurred efforts to replace proteinaceous elements of the medium formulation with non-protein substitutes with equivalent bioactivity. Our results demonstrate the potential of using non-proteinaceous insulin-mimetic trace metals to replace insulin in mammalian cell cultures.

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