Evaluation of recombinant human transferrin (DeltaFerrin™) as an iron chelator in serum-free media for mammalian cell culture

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Abstract DeltaFerrin™, a yeast-derived recombinant human transferrin produced by Delta Biotechnology Ltd. (Nottingham UK), was found to be a suitable replacement for holo human transferrin in serum-free culture media of the MDCK cell line (chosen because of its transferrin dependence) in short-term screening assays. Long-term subculture was achieved with DeltaFerrin™ supporting growth equivalent to that of holo human transferrin. DeltaFerrin™ and a selection of chemical iron chelators were found in short-term assays to be equivalent to holo human transferrin in supporting growth of MDCK, BHK-21-PPI-C16 and Vero-PPI. In long-term subcultures, however, only DeltaFerrin™ was found to support cell growth in a manner essentially equivalent to holo human transferrin in all three cell lines. For both BHK and Vero variants tested, recombinant preproinsulin production was unaltered by replacing holo human transferrin with DeltaFerrin™. As such, this is the first report of a recombinant human transferrin pro-

duced under animal-free conditions that can act as a universal iron chelator for cells grown in serum-free media (SFM).

Keywords Cell culture · Iron chelator · DeltaFerrin™ · Recombinant human transferrin · Serum-free media

Introduction

The development and routine use of serum-free media (SFM) is a high priority for cell culture from a scientific point of view where components in traditional sera may complicate research (Salis et al. 2002). Industrially, SFM is very important from a regulatory view to reduce or eliminate the use of animal-derived components (Castle and Robertson 1999) thus avoiding contamination with viruses, mycoplasma or prions and for better control of batch-to-batch variation and downstream processing (Chu and Robinson 2001).

Two of the most common supplements added to basal media for serum-free development are insulin and transferrin (Fitzsimmons et al. 2004; Barnes 1987). Alternatives to animal-derived insulin include recombinant insulin (e.g. VP-SFM from Invitrogen) and recently zinc has been suggested as an alternative for hybridoma, myeloma and CHO-K1 cells (Wong et al. 2004).

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Transferrin is the most commonly used iron chelator and regulates the bioavailability of iron to cells. Iron supports metabolic processes such as oxygen transport, DNA synthesis and electron transport (Richardson and Ponka 1997) and is closely linked with cell growth (Laskey et al. 1988). However, non-protein bound iron can lead to free radical formation and tissue damage (Conrad et al. 1994). Some cells produce their own transferrin (Vyhldal et al. 2002; Shintani et al. 1994), while many cell lines depend on an exogenous source of transferrin (Yamada et al. 1990; Okamoto et al. 1996). Some iron chelators can replace transferrin (Darfler 1990; Keenan and Clynes 1996; Neumannova et al. 1995) for specific cell lines but none can support the universal activity of transferrin (Sanders and Cheung 1988). For a particular cell line, screening and optimisation is often required to select a suitable chemical iron chelator, followed by long-term subculturing to confirm continued activity. To date, a recombinant source of human transferrin has not been readily available. A recombinant human transferrin with activities of the native transferrin would provide a universal iron chelator produced under animal-free conditions and would obviate lengthy screening and optimisation. From an industrial view, this would also satisfy the increasing stringency for the manufacture of safe biotherapeutics.

In this paper, we compared the growth promoting activities of a variety of iron chelators to DeltaFerrinTM and holo human transferrin in three industrially relevant cell lines namely, MDCK, Vero and BHK.

Materials and methods

Cells

MDCK (ATCC CCL34) were obtained from the American Type Culture Collection. BHK-21-PPI-C16 (Gammell et al. 2003) and Vero-PPI (O'Driscoll et al. 2002) used here were stably transfected previously to express human prepro-insulin. MDCK were routinely grown in DME:Ham's F12 (1:1 vol/vol) supplemented with 5% Fetal Calf Serum (FCS) at 37°C and 5% CO₂.

BHK-21-PPI-C16 and Vero-PPI were routinely grown in MEM supplemented with 5% FCS and 1% non-essential amino acids at 37°C and 5% CO₂. All cell lines tested negative for mycoplasma contamination.

Materials

Media (DME, Ham's F12 and MEM) L-glutamine and trypsin were supplied by Gibco. All components for SFM, trypsin inhibitor and iron chelators (ferrous sulphate (Fe₂SO₄), ferric ammonium sulphate (FAS), ferredoxin (FeDox), sodium nitroprusside (SNP), and glycine hydroxymate (GlyHy)) were supplied by Sigma (Poole, UK). Holo human transferrin (T0665) and bovine transferrin (T1283) were supplied by Sigma (Poole UK). DeltaFerrinTM, a recombinant human transferrin, was supplied by Delta Biotechnology Ltd. (Nottingham, UK). An iron chelate sample kit with components labelled A–E was supplied by Invitrogen. The MDCK SFM (Taub et al. 1979) was used (DME:Ham's F12 supplemented with 5 µg ml⁻¹ bovine insulin, 5 µg ml⁻¹ holo human transferrin, 25 ng ml⁻¹ prostaglandin E1, 10 nM sodium selenite, 5 pM Tri-iodothyronine and 50 nM Hydrocortisone). For Vero-PPI and BHK-21-PPI-C16 the original SFM designed by Bradshaw et al. (1994) was used as a basis (DME:Ham's F12 with 4 mM L-glutamine, 2.5 µg ml⁻¹ insulin, 5 µg ml⁻¹ transferrin). In addition for Vero-PPI, 5 pM Tri-iodothyronine and 5 µg ml⁻¹ fibronectin were included and was found to support limited growth over 5 subcultures. For BHK-21-PPI-C16, 25 ng ml⁻¹ prostaglandin E1, 50 nM Hydrocortisone and 5 µg ml⁻¹ fibronectin were included and was found to support limited growth over 5 subcultures.

Transferrin/iron chelator screen

All transferrins or iron chelators were diluted to 2× concentration in DME:Ham's F12 (Fe₂SO₄, FAS and SNP at 1 µg ml⁻¹; transferrins at 10 µg ml⁻¹). Cells in log phase were washed three times and incubated in DME:HF12 (no serum, no transferrin). After 24 h incubation, cells were trypsinised and set up in 96-well plates. MDCK were plated at 2 × 10³ cells/well. Vero-PPI were plated at 1 × 10³

cells/well and BHK-21-PPI-C16 at 4×10^3 cells/well. After incubation at 37°C and 5% CO₂ for 5–7 days when the most actively growing cells were almost confluent, the assay was terminated and cell growth measured using acid phosphatase activity as the end point (Martin and Clynes 1993).

Subculture experiments

Subculture experiments were used to test the long-term effectiveness of transferrins and iron chelators. Cells in log phase were washed three times and incubated in DME:Ham's F12 (no serum, no transferrin) overnight. Cells were then set up in SFM with the selected iron chelators in duplicate for each condition and with three separate experiments. At each subculture fresh medium was prepared and fresh flasks were used. MDCK were set up at 1.5×10^5 cells/flask and subcultured every 4 days. BHK-PPI-C16 and Vero-PPI cells were set up at 3.0×10^5 cells/flask and 2.5×10^5 cells/flask, respectively, and were subcultured every 5 days.

When trypsinising, equivolume trypsin inhibitor was added and the cell suspension diluted to 10 ml with DME:Ham's F12 before centrifugation at 1000 rpm for 5 min. Resuspended in basal medium, the cells were counted in duplicate by haemocytometer and used to reseed a fresh flask for the next subculture. Due to trypsin susceptibility of BHK in SFM (Bradshaw et al. 1994), trypsin and trypsin inhibitor were diluted after the first subculture.

Insulin determination

Insulin production was determined using the Rat Insulin ELISA supplied by Mercodia (Cat. No. 10-1124). Briefly, Vero or BHK supernatants were removed after 1 day or 5 days incubation in insulin-free medium and centrifuged to remove any residual cells. Samples were frozen before analysis. Supernatants were then tested according to the manufacturers instructions.

Determination of specific growth rate

The specific growth rate μ (h⁻¹) was estimated using the equation:

$$\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$$

where X is cell number (initial inoculation densities and final cell number) and t is the time in hours for two sampling points (start and end of subculture).

Results

Comparison of DeltaFerrin™ to native transferrins

Screening DeltaFerrin™ with native, plasma-derived transferrins (Fig. 1), showed DeltaFerrin™ to support $95 \pm 5\%$ of the growth with holo human transferrin ($100 \pm 12\%$) at $5 \mu\text{g ml}^{-1}$, while holo bovine transferrin was slightly lower at $88 \pm 8\%$. These results suggest DeltaFerrin™ may replace native transferrins in the support of cell growth and at concentrations equivalent to those used for the plasma-derived analogues.

To ensure that the effects seen were not due to carry-over of the bovine transferrin present in serum-supplemented medium and to evaluate if the recombinant version could support longer term cell proliferation, MDCK cells were grown using DeltaFerrin™ and native holo human transferrin for ten passages (Fig. 2). Both

Fig. 1 Screen of transferrins at 5, 25 and 50 $\mu\text{g ml}^{-1}$ on MDCK cells grown in SFM. Results are expressed as the percentage growth relative to control (no transferrin in SFM) and are the average $\pm$ standard deviation (four assays with 8 repeats per assay). The screen was carried out in miniaturised assays with acid phosphatase activity as the end point

Fig. 2 Subculture of MDCK cells in SFM with either $5 \mu\text{g ml}^{-1}$ holo human transferrin or DeltaFerrinTM over 10 subcultures. Results are expressed as the average cell number per flask $\pm$ standard deviations (three separate assays and three flasks per variable). Duplicate counts with a haemocytometer were made per flask. MDCK were set up 1.5×10^5 – 2×10^5 cells/flask and subcultured every 4 days

transferrins continued to support good growth throughout the ten passages with DeltaFerrinTM achieving $99 \pm 7\%$ of the growth seen with holo human transferrin ($100 \pm 10\%$). Variation in total cell numbers for both transferrin within the first 4 subcultures were likely due to the inoculation density being slightly too high (initially at 2×10^5 cells/flask), so this was reduced in subsequent subcultures.

Iron chelator screen

A panel of iron chelators (Invitrogen samples A–E; DeltaFerrinTM; holo human transferrin; Fe_2SO_4 ; FAS; SNP; Fedox and Glyhy) were evaluated for growth stimulation of MDCK, BHK-21-PPI-C16 and Vero-PPI (Fig. 3). Transferrins were tested at $5 \mu\text{g ml}^{-1}$, A–E at $1\times$ and the other iron chelators at $0.5 \mu\text{g ml}^{-1}$.

For BHK-21-PPI-C16, the inclusion of holo human transferrin or DeltaFerrinTM showed averages of 2.0-fold and 2.4-fold higher, respectively, compared to the control without transferrin. Of the components in the sample kit, only C (96%) and E (85%) supported growth close to holo human transferrin. Ferredoxin and glycine hydroxymate were less effective than the transferrins. Fe_2SO_4 (1.2-fold increase), FAS (1.1-fold increase) and SNP (1.2-fold increase) were as effective as holo human transferrin.

Fig. 3 Screening Vero-PPI, MDCK and BHK-21-PPI-C16 for growth with iron chelators. Results are expressed as the percentage growth of the control (SFM without transferrin) and are taken as the average $\pm$ standard deviation (three separate assays with 8 replicates per plate). The screen was carried out in miniaturised assays with acid phosphatase activity as the end point. Transferrins were tested at $5 \mu\text{g ml}^{-1}$, Invitrogen iron chelator samples A–E at $1\times$ and the other iron chelators at $0.5 \mu\text{g ml}^{-1}$. Abbreviations: hH, holo human transferrin; DF, DeltaFerrinTM; A–E, Invitrogen iron chelator kit components; Fe_2SO_4 , ferrous sulphate; FAS, ferric ammonium sulphate, SNP, sodium nitroprusside; Fedox, ferredoxin; GlyHy, glycine hydroxymate

For Vero-PPI, no significant benefit was seen on the addition of holo human transferrin to the SFM but DeltaFerrinTM performed as well as the human transferrin. Only sample B and FAS showed growth of about 20% above holo human transferrin.

MDCK cells were most dependent on transferrin with at least a 5-fold increased growth on inclusion into the SFM (Fig. 2). Only sample B showed promise but the activity was quite variable between four separate assays ($102 \pm 60\%$ compared to holo human transferrin). FAS was as stimulatory as transferrin.

Extended subculture with selected iron chelators in SFM

The most promising chelators identified in the short-term culturing screen were tested for their ability to support extended growth of BHK-21-PPI-C16, Vero-PPI and MDCK in SFM. Only with repeated subculturing would it be possible to eliminate the impact of bovine transferrin masking in the short screen experiments.

MDCK were subcultured with sample B, Fe_2SO_4 , FAS or DeltaFerrinTM (Fig. 4). Holo human transferrin was not included as DeltaFerrinTM was earlier shown to be equivalent in long-term subculture to holo human transferrin and Fe_2SO_4 was included as it was previously found to be a suitable replacement for MDCK (Keenan and Clynes 1996). Of the five chelators tested, DeltaFerrinTM performed the best overall. The Invitrogen chelator B was almost as good as the DeltaFerrinTM (93%) while Fe_2SO_4 was not as consistent (78%), but was more stimulatory than in the screen. Consistently, FAS performance was lower than the others (48% of the growth achieved by DeltaFerrinTM), which did not reflect the growth seen in the screen and its growth promoting activity decreased with increasing subcultures. For this subculture experiment, the average specific growth rates of MDCK in SFM with DeltaFerrinTM and Invitrogen chelator B were 0.0296 h^{-1} and 0.0275 h^{-1} , respectively. For Fe_2SO_4 and FAS, the specific growth rates were lower at 0.025 h^{-1} and 0.0196 h^{-1} , respectively.

BHK-21-PPI-C16 was subcultured with Fe_2SO_4 , FAS, DeltaFerrinTM or holo human transferrin (Fig. 5). DeltaFerrinTM performed well, achieving 92% of the growth of holo human transferrin, except for the last subculture where the recombinant out-performed the native transferrin (last subculture was insulin-free). Ferrous sulphate achieved 85% of the growth obtained

Fig. 4 Subculture results for MDCK with a variety of iron chelators over 5 subcultures. Results are shown here as the average $\pm$ standard deviation (3 separate subcultures with 3 flasks per subculture). Duplicate counts with a haemocytometer were made per flask. MDCK were set up 1.5×10^5 cells/flask and subcultured every 4 days. DeltaFerrinTM was tested at $5 \mu\text{g ml}^{-1}$, Invitrogen B at $1 \times$ and Fe_2SO_4 and FAS at $0.5 \mu\text{g ml}^{-1}$

Fig. 5 Subculture results for BHK-21-PPI-C16 with a variety of iron chelators over 5 subcultures. Results are shown here as the average $\pm$ standard deviation (3 separate subcultures with 3 flasks per subculture) and two cell counts per flask. BHK-PPI-C16 were set up at 3.0×10^5 cells/flask and were subcultured every 5 days. Transferrins were tested at $5 \mu\text{g ml}^{-1}$ and Fe_2SO_4 and FAS at $0.5 \mu\text{g ml}^{-1}$

with holo human transferrin while FAS was not able to support growth beyond the first passage. For this subculture experiment, the average specific growth of BHK-21-PPI-C16 determined over 4 subcultures in SFM was 0.0043 h^{-1} and 0.0036 h^{-1} , respectively, for holo human transferrin and DeltaFerrinTM. For Fe_2SO_4 , the average specific growth rate was 0.0025 h^{-1} .

Vero-PPI were subcultured with holo human transferrin, DeltaFerrinTM, sample B, without transferrin (blank) or FAS (Fig. 6). Why the growth decreased in subcultures 3 and 4 is not clear? The fact that growth decreased across all iron chelators, in triplicate repeats and in control SFM (without iron chelator) suggests the reduction is not due to specifically the iron chelators but to a deficiency in the medium that is not addressing completely the requirements of these cells. In support of this, a previous SFM subculture experiment showed the same growth reduction.

The iron chelator blank was included to see if Vero-PPI could continue to grow without additional iron chelators. Interestingly, incorporation of holo human transferrin was beneficial in subculture experiments, contrasting the results in the screening assays. None of the other chelators were as good as holo human transferrin. DeltaFerrinTM and FAS at 88% and 81%, respectively, of the growth seen with the native transferrin, were the next best. However, DeltaFerrinTM

Fig. 6 Subculture results for Vero-PPI with a variety of iron chelators over 5 subcultures. Results shown here are the average $\pm$ standard deviation in error bars for 3 separate subcultures with 3 flasks per subculture and two cell counts per flask. Vero-PPI cells were set up at 2.5×10^5 cells/flask and were subcultured every 5 days. Transferrins were tested at $5 \mu\text{g ml}^{-1}$, Invitrogen B at $1 \times$ and FAS at $0.5 \mu\text{g ml}^{-1}$

appeared to be more consistent as evidenced by the lower deviations.

For this subculture experiment, the specific growth of Vero-PPI determined over 4 subcultures in SFM was 0.015 h^{-1} and 0.014 h^{-1} , respectively, for holo human transferrin and DeltaFerrinTM.

The overall ability of the selected iron chelators to support extended growth in the three cell lines shows DeltaFerrinTM supported growth on average 95% of the growth achieved with holo human transferrin and was the only one to consistently support growth equivalent to that seen with human transferrin (Fig. 7).

Fig. 7 Combined results from subculture experiments. Results are taken as the average of the 5 subcultures $\pm$ the standard deviations expressed as the percentage growth relative to the growth obtained with holo human transferrin over the 5 subcultures

Recombinant preproinsulin production in SFM

Production of recombinant preproinsulin in BHK-21-PPI-C16 and Vero-PPI was measured during the final subculture of the extended subculture experiments described above. Because both cell lines showed requirements for exogenous insulin under serum-free conditions, it was not possible to assess production of recombinant preproinsulin during the subculture experiment. On the fifth subculture, insulin was omitted from the serum-free media, allowing the preproinsulin produced to be measured. Insulin-free medium was used to assess the impact of the iron chelators on insulin production.

After 5 days growth, BHK-21-PPI-C16 subcultured in DeltaFerrinTM or Fe_2SO_4 produced similar preproinsulin (21 ± 3.1 and 20 ± 1.9 ng per 10^6 cells per 5 days, respectively) to holo human transferrin (17 ± 4.3 ng per 10^6 cells per 5 days). While the production after 5 days was lower than that achieved in 12 h (36.9 ng per 10^6 cells per 12 h), the results show that both DeltaFerrinTM and Fe_2SO_4 could each support preproinsulin production for the BHK-21-PPI-C16 similar to that found with the native holo human transferrin. For Vero-PPI cells, there were no measurable levels of preproinsulin production after extended subculturing in SFM. In a separate subculture of Vero-PPI set up with insulin-free

SFM, preproinsulin production for holo human transferrin and DeltaFerrinTM were similar at 6.6 ng and 6.5 ng per 10⁶ cells per 5 days. These results suggest that the level of preproinsulin production was low to start with and may explain why there were negligible levels of preproinsulin after 5 subcultures in SFM.

Discussion

In the field of biotechnology, the number and volumes of protein therapeutics (such as recombinant proteins, monoclonal antibodies and viruses) produced by mammalian cells is growing significantly.

As these materials are destined for human use, producers are encouraged to reduce or eliminate animal-derived components in their production to prevent contamination with viruses, mycoplasma or prions (Castle and Robertson 1999). As a result, much effort has gone to developing serum-free and protein-free media to support growth and production of a variety of cell lines. Of the two most common components of SFM, bovine insulin has been replaced by recombinant insulin but until now there has been no recombinant transferrin. A variety of iron chelators have been used as successful replacements for transferrin but none have been found that are universally active. These iron chelators include ferrous sulphate, ferric ammonium sulphate, Fe-PIH, Tropolone and Fe(III)-glycylglycine (Jayme 1999; Neumannova et al. 1995; Keenan and Clynes 1996; Yabe et al. 1987; Metcalfe et al. 1994). As the chemical iron chelators did not have the universal activity of transferrin, it has become necessary to screen and optimise conditions for a given cell line of interest and then validate the chelator with subculture experiments.

The results presented here show that the yeast-derived recombinant human transferrin was capable of replacing holo human transferrin in SFM for growth and production in three industrially important cell lines; MDCK, Vero and BHK-21. MDCK, a transferrin-dependent cell line was initially screened and then subcultured with DeltaFerrinTM and compared favourably to human transferrin. MDCK has been previously

subcultured successfully with iron chelators ferric citrate (Merten 2002), ferrous sulphate or ferric ammonium sulphate (Keenan and Clynes 1996).

The universal ability of DeltaFerrinTM to support growth was shown in a short-term screening assay with two variants of BHK-21 and Vero producing recombinant preproinsulin. DeltaFerrinTM supported growth at 116% and 90% of that obtained with native transferrin for BHK-21-PPI-C16 and MDCK, respectively, while Vero-PPI displayed little requirement for transferrin. Of the other chelators tested in the short-term screen, FAS was most consistent for the three cell lines.

In long-term subcultures, only DeltaFerrinTM consistently performed as well as holo human transferrin in all three cell lines (Fig. 7). FAS could support growth of Vero-PPI but for MDCK, in contrast to previous findings (Keenan and Clynes 1996) FAS gradually became less stimulatory as the cells were subcultured. For BHK-21-PPI-C16, FAS was not able to support growth beyond the first subculture. Invitrogen sample B and Fe₂SO₄ could support growth in two of the three cell lines but to a lower degree than holo human transferrin or DeltaFerrinTM and were also more variable in their growth stimulation. The lack of universal application of iron chelators has been previously reported (Yabe et al. 1987; Metcalfe et al. 1994). For example, while iron choline citrate was able to support the growth of Vero (Litwin 1992) it could not support long-term growth of MDCK cells (Keenan and Clynes 1996).

Insulin production in Vero-PPI and BHK-21-PPI-C16 was similar when DeltaFerrinTM replaced holo human transferrin. Given the limited production of preproinsulin by these cell lines in SFM, further studies with higher recombinant protein producing cells would fully validate the ability of the DeltaFerrinTM to support recombinant protein production.

For Vero, BHK-21 and MDCK, growth and biological production has been previously investigated in a variety of SFM (Merten et al. 1996; Perrin et al. 1995; Kallel et al. 2002). Merten et al. (1999) looked at all three cell lines with MDSS2N medium. While MDSS2N is devoid of animal-derived proteins, it contains plant extracts (soy bean peptone). For MDCK, the specific

growth rate was 0.0248 h^{-1} with MDSS2N and using the SFM described here with DeltaFerrinTM was 0.029 h^{-1} so a better growth rate could be achieved with a completely chemically defined SFM. For Vero, the specific growth rates in MDSS2N and the SFM described here with holo human transferrin or DeltaFerrinTM were 0.0166, 0.015 and 0.014 h^{-1} , respectively, where the cells were grown on microcarriers in MDSS2N. For the BHK, the specific growth rate in the SFM described here was much lower at 0.0043 h^{-1} and 0.0036 h^{-1} for holo human transferrin and DeltaFerrinTM, respectively, in comparison to 0.0197 h^{-1} in MDSS2N (suspension). The anchorage independent status of this variant was not responsible for the lower growth rate as this variant did not grow faster in suspension (i.e. when grown in the SFM without fibronectin). In part, it may reflect a slow growing BHK variant as this cell line grows slowly in serum-supplemented medium (specific growth rate in serum-supplemented medium of 0.022 h^{-1}).

In addition, the lower specific growth rate may be due to some essential component missing from the SFM or to the adaptation to SFM that BHK-21 appears to require in MDSS2N media and elsewhere (Cruz et al. 1998; Kallel et al. 2002).

The ability of DeltaferrinTM to perform equivalent to holo human transferrin impacts on its potential use in SFM. As it provides a universal iron chelator, it removes the need to screen and optimise for a given cell line and there is no necessity to validate its long-term effectiveness. In addition, that DeltaferrinTM is produced in animal-free conditions, its incorporation into SFM reduces contamination issues for therapeutic products.

Conclusion

From these investigations it can be concluded that DeltaFerrinTM is a suitable replacement for holo human transferrin and is more universally suitable than any of the common iron chelators tested in this study. Limited results on insulin production in the BHK-21-PPI-C16 and Vero-PPI suggest that DeltaFerrinTM can also support recombinant protein production to the same degree as holo human transferrin.

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