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Delivery of iron to human cells by bovine transferrin

Implications for the growth of human cells *in vitro*

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Following suggestions that transferrin present in fetal-bovine serum, a common supplement used in tissue-culture media, may not bind well to human cells, we have isolated the protein and investigated its interaction with both human and bovine cells. Bovine transferrin bound to a human cell line, K562, at 4 °C with a k_d of 590 nM, whereas human transferrin bound with a k_d of 3.57 nM, a 165-fold difference. With a bovine cell line, NBL4, bovine transferrin bound with the higher affinity, k_d 9.09 nM, whereas human transferrin bound with a k_d of 41.7 nM, only a 5-fold difference. These values were reflected in an 8.6-fold difference in the rate of iron delivery by the two proteins to human cells, whereas delivery to bovine cells was the same. Nevertheless, the bovine transferrin was taken up by the human cells by a specific receptor-mediated process. Human cells cultured in bovine diferric transferrin at 40 μg/ml, the concentration expected in the presence of 10% fetal-bovine serum, failed to thrive, whereas cells cultured in the presence of human transferrin proliferated normally. These results suggest that growth of human cells in bovine serum could give rise to a cellular iron deficiency, which may in turn lead to the selection of clones of cells adapted for survival with less iron. This has important consequences for the use of such cells as models, since they may have aberrant iron-dependent pathways and perhaps other unknown alterations in cell function.

INTRODUCTION

Advances in cell-culture techniques have allowed the identification of many of the nutrients and growth factors necessary to maintain mammalian cells in continuous long-term culture, and this has led to the development of artificial tissue-culture media. One of these, which is widely used (Murakami *et al.*, 1982), supplements the usual amino acid/vitamin mixture with ethanolamine, insulin, selenium and transferrin to allow normal growth in the absence of serum. However, most media are still supplemented with fetal-bovine serum because this appears to supply the nutrients required by most cell lines. Since transferrin is a major constituent of serum, it is thought that the requirement for serum reflects to a large degree the dependence of growth on the presence of transferrin and its passenger iron (Brock, 1981; Anderson *et al.*, 1982). A number of reports (Galbraith *et al.*, 1980; Ward *et al.*, 1982), however, have suggested that bovine transferrin does not bind to the human transferrin receptor and therefore fails to deliver iron to human cells (Sephton & Kraft, 1980). Others have shown, using competition studies, that bovine transferrin probably does bind to the human transferrin receptor, but only weakly (Tsavaler *et al.*, 1986). Since almost all cells require transferrin for growth (Barnes & Sato, 1980) the absence of binding of bovine transferrin would imply that human cells cultured in fetal-bovine serum may be able to acquire iron, and other trace elements that may be carried by transferrin, by a mechanism other than the normal receptor-mediated endocytosis of transferrin. The inability of bovine transferrin to bind to human cells could be due to a simple species difference in affinity for the receptor, but an alternative explanation could be that the internal proteolytic cleavage, which can occur in bovine transferrin (Maeda *et al.*, 1980), could decrease the binding affinity.

We have isolated transferrin from fetal-bovine serum and studied its interaction with transferrin receptors on both human and bovine cells in culture, and its delivery of iron to the cells. We found that bovine and human transferrin bind to bovine cells and deliver iron at a similar rate. Their interaction with human cells is, however, very different, with a k_d for binding of bovine transferrin much lower than that of human transferrin, with a subsequent difference in the rate at which iron is delivered to the cells.

MATERIALS AND METHODS

Materials

Minimum Essential Medium (Dulbecco's modification), RPMI 1640, tissue-culture plastics and several different batches of fetal-bovine serum were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. $Na^{125}I$ and $^{59}FeCl_3$ were bought from Amersham International, Amersham, Bucks., U.K., and bovine serum albumin (fraction V) from Sigma.

Transferrin preparation and labelling

The methods to purify transferrin from pooled human plasma and to label it with ^{59}Fe and ^{125}I have been described elsewhere (Young *et al.*, 1984). Bovine transferrin was prepared from fetal-bovine serum and labelled by the same methods.

Cell culture

Bovine embryonic tracheal cells (NBL4) and human fibroblasts (Flow 5000) were obtained from Flow Laboratories. NBL4 cells, Flow 5000 and human

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hepatoblastoma cells (HepG2) (Knowles *et al.*, 1980) were maintained in exponential growth in Minimum Essential Medium (Dulbecco's modification) and 10% (v/v) fetal-bovine serum. The medium for the NBL4 cells was also supplemented with 1% non-essential amino acids (Flow Laboratories). Human myeloid leukemia cells (K562) were grown in RPMI 1640 with 10% fetal-bovine serum. The adherent cells were cultured in 10 cm^2 dishes and the K562 cells in 75 cm^2 flasks (Flow).

Cell-growth studies

In order to study the ability of human and bovine transferrins to support growth of human cells, it was necessary to be able to alter just the transferrin species and not other growth factors. To do this K562 cells were adapted for growth in serum-free medium containing human diferric transferrin (25 μg/ml), ethanolamine (5 μM), bovine insulin (5 μg/ml) and sodium selenite (2.5 nM) as described by Taetle *et al.* (1985), by decreasing the concentration of serum over a period of 6 months. The cells were carefully washed to remove endogenous human transferrin (Hemmaplardh & Morgan, 1974) and then cultured in quadruplicate flasks in the same medium with either bovine or human diferric transferrin at 40 μg/ml. Each day, half of the cell suspension was replaced with medium and the cells were counted in a haemocytometer.

Titrations

The adherent cells were released from the dishes by exposure to trypsin/EDTA (0.2% and 0.02% w/v respectively) for 15 min and put into six-well plates (10 cm^2/well) at a density of (0.8–1.2) $\times 10^6$ cells/well, and were used after a further 24 h in culture. Cells were washed three times in phosphate-buffered saline (Dulbecco & Vogt, 1954) at 20 °C and then incubated for three periods of 15 min at 37 °C in Minimum Essential Medium + 1% (v/v) bovine serum albumin to allow the release of intracellular and surface transferrin (Hemmaplardh & Morgan, 1974). They were then cooled to 4 °C, and ice-cold medium (1 ml) containing increasing concentrations (2 nM–2 μM) of ^{125}I-labelled diferric transferrin was added. Each sample was duplicated, and an additional sample containing a large excess (up to 25 μM) of unlabelled transferrin was also prepared. After 90 min at 4 °C the dishes were gently washed four times with ice-cold phosphate-buffered saline (2 ml). NaOH (1 M, 1 ml) was added to dissolve the cells and the solution was removed for γ-radiation-counting in an LKB Multigamma counter. A further four wells were taken through the same procedure, but the cells were released into suspension with trypsin/EDTA for cell counting. Cells in suspension (K562) were treated as above, but washing was performed by centrifugation (400 g_{av}) for 5 min.

Iron and transferrin uptake

Cells prepared as described above were incubated for up to 6 h in Minimum Essential Medium + 1% bovine serum albumin with 40 μg of ^{59}Fe or ^{125}I diferric transferrin/ml. Cells were incubated at 37 °C, and at various times the labelled transferrin was added. At the end of the incubation all of the wells were cooled by the addition of ice-cold phosphate-buffered saline and then washed and prepared for γ-radiation counting as above. A parallel incubation in the presence of excess (2 mg/ml) unlabelled transferrin was also performed. ^{59}Fe samples were counted in a Packard Autogamma counter with a 7.6 cm-diameter (3 inch) crystal detector.

Serum iron and iron-binding capacity

These were determined by using the method of Ruuto (1975), making use of the reaction of iron with the iron indicator ferrozine. Analyses on batches of serum were performed in quadruplicate.

RESULTS

The binding of bovine and human ^{125}I-diferric transferrin to two human cell lines, namely an adherent fibroblast line, Flow 5000, and a line grown in suspension, K562, was investigated. Similar results were obtained with both lines: bovine transferrin bound, but with a very low affinity. Figs. 1(*a*) and 1(*b*) show a Scatchard analysis of the binding of the two transferrins to human cells (K562) in suspension. The k_d derived from the slopes of the curves were 3.03 nM and 3.3 μM respectively for human and bovine transferrin. Extrapolating the line to the intercept on the *x*-axis showed that both transferrins were binding to about 400000 sites on the cells at 4 °C. A series of experiments gave mean k_d values

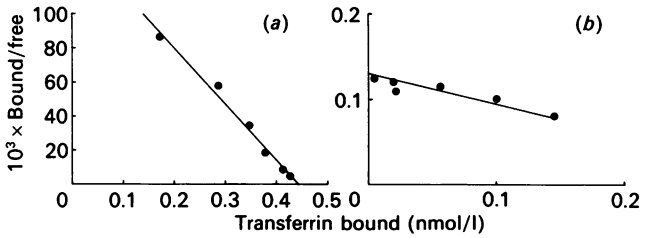

Fig. 1. Scatchard analysis of the binding of bovine (*b*) and human (*a*) transferrins to human cells at 4 °C

Washed K562 cells (1.3 $\times 10^6$/ml) were incubated at 4 °C for 90 min with up to 2 μM bovine transferrin or 100 nM human transferrin labelled with ^{125}I and then washed three times at 4 °C in phosphate. Non-specific binding was estimated in the presence of 25 μM unlabelled transferrins.

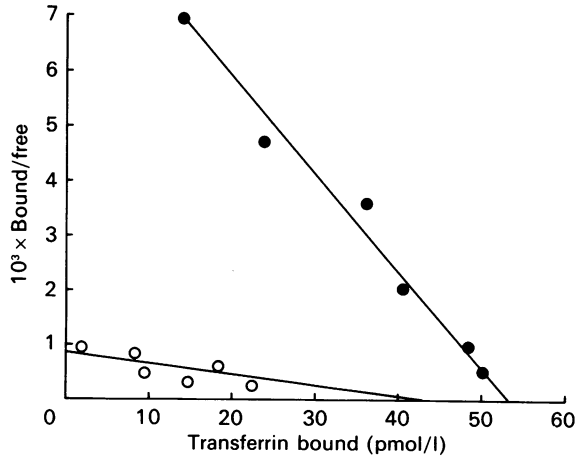

Fig. 2. Scatchard analysis of the binding of bovine and human transferrins to bovine cells at 4 °C

Washed NBL4 cells (0.8 $\times 10^6$/well) were incubated with bovine (●) or human (○) transferrin as in Fig. 1.

Table 1. Analysis of fetal-bovine serum for iron and iron-binding capacity

Five different batches of fetal bovine serum were analysed for iron and iron-binding capacity by using the iron-chelating dye ferrozine (Ruutu, 1975).

Serum batch	Iron-binding capacity (μM)	Unsaturated iron-binding capacity (μM)	Total iron (μM)	Saturation (%)
1	33.3	105.8	139.1	23.9
2	45.5	99.5	145.0	31.4
3	36.1	100.7	136.8	26.4
4	34.4	104.1	138.5	24.8
5	36.6	105.8	142.4	25.7
Mean...	37.2	103.0	140.4	26.4
S.D....	4.8	2.8	3.3	2.9

of 3.57 ± 0.33 nM (mean $\pm$ S.E.M.; $n = 3$) for human transferrin and 0.59 ± 0.71 μM ($n = 4$) for bovine transferrin, a 165-fold difference in affinity.

To eliminate the possibility that this discrepancy in the binding could have been due to an abnormality in the preparations of bovine transferrin used, the binding of both proteins to a bovine cell line, NBL4, was investigated. With these cells the bovine transferrin bound with a higher affinity (Fig. 2), but the discrepancy between the affinities was much less than with the human cells. In the experiment shown, the k_d for bovine transferrin was 5.88 nM and for human transferrin, 27.0 nM. A series of experiments gave values of 9.09 ± 0.5 nM (mean $\pm$ S.E.M.; $n = 4$) for bovine transferrin and 45.3 ± 7.4 nM ($n = 4$) for human, a 5-fold difference in affinity.

To be certain that the diminished difference in affinities was real and not merely due to the use of the adherent bovine cell line, experiments were repeated with an adherent human line. The difference was still observed when using an adherent human fibroblast cell line (Flow 5000), when a k_d value of 3.45 nM was found for human transferrin and a value of 1.1 μM for bovine transferrin.

In order to assess the level of bovine diferric transferrin to which cells cultured in 10% fetal-bovine serum are exposed, serum iron and total iron-binding capacity of five batches of fetal-bovine serum were measured. As Table 1 shows, the results for both serum iron and binding capacity for the five batches were tightly grouped with mean values of 140.4 and 37.2 μM respectively. From these values it was possible to calculate that the serum was on average 26.4% saturated with iron. Making the assumption that the iron was randomly distributed on the transferrin iron-binding sites (Lane, 1975), it is possible to calculate the amount of diferric transferrin present. At the saturation of the serum found, 7% of the transferrin would have been diferric, which corresponds to approx. 40 μg of diferric bovine transferrin/ml. Although iron transferrin would also have been present with only one iron-binding site occupied, we have shown previously that monoferric transferrins bind to the receptor considerably less avidly than the diferric molecule (Young *et al.*, 1984). For this reason the ability of both bovine and human transferrin to bind to and deliver iron to human and bovine cells was assessed by using a diferric transferrin concentration of 40 μg/ml.

The low affinity of the bovine transferrin led to low uptake by human cells. When K562 cells were incubated at 37 °C with diferric transferrin at 40 μg/ml, uptake reached a maximum after 15 min with both proteins, but the uptake of bovine transferrin was only 25% that of the human protein (Fig. 3). This uptake was largely abolished by the addition of a large excess of unlabelled transferrin, indicating that the uptake was specific and via the transferrin receptor, rather than by pinocytosis. When ^{59}Fe-labelled human and bovine transferrins were incubated with human and bovine cells, a difference in iron delivery was observed with the human cells. As shown in Fig. 4, iron uptake from both proteins by HepG2 cells was linear over a period of 6 h, but the rate of uptake from the human transferrin was 8.6 times greater. In contrast the delivery of iron to the bovine cells was the same from both proteins (Fig. 5).

The effect of this diminished binding and iron delivery on cell growth was assessed. K562 cells were adapted for

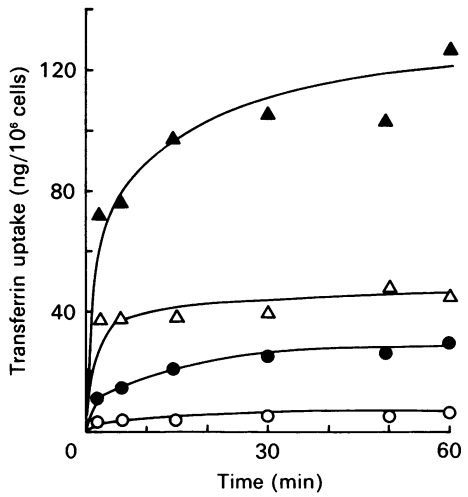

Fig. 3. Uptake of human and bovine transferrins by human cells at 37 °C

Washed K562 cells (1.9×10^6/ml) were incubated at 37 °C with ^{125}I-human or -bovine transferrin (40 μg/ml) with or without unlabelled transferrin (1 mg/ml) for estimation of non-specific uptake. At intervals cells (0.4×10^6) were removed, washed by centrifugation through dibutyl phthalate and counted for radioactivity in a γ-radiation counter. Specific (●, ▲) and non-specific (○, △) uptake of human (▲, △) and bovine (●, ○) transferrin is shown.

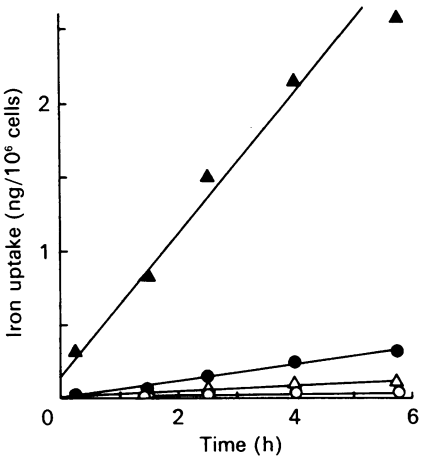

Fig. 4. Uptake of ^{59}Fe by human cells at 37 °C from human and bovine transferrin

Washed HepG2 cells (3×10^6/well) were incubated in duplicate with ^{59}Fe-labelled human or bovine transferrin (40 μg/ml) with or without unlabelled transferrin (1 mg/ml). At intervals the medium was removed and the cells were washed three times with ice-cold phosphate-buffered saline. Cells were removed from the dish with NaOH, and the radioactivity in them was determined in a γ-radiation counter. Specific (●, ▲) and non-specific (○, △) uptake of iron from human (▲, △) and bovine (●, ○) transferrin is shown.

growth in serum-free medium containing only transferrin, ethanolamine, selenium and insulin (Taetle *et al.*, 1985) in order that the species of transferrin could be changed without effecting the other nutrients. The cells were cultured for a period of days in this medium containing either diferric human or bovine transferrin (both at 40 μg/ml). As Fig. 6 shows, cells proliferated in the presence of human transferrin, but in bovine transferrin this level of protein was unable to support cell growth.

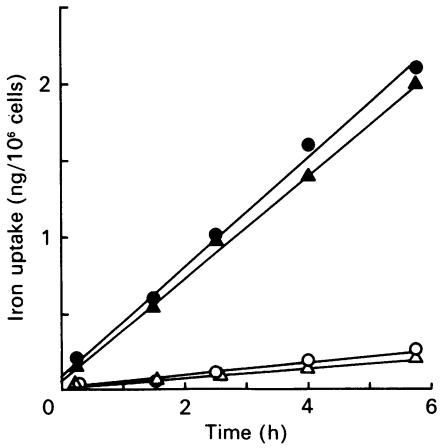

Fig. 5. Uptake of ^{59}Fe by bovine cells at 37 °C from human and bovine transferrin

Washed NBL4 cells (0.8×10^6/well) were incubated as described in Fig. 4. Specific (●, ▲) and non-specific (○, △) uptake of iron from human (▲, △) and bovine (●, ○) transferrin is shown.

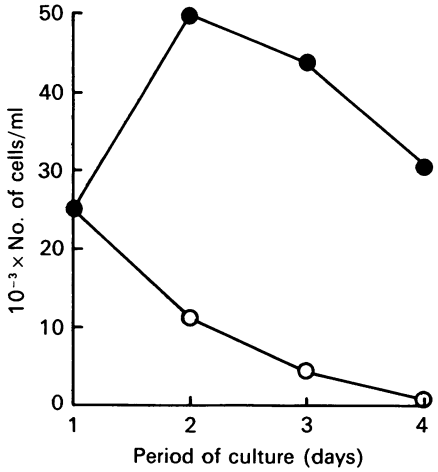

Fig. 6. Growth of human cells in serum-free medium with bovine or human transferrin

K562 cells, adapted for growth in serum-free medium, were washed free of human transferrin and then cultured in serum-free medium supplemented with bovine (○) or human (●) diferric transferrin at 40 μg/ml. Each day half of the suspension was replaced with fresh medium and the cells were counted.

DISCUSSION

Transferrin appears to be vital for the survival of cells in culture, and although several attempts have been made to ascribe alternative functions to the protein, its most vital role is in the delivery of iron to cells (Brock & Mainou-Fowler, 1983). Continuously proliferating cells, or those induced to proliferate, for instance lymphocytes, express transferrin receptors and take up iron (Galbraith *et al.*, 1980; Bomford *et al.*, 1983). If the cells are deprived of iron they respond by up-regulating receptor expression (Bridges & Cudkowitz, 1984; Bomford *et al.*, 1986) and ultimately proliferation is halted while the cells are in S-phase (Lederman *et al.*, 1984; Bomford *et al.*, 1986). This stasis can be induced by iron chelators such as desferrioxamine (Bridges & Cudkowitz, 1984; Bomford *et al.*, 1986) and the mode of action, in this case, is thought to be via the depletion of the iron-dependent enzyme ribonucleotide reductase (Hoffbrand *et al.*, 1976).

From the studies described here, it seems likely that human cells grown in fetal-bovine serum could be in a state of iron deprivation, with many consequences for their function. Human cells did not survive when grown in serum-free medium with bovine transferrin at 40 μg/ml, and the explanation for this comes from the observation that bovine transferrin bound to the transferrin receptor at 4 °C with an affinity of only 1/165th of that of human transferrin. This led to decreased uptake of the protein, and iron uptake at 37 °C only 10% of the rate from human transferrin. Assuming pseudo-first-order binding of transferrin (TF) to the receptor (R), it is possible to calculate the receptor occupancy:

$$TF + R \rightleftharpoons TFR$$

$$k_d = [R]/[TFR] \times [TF]$$

$$[TFR]/[R] = [TF]/k_d = 500 \times 10^{-9}/3.57 \times 10^{-9}$$

(for human cells and human transferrin at 40 μg/ml)

$$[TFR]/[R] = 140.0 \text{ (i.e. } 99.3\% \text{ of the receptors will be occupied)}$$

A similar calculation for bovine transferrin indicates that only 45.9% of the receptors on human cells will be occupied in the presence of the protein at 40 μg/ml. Therefore a diminished delivery of iron from the bovine transferrin would be expected. Of necessity the titrations were done at 4 °C and the iron-uptake studies at 37 °C, and so a better correlation between the diminished uptake of iron (8.6-fold) and the affinity for the receptor (165-fold) could not really be expected. Tsaveler *et al.* (1986) were unable to detect directly binding of bovine transferrin to human cells, but did show that bovine transferrin would compete with human transferrin for binding. The direct measurement of the dissociation constants reported here supports their finding of a large discrepancy in the binding of the two ligands to the human receptor. However, since the binding and transport of iron to bovine cells by the bovine transferrin was essentially the same as for the comparable human system, this suggests that there was no intrinsic defect in the bovine transferrin. The discrepancies observed were likely to be due to structural differences in the bovine transferrin. The bovine receptor bound the human protein with a slightly lower affinity (5-fold), indicating that the structural differences between the two species of receptor are likely to be less marked than those of the transferrins.

Cells in culture are obviously in an artificial environment and so presumably adapt in order to survive. Cells in fetal-bovine serum probably express more transferrin receptors than they would normally. Support for this comes from the finding that HeLa cells, when grown in human transferrin, can be made to down-regulate transferrin receptors, presumably because of the increase in available iron (Ward *et al.*, 1982). However, primary cultures of non-malignant human cells appear to have a limited ability to adapt to bovine transferrin, since Gaston *et al.* (1987) have shown that bovine serum alone will not support the outgrowth of human T-cell lines. Supplementation with a low concentration of human transferrin or human serum was necessary for successful growth to occur. Tsavaler *et al.* (1986) found little difference between growth of human cells in the presence of bovine or human transferrin. However, they used a diferric transferrin concentration of 300 μg/ml, which is a much higher concentration than is found in medium containing the usual 10% fetal-calf serum (Table 1) and which might have overcome the effect of the low binding constant.

It is not unlikely that, during the initial derivation of cell lines from human primary tissues and in the case of long-term culture in fetal-bovine serum, selection occurs of those clones whigh grow best in the effectively iron-deficient medium provided. Surviving clones may include those with either greater numbers of transferrin receptors or, perhaps more importantly, with metabolic pathways involving iron-dependent enzymes altered to make them less critical for growth. The involvement of iron in important enzyme systems, such as the cytochromes aconitase and ribonucleotide reductase, suggest that such alterations could have important implications for the use of such cells in the study of biochemical and cell functions.

These potential changes could be avoided by the simple expedient of the addition of human transferrin to the medium, as occurs in the formulation of many serum-free media. An alternative would be to increase either the concentration of bovine serum used or the saturation of fetal-bovine-serum transferrin with iron. A calculation, as given above, of the occupancy of the receptors in the presence of 20% fetal-bovine serum indicates that 62.8% of the receptors will be occupied, a value still not approaching that with human transferrin. Similarly if 10% fetal-bovine serum was saturated with iron, this would lead to 92.2% occupation of the receptors, which may prove adequate for unimpeded cell growth.

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