Modulation of transferrin-receptor activity and recycling after induced differentiation of BeWo choriocarcinoma cells

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BeWo human choriocarcinoma cells normally grow as cytotrophoblast cells. However, in the presence of 100 μ M-forskolin or 5 mM-theophylline, these cells form syncytia similar to morphologically well differentiated syncytiotrophoblasts. We have examined the effect of syncytia formation on transferrin-receptor activity and recycling. Although cellular proliferation stops upon growth in the presence of forskolin or theophylline, the number of cell-surface transferrin-receptors unexpectedly increased 2-fold, whereas the total cellular number increased at most 15%. The rate of biosynthesis of the transferrin receptor as well as class I MHC glycoprotein did not change measurably during syncytium formation. The biosynthesis of human chorionic gonadotropin increased 35-fold after 30 h of growth in the presence of theophylline. The redistribution of the transferrin receptor in syncytia is maintained by a decreased rate constant of endocytosis (0.141 min⁻¹ compared with 0.231 min⁻¹ for control cells) and an increased rate constant of externalization (0.122 min⁻¹ compared with 0.060 min⁻¹ for control cells). These altered rates of endocytosis and externalization resulted in an increased rate of iron accumulation in the syncytia. Furthermore, the recycling time of the transferrin receptor decreased in cells grown in the presence of theophylline (14.6 min compared with 21.2 min in control cells).

INTRODUCTION

Transferrin, a plasma glycoprotein, delivers iron to cells via receptor-mediated endocytosis. This transport protein [with two iron(III) atoms bound in its saturated form] binds the transferrin receptor at the cell surface and is subsequently taken up by the cell (Aisen & Listowski, 1980; Young & Aisen, 1981). Intracellularly the iron moiety dissociates from the transferrin polypeptide within an acidic compartment. The apotransferrin-transferrin-receptor complex remains intact at pH below 6.5 and recycles to the cell surface, where it dissociates and the apotransferrin is released into the medium at neutral pH (van Renswoude *et al.*, 1982; Dautry-Varsat *et al.*, 1983; Ciechanover *et al.*, 1983; Klausner *et al.*, 1983).

Transferrin is essential for cellular growth *in vitro* (Hemmaplardh & Morgan, 1974; Barnes & Sato, 1980). Proliferating cells and other cells which require vast amounts of iron (e.g. for iron transport) show an enhanced expression of transferrin receptors [see, for a review, Hanover & Dickson (1985)]. By contrast, down-regulation of surface transferrin-receptor activity occurs in cells differentiating to a terminal stage, which is thought to be a consequence of cessation of cell proliferation. Examples are the maturation of Friend-virus-infected erythroid cells by erythropoietin (Sawyer & Krantz, 1986), the differentiation of murine erythroleukaemic (MEL) cells *in vitro* (Mulford & Lodish, 1988) and the differentiation of HL-60 leukaemias (Yeh *et al.*, 1982).

Under basal conditions, BeWo choriocarcinoma cells display abundant transferrin receptors (3.4×10^6 binding sites/cell). Furthermore, the kinetics of transferrin-receptor recycling proceeds normally in these cells. However, BeWo cells are capable of releasing a substantial amount of their accumulated iron, which may function in iron transport through the trophoblast (van der Ende *et al.*, 1987, 1989). We have recently defined conditions under which BeWo cells are induced to differentiate

into a syncytium (Wice *et al.*, 1990). We demonstrated that, within 72 h, almost all cells fused and formed syncytia in the presence of 100 μ M-forskolin or 5 mM-theophylline. Morphological differentiation was complete, although the underlying mechanism(s) is not clear.

In the present study we describe the alterations in transferrin-receptor expression on the cell surface during theophylline- and forskolin-induced syncytium formation. We find an increased transferrin-receptor activity at the cell surface as well as a slightly increased total transferrin-receptor activity. The altered ratio of surface and intracellular transferrin receptor is maintained by an increased externalization rate constant.

MATERIALS AND METHODS

Cell and culture conditions

A clone of the choriocarcinoma cell line BeWo (originally isolated by Patillo & Gey, 1968), b24, was cultured in either the presence or absence of 5 mM-theophylline or 100 μ M-forskolin as described by van der Ende *et al.* (1987) and Wice *et al.* (1990).

Labelling of transferrin

Transferrin and protein A were iodinated with ¹²⁵I as described previously (van der Ende *et al.*, 1987). For loading transferrin with ⁵⁹Fe, the procedure of van der Heul (1978) was used.

Binding assays

Binding assays were carried out as described previously (Ciechanover *et al.*, 1983; van der Ende *et al.*, 1987), generally in 12-well plates. The ratio of surface transferrin receptor and total cellular transferrin receptor was determined in 35 mm-diameter dishes. Cells were incubated with ¹²⁵I-ferrotransferrin (20 μ g/ml)

in binding medium (Eagle's minimal essential medium buffered with 20 mM-sodium Hepes, pH 7.4) for 1 h at 37 °C, and subsequently the cells were washed three times with binding medium at 4 °C. The cells were then washed for 10 min with sodium acetate buffer, pH 4.5, and thereafter for 10 min with binding medium at 4 °C. This wash procedure was repeated twice. After washing, the cells were dissolved in 1 M-NaOH. The combined acid/neutral washes and the dissolved cells were counted separately in a γ -radiation counter. Surface-bound transferrin was determined as acid-wash-labile radioactivity, intracellular transferrin as acid-resistant radioactivity. Non-specific binding, determined as binding in the presence of a 50-fold excess of unlabelled ferrotransferrin, was subtracted in all binding experiments. Single-cycle experiments were performed as described previously (van der Ende *et al.*, 1987). Cells were incubated with saturating amounts (20 μ g/ml) of [^{59} Fe]ferrotransferrin for 2 h at 4 °C. After removal of the excess labelled transferrin, the cells were reincubated in prewarmed minimal essential medium (MEM)/Hepes, pH 7.4. At the indicated times, medium was collected and the cells were quickly chilled by immersion in ice-cold phosphate-buffered saline (PBS). Cell-surface radioactivity was found in the acid/neutral wash. Intracellular radioactivity was determined as the amount of radioactivity resistant to acid/neutral wash. For the determination of the externalization rate constant, the cells were incubated with [^{125} I]-ferrotransferrin (20 μ g/ml; in MEM/Hepes, pH 7.4) for 1 h at 37 °C. After removal of excess and surface-bound labelled transferrin at 4 °C, the cells were reincubated in MEM/Hepes supplemented with an excess of unlabelled ferrotransferrin (100 μ g/ml) at 37 °C. After incubation the medium was collected and the cells were washed again according to the acid/neutral-wash procedure. This acid/neutral wash was also collected and counted for radioactivity separately. Finally the cells were dissolved in 1 M-NaOH and counted for radioactivity in a γ -radiation counter.

Immunoprecipitations

Cells (in 35 mm-diameter dishes) were labelled with [^{35} S]methionine (60 μ Ci/ml; 800 Ci/mmol) for 2 h. The cells were lysed with 1 ml of lysis mix (1 % Triton X-100 in PBS). Aliquots of each lysate were precipitated with trichloroacetic acid and counted for radioactivity in a liquid-scintillation counter. Equal amounts of radioactivity for each sample were used for immunoprecipitation. For immunoprecipitation, samples (250 μ l) were mixed with an equal volume of immunomix [1 % (w/v) Triton X-100/0.5 % deoxycholate/1 % (w/v) SDS/1 % (w/v) BSA in PBS]. After addition of the antibody in excess and incubation overnight the samples were incubated with 50 μ l of fixed *Staphylococcus aureus* (IgG-sorb; The Enzyme Center, Malden, MA, U.S.A.) for another 1 h. The bacteria were pelleted and the pellet was washed three times in immunomix and once in PBS. The pellet was finally resuspended in sample buffer (Laemmli, 1970) and run on SDS/10 (or 15) % (w/v) PAGE gels. Radioactive proteins were detected by autoradiography using preflashed films (Kodak X-OMAT S; Kodak France). Protein bands on the original films were quantified with an LKB Ultrascan laser densitometer.

Anti-hCG antibody was purchased from Cappel (Organon Teknika Corp., West Chester, PA, U.S.A.). Anti-(MHC class I heavy chain) antiserum was a gift from Dr. H. Ploegh, The Netherlands Cancer Institute, Amsterdam, The Netherlands. Anti-(transferrin receptor) antibodies were raised against the transferrin receptor purified from human placenta as described by Bleil & Bretscher (1982).

RESULTS

Binding of transferrin to BeWo cells at different stages of syncytium development

In order to assess the total number of binding sites, we examined transferrin binding at 37 °C by BeWo cells during growth in the presence of theophylline or forskolin. After incubation with [^{125} I]-ferrotransferrin the cells were treated with sequential acid and neutral washes. The amount of surface binding was determined from the acid-sensitive radioactivity. As shown in Fig. 1, the amount of surface binding as a percentage of the total binding increases only after a lag of about 24 h. The surface binding increases 2-fold, whereas the total binding increases at the most 15 % above the initial value (results not shown). Maximal binding is observed after about 40 h growth in the presence of theophylline. This value is maintained until at least 76 h. Similar results are seen when cells are grown in 100 μ M-forskolin (Fig. 1).

To determine whether the increase in transferrin binding is due to an altered dissociation constant or to an increased number of transferrin receptors, we analysed, by means of Scatchard (1949) plots, the transferrin binding of normal cells and cells grown for 48 h in the presence of theophylline (under which conditions 60–80 % of the nuclei are in syncytia; Wice *et al.*, 1990). As Fig. 2 shows, the dissociation constant for transferrin binding is identical in control cells and cells grown in the presence of theophylline (1.4×10^{-8} M and 1.5×10^{-8} M at 4 °C and 37 °C respectively). The number of cell-surface and total cellular transferrin-binding sites is $(1.9 \pm 0.5) \times 10^{12}$ ($n = 6$) and $(5.4 \pm 1.0) \times 10^{12}$ ($n = 6$) per mg of cellular protein respectively for control cells. However, for cells grown in the presence of theophylline, the number of surface binding sites is doubled, i.e. $(3.5 \pm 0.7) \times 10^{12}$ ($n = 6$)/mg of cellular protein, whereas the number of total binding sites is only increased 15 %, i.e. $(6.3 \pm 1.0) \times 10^{12}$ ($n = 6$)/mg of cellular protein. The results obtained with cells grown in the presence of theophylline for 72 h or in the presence of forskolin for 48 h were nearly identical (results not shown).

From these results we conclude that the number of cell-surface transferrin receptors increases about 2-fold during syncytia development, whereas the number of total cellular transferrin receptors increases only 15 %. The net result is a more-than-2-fold increase in the ratio of cell surface to intracellular transferrin

Fig. 1. Effect of theophylline (○) and forskolin (●) on the ratio of surface/total transferrin receptors in BeWo cells

Cells were grown in the presence of 5 mM-theophylline or 100 μ M-forskolin for the indicated times. The ratio of surface/total transferrin receptors was determined as described in the Materials and methods section.

Fig. 2. Scatchard analysis of transferrin binding to control (○, ●) BeWo cells and cells grown in the presence of theophylline for 48 h

Cells were incubated with increasing amounts of ^{125}I -labelled ferrotransferrin for 2 h at 4 °C (□, ○) or 37 °C (■, ●). After incubation, cells were washed three times with PBS and subsequently dissolved in 1 M-NaOH. Bound radioactivity was analysed as described in the text.

receptors. This redistribution of the transferrin receptor was not observed in hepatoma Hep G2 cells exposed to theophylline for 48 h under identical conditions (results not shown).

Synthesis of the transferrin receptor, class I MHC and hCG (human chorionadotropin) during syncytium formation by BeWo cells

The biosynthesis of the transferrin receptor was examined during syncytia development in BeWo cells. Cells were grown in the presence of 5 mM-theophylline for increasing times. The cells were labelled for 2 h with [^{35}S]methionine as described in the Materials and methods section. Equal amounts of trichloroacetic acid-precipitable ^{35}S radioactivity (this was linearly related to the amount of cellular protein) were immunoprecipitated with anti-(transferrin receptor) antibody and analysed by SDS/PAGE and autoradiography. Fig. 3 shows that, although there is some random variation in the amount of transferrin receptor precipitated (less than 25% as measured from the original film), the results suggest that the biosynthesis of the transferrin receptor was not significantly altered during syncytia formation. This is not unexpected, since the present methods used to determine rates of biosynthesis would not detect changes of less than 25% during the observation period. Thus small changes sustained over the time course of fusion (i.e. 48 h) could easily result in the changes in total receptor content seen above. In addition, the synthesis of class I MHC (or HLA) and hCG were also studied. As Fig. 3 shows, the synthesis of class I MHC heavy chain (molecular mass 47 kDa) at different stages of syncytia formation was unaltered, similarly to the synthesis of the transferrin receptor.

However, the synthesis of hCG was significantly increased during growth in the presence of 5 mM-theophylline. Bands of molecular mass 15, 18, 22 and 24 kDa were observed, consistent with the molecular mass of the α - and β -subunit precursors and the mature α -form (Peters *et al.*, 1984). In these cells the ratio α/β for hCG is about 10, as has been observed previously in placental cells (Vaitukaitis, 1974) and because the α -subunit has three methionine subunits whereas the β -subunit has only one, the precursor of β (24 kDa) is hardly observed.

As Fig. 3 shows, the synthesis of hCG increases in cells grown in the presence of theophylline after a lag period of about 4–6 h. The maximal increase (i.e. about 35-fold the control level) is

Fig. 3. Effect of theophylline on the biosynthesis of the transferrin receptor (Tf-R; □), class I MHC glycoprotein (MHC I; △) and hCG (○) by BeWo cells

Cells were grown in theophylline for the indicated times and labelled with [^{35}S]methionine for 2 h. After labelling, the cells were lysed and processed via immunoprecipitation as described in the Materials and methods section. (a) The immunoprecipitates were analysed by SDS/PAGE and autoradiography. Molecular masses were determined from ^{14}C -labelled standard proteins run on each gel. (b) The amount of immunoprecipitated protein was quantified by optical densitometry. The amount of each immunoprecipitated protein at each time point of growth in the presence of theophylline was related to the amount of immunoprecipitated protein synthesized in control cells at zero time.

reached after about 30 h of growth in theophylline. After longer periods of growth in the presence of theophylline the synthesis of hCG decreases somewhat, but remains 10–20-fold greater than the control even after 72 h. These results suggest that stimulation of hCG synthesis is concomitant with syncytia development. In addition, pulse-chase experiments using control cells and cells grown in the presence of theophylline for 30 h demonstrate that the rate of secretion is nearly identical in the theophylline-treated cells [half-time ($t_{1/2}$) 60 min] (A. van der Ende, A. du Maine, A. L. Schwartz and G. J. Strous, unpublished work).

Rate of iron uptake

Redistribution of the transferrin receptor may result from alteration in either the rate constant of endocytosis (k_{endo}) and/or the rate constant of externalization (k_{exc}) (Tanner & Lienhard, 1987; Mulford & Lodish, 1988). Furthermore, changes in the rate constants of endocytosis and externalization may also affect the overall recycling time of transferrin-transferrin receptor. In order to determine the overall recycling time of the transferrin-transferrin-receptor complex in both normal cells and cells grown in the presence of theophylline, we incubated cells with saturating amounts of [^{59}Fe]ferrotransferrin. Both

control cells and cells grown in the presence of theophylline accumulated ^{59}Fe in a linear fashion (Fig. 4). The recycling time can be calculated from the ratio of the total number of transferrin receptors and the number of Fe atoms taken up/min (i.e. the slope of the ^{59}Fe uptake rate in Fig. 4). As we determined previously, the recycling time for control cells is 21.2 min (van der Ende *et al.*, 1987). However, for cells grown in the presence of theophylline, the recycling time is reduced to 14.6 min.

Rate of endocytosis

The reduction of the recycling time of the transferrin receptor in syncytia suggests that either the rate constant of endocytosis and/or the rate constant of externalization is altered. In order to determine the rate constant for endocytosis (k_{endo}) of the transferrin–transferrin-receptor complex, a single-cycle cohort analysis using [^{59}Fe]ferrotransferrin was performed. The rate of internalization is equivalent to the rate of acquisition of 'acid/neutral-wash-resistance' of a ligand prebound at 4 °C, and thereafter warmed to 37 °C. Since the dissociation of the iron moiety occurs inside the cell and since the free iron remains within the cell, we measured the net accumulation of ^{59}Fe within the cell in a single-cycle experiment using [^{59}Fe]ferrotransferrin. Upon warming to 37 °C 50% of the prebound [^{59}Fe]ferrotransferrin is internalized within 10 min (Fig. 5a).

The $t_{1/2}$ for internalization was 3.0 min (Fig. 5b). However, for

Fig. 4. Continuous uptake of ^{59}Fe into control BeWo cells (○) and cells grown in the presence of theophylline (●).

Cells were incubated with saturating amounts of [^{59}Fe]ferrotransferrin (1 μM) for the indicated times. After incubation, cells were processed as described in the Materials and methods section.

cells grown in the presence of theophylline, only 35% of the ^{59}Fe was found intracellularly within 20 min (Fig. 5a). The $t_{1/2}$ for internalization under these conditions was 4.9 min (Fig. 5b). The decreased percentage of ^{59}Fe found intracellularly in cells grown in the presence of theophylline can be explained by the decreased rate of endocytosis, such that during the incubation at 37 °C relatively more transferrin dissociates directly into the media.

Rate of externalization

Since the rate constant of transferrin endocytosis is decreased in cells grown in the presence of theophylline, the externalization rate constant of the transferrin–transferrin-receptor complex must be markedly increased relative to control cells in order to result in an overall reduction in the recycling time. In order to determine the rate of externalization, cells were incubated with saturating concentrations of ^{125}I -labelled ferrotransferrin at 37 °C. After incubation to equilibrium such that all transferrin receptors were occupied, the cells were rapidly chilled to 0 °C. At this temperature the surface-bound transferrin was removed by sequential acid and neutral washes. The cells were then incubated at 37 °C for increasing periods of time in the presence of excess unlabelled ferrotransferrin. The rate of decrease of the intracellular ^{125}I label (i.e. resistant to acid/neutral wash) is equivalent to the rate of externalization. About 60% of the transferrin is rapidly released from the cells within 30 min (Fig. 6a). When the results were plotted semi-logarithmically (and corrected for the 27% of the label that is not released after 60 min), it is apparent that 60% of the transferrin is externalized by a pseudo-first-order process with a $t_{1/2}$ of 11.5 min (Fig. 6b).

The rate constant of externalization of transferrin in cells grown in the presence of theophylline is increased compared with control cells, i.e. the $t_{1/2}$ for transferrin externalization is 5.7 min (Fig. 6b). Similar results were obtained with cells grown in the presence of forskolin. This increased rate of transferrin externalization compensates for the decreased rate of endocytosis and results in a net reduction in the recycling time, consistent with the results of the continuous [^{59}Fe]ferrotransferrin-uptake experiments (Fig. 4).

The kinetics of development of this process are seen in Table 1. In cells grown in the presence of theophylline for 24 h, the externalization rate constant is unchanged compared with control cells. However, by 48 h of growth in the presence of the drug, the externalization rate constant is increased 2-fold and remains at that level after 72 h. This is consistent with the data of Fig. 1, which demonstrates that more than 24 h of growth in the presence of theophylline or forskolin are required in order to observe redistribution of the transferrin receptor. Furthermore,

Fig. 5. Single cycle of [^{59}Fe]ferrotransferrin in control BeWo cells (○) and cells grown in the presence of theophylline (●).

For experimental details, see the Materials and methods section. (a) Intracellular radioactivity was determined as the amount of radioactivity resistant to acid/neutral wash. The values were corrected for the background of acid/neutral-wash-resistant radioactivity at zero time. (b) The accumulation of ^{59}Fe in the cell was plotted semi-logarithmically as $1 - (B_t/B_{\text{max}})$, where B_t is the amount at time t and B_{max} , the maximum amount in the cell. Data from two independent experiments were plotted.

Fig. 6. (a) Externalization of ¹²⁵I-ferrotransferrin in control BeWo cells (○) and cells grown in the presence of theophylline (●)

For experimental details, see the Materials and methods section. (b) Data from three independent experiments were plotted semi-logarithmically after correction (for the 27% the radioactivity not released after 1 h of incubation).

Table 1. Effect of theophylline on the externalization rate in BeWo cells

The externalization rate of ¹²⁵I-ferrotransferrin was determined in BeWo cells, which were grown in the presence of 5 mM-theophylline for the indicated times. The *t*_{1/2} values for externalization were determined as described in Fig. 6.

Period of incubation with theophylline (h)	<i>t</i> _{1/2} (min)	<i>k</i> _{exo.} (min ⁻¹)
0	11.5	0.060
24	10.0	0.069
48	5.7	0.122
72	5.5	0.126

redistribution of the transferrin receptor is maximal after about 40 h of growth in the presence of the drugs and remains constant upon prolonged incubation.

DISCUSSION

The present study describes the effect of BeWo choriocarcinoma syncytium formation by forskolin and theophylline on the transferrin-receptor dynamics. This is of interest, because the syncytium in the human placenta is a specialized epithelium and develops the physical barrier between the blood circulations of the mother and the developing fetus. However, the syncytia not only serves as an obligate barrier to most circulating substances (e.g. blood proteins, hormones and transferrin), but also serves as a selective transporting structure for selected nutrients (e.g. iron). Exposure of the cytotrophoblast-like choriocarcinoma cells to 100 μM-forskolin or 5 mM-theophylline induces the formation of syncytia after 24–48 h (Wice *et al.*, 1990). In the present study we demonstrate a marked redistribution of transferrin receptor in BeWo cells grown in the presence of theophylline or forskolin (i.e. during syncytium formation). Although the cellular proliferation ceases, the amount of receptor at the cell surface surprisingly increases at least 2-fold, whereas the total cellular receptor population increases at the most 15% after 40 h of growth in the presence of theophylline or forskolin. Under these conditions, the rate of biosynthesis of the transferrin receptor is not detectably altered. However, the recycling time of the transferrin–transferrin-receptor complex is reduced to 68% of that found in control cells. This redistribution of receptor is maintained by a decreased rate of endocytosis and a concomitant increased rate of externalization. From the experimentally

obtained *t*_{1/2} values for each process, we can calculate the appropriate rate constants according to the equation:

$$t_{1/2} = \ln 2/k \text{ (Table 2)}$$

The receptor recycling time can be calculated according to

$$T_c = (k_{\text{bind.}} \cdot L)^{-1} + (k_{\text{endo.}})^{-1} + (k_{\text{exo.}})^{-1} + (k_{\text{diss.}})^{-1}$$

where *k*_{bind.} is the rate constant for ferrotransferrin binding, *k*_{endo.} the rate constant for internalization, *k*_{exo.} the rate constant for externalization, *k*_{diss.} the rate constant for dissociation of the apotransferrin at the cell surface and *L* is the ligand (ferrotransferrin) concentration (Ciechanover *et al.*, 1983). We did not determine directly the rate constants for ferrotransferrin binding and apotransferrin dissociation, as these terms contribute little to the *T*_c values. They have been obtained previously (*k*_{bind.} = 3.0 × 10⁶ M⁻¹·min⁻¹ and *k*_{diss.} = 2.6 min⁻¹) (Ciechanover *et al.*, 1983). We assumed that these rate constants for the transferrin receptor in BeWo cells are the same under both growth conditions. Using these and the experimentally obtained rate constants in the equation cited above, we can calculate the recycling time for the transferrin–transferrin-receptor complex. The values are 22.7 min and 16.9 min for control cells and cells grown in the presence of theophylline respectively. These values are in close agreement with the experimentally obtained values for recycling times (Table 2).

The modulation of surface transferrin activity, concomitant with the developmental event in BeWo cells is distinct from the rapid modulation induced by certain growth factors or other agents (like phorbol esters) in several cell lines. For example, 3T3 cells exposed to growth factors (like platelet-derived growth factor or epidermal growth factor) and exposure of macrophages to phorbol esters result in a rapid increase of surface transferrin receptors (Buys *et al.*, 1984, 1987; Davis & Czech, 1986; Tanner & Lienhard, 1987). In general, the effect on the transferrin-receptor activity of these agents occurs very rapidly (within a few minutes).

There are few examples of cellular differentiation accompanied by alteration in receptor expression. For example, the human leukaemic cell line HL60 undergoes differentiation over a process of many hours when grown in the presence of phorbol esters; however, the transferrin receptor rapidly redistributes (within minutes), resulting in more intracellular transferrin receptors (May *et al.*, 1984). Thus the relationship between differentiation of these cells and receptor modulation is not clear, and down-regulation of the receptor might be independent of the maturation process.

A relationship between gradually altered receptor distribution and cellular differentiation has been observed in erythropoietin-

Table 2. Effect of theophylline on the parameters of transferrin binding and recycling in BeWo cells

Tf - R_t/Tf - R_s is the ratio of total and surface transferrin receptors.

	Control cells	Syncytium*
10 ⁻¹² × Transferrin bound (molecules/mg of protein)		
Total	5.4 ± 1.0	6.3 ± 1.0
Cell surface	1.9 ± 0.5	3.5 ± 0.7
Tf - R _t /Tf - R _s	1.8	0.8
t _{1/2} endo. (min)	3.0	4.9
k _{endo.} (min ⁻¹)	0.231	0.141
t _{1/2} exo. (min)	11.5	5.7
k _{exo.} (min ⁻¹)	0.060	0.122
Recycling time (min)		
Experimental	21.2	14.6
Calculated	22.7	16.9

* Data were obtained from cells which were grown in the presence of theophylline for 48 h.

induced maturation of Friend-virus-infected erythroid cells. Although the surface transferrin-receptor activity increases 2-fold during the first 24 h, at the stage of terminal differentiation, when proliferation stops, the surface transferrin-receptor activity is reduced again to the level of that in uninduced cells (Sawyer & Krantz, 1986). Fairly recently, Mulford & Lodish (1988) reported, that during the differentiation of murine erythroleukaemic (MEL) cells, the number of surface transferrin receptors decreased 4-fold in 4 days, whereas the total number of transferrin receptors remained constant. From single-cycle experiments it was calculated that the rate constant of endocytosis increased about 2.5-fold. The molecular mechanism responsible for those responses still remains to be established.

These examples of erythroid differentiation demonstrate a decrease of the number of surface transferrin receptors when cellular proliferation stops. This relationship between transferrin-receptor number and cell growth has also been observed in other cell types (Sutherland *et al.*, 1981; Trowbridge & Lopez, 1982). In contrast with this, the surface transferrin-receptor activity is gradually increased during monocyte-macrophage maturation. This increase in surface transferrin receptors is not associated with cell proliferation (Testa *et al.*, 1989). Surprisingly the expression of the transferrin receptor on the cell surface of BeWo cells increases, although the proliferation terminates upon growth in the presence of theophylline or forskolin. Thus it appears that the increased expression of cell-surface transferrin receptors may be a specific function of the placental syncytium, i.e. associated with iron transport directed from the maternal blood circulation to that of the developing fetus.

The peptide hormones human placental lactogen and hCG are produced mainly by the syncytium within the placenta (Thiede & Choate, 1963; Sciarra *et al.*, 1963) and are often used as markers of syncytium formation. In the present study we observed that the synthesis of hCG (both α - and β -subunits) increases 35-fold when the BeWo cells are grown in theophylline for 30 h. This is consistent with previous observations with both BeWo cells as well as other human cytotrophoblast cells, which have demonstrated that hCG synthesis and secretion can be markedly stimulated by cyclic AMP analogues (e.g. dibutyryl cyclic AMP) (Jameson *et al.*, 1986; Ritvos *et al.*, 1987). These data raise the possibility that the synthesis and secretion of hCG may not be an ideal biochemical marker for syncytium formation and that

transferrin-receptor distribution may therefore be a better biochemical parameter. It will be of interest to determine if the expression of surface transferrin receptor is increased during differentiation of freshly isolated cytotrophoblasts into syncytia (Kliman *et al.*, 1986). Findings with a similar system (Bierings *et al.*, 1988) indicate that this might be the case.

Several interesting cell-biological and physiological questions now present themselves in this system of placental differentiation. Are the intracellular routes of the transferrin receptor different in cells grown in the presence of theophylline compared with control cells? Does the syncytium function as a polarized epithelium with regard to transferrin and transferrin-receptor dynamics?

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