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Original Article

Transferrin receptor expression and the regulation of placental iron uptake

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Abstract

Placental transferrin receptors, located at the apical side of syncytiotrophoblast, mediate placental iron uptake. Regulation of transferrin receptors on the fetal-maternal exchange area could be a major determinant in the regulation of trans-placental iron transport.

Transferrin receptor expression in cultured human term cytotrophoblasts is on a much lower level than in choriocarcinoma cells, with a higher proportion of receptors located on the cell surface. Differentiation of cells, either due to longer culture periods or to 8-bromo-cAMP treatment does not lead to an increase of transferrin receptor expression. *In vitro,* the level of expression is largely regulated by the cellular density in the culture dishes. Low cellular occupancy of the dish leads to a high level of transferrin receptors. Treatment with iron-sources results in a down regulation of transferrin receptors.

Thus, though the level of transferrin receptors in cultured normal trophoblast is at a constant level, unaffected by differentiation, high levels of maternal transferrin-iron availability can lead to a decrease in placental iron uptake. This feed-back mechanism makes placental iron uptake independent of maternal iron stores.

Abbreviations: hCG – human Chorionic Gonadotrophin, TfR – Transferrin Receptor

Introduction

Regulation of transferrin receptor (TfR) expression on the maternal-fetal exchange area, the so-called apical side of syncytiotrophoblast could be a major determinant in the regulation of placental iron uptake. This paper describes experiments on the regulation of TfR expression in cultured human term cytotrophoblasts. In recent years, a well defined method to isolate human term cytotrophoblasts has become available [1]. We have previously shown that the level of TfR expression on freshly isolated cytotrophoblasts is lower than on the small amounts of monocytes in these cell suspensions [2]. In culture, cytotrophoblasts tend to fuse and start producing Schwangerschafts-protein 1 (SP-1), human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) [1–3]. In the first 40 hours *in vitro* both mono- and multinucleated (non-proliferative) cells start expressing TfR's [2]. Thus, *in vitro* both mono- and multinuclear cells express syncytiotrophoblast characteristics.

^{125}I-transferrin binding studies show that the level of TfR's at the cell surface is approximately 3×10^4 binding sites per cell after 40 hours *in vitro* [4]. This is considerably lower than the level of TfR expression in malignant trophoblast cell lines [5, 6]. The level of cellular TfR expression – an important regulating factor in cellular iron uptake [7] – is generally regulated by a number of mechanisms:

Cellular differentiation influences TfR expression. Biochemical differentiation of cultured cytotrophoblasts can be induced by either increased culture periods or the addition of cAMP-analogons to the culture medium [8–11].

Proliferation is a second well-known trigger for cellular TfR expression, e.g. in lymphocytes [12].

Thirdly, the genes for both ferritin and TfR are regulated by iron, through iron-responsive elements [13]. TfR mRNA levels can be modulated by treatment with iron chelators and iron sources. Finally insulin and growth factors like Epidermal Growth Factor (EGF) have been shown to cause a redistribution of TfR's over various pools of the endocytotic pathway, resulting in an increase of cellular surface TfR level [14, 15]. The effects of proliferation, differentiation and iron availability on TfR-expression in cultured term cytotrophoblasts will be discussed in this paper.

Materials and methods

Isolation of cells from human term placentas

Normal term human placentas were obtained within half an hour of spontaneous vaginal delivery. A total of 35 gram (wet weight) of villous tissue was cut out from the maternal side. Villi were washed extensively in cold 0.15 M NaCl, minced and subjected to three 30 min trypsin-DNase digestions using respectively 5, 3.3 and 2.5 ml/g tissue of the enzyme solution. The enzyme solution contained 1250 BAEE units trypsin (1 : 250, t.c., Sigma) and 475 Kunitz Units DNase 1, grade II (Boehringer-Mannheim) per millilitre Calcium- and magnesium- free solution of Earle's balanced salts (CMFS, Flow labs). 1 mM $CaCl_2$ and 0.8 mM $MgSO_4$ was added to the trypsin solution. These additions lead to improved cell yields and viability (de Groot, oral communication, 11th Rochester Trophoblast Conference, 1988, Rochester (NY)).

This results in cell suspensions with over 95 per cent cytotrophoblasts [4]. At the end of each trypsinization 6 fractions of 13.5 ml supernatant were each layered over 1.5 ml fetal calf serum (Flow labs). Pellets were spun down at 1000 g for 5 min. They were resuspended in 1 ml Dulbecco's Modification of Eagle's Medium with 20 mM HEPES (DMEM-H, Flow labs) and collected. After the third trypsinization the three pellets from consecutive enzyme digestions were spun down at 1000 g for 10 min, resuspended and collected in 4 ml DMEM-H. This suspension was carefully layered on a preformed Percoll gradient, 5 to 70 per cent Percoll (osmolality adjusted to 300 mosmol/kg by the addition of 1.5 mM NaCl) with CMFS in 5 per cent steps of 3 ml each, and centrifuged for 20 min at 1200 g at room temperature. The gradient was then fractionated by inserting a glass capillary from the top to the bottom of the tube, subsequently collecting 1 ml fractions. Fractions with densities of 1.048–1.062 g/ml washed once, using DMEM-H and checked under the microscope. Mononuclear cell fractions, virtually free of tissue fragments and cellular debris were used for cell culture.

Cells were counted and diluted to 6×10^5 cells/ml, unless otherwise indicated. 2.5 ml of this suspension was plated out in 35 mm Falcon culture dishes (Becton and Dickinson, USA). For immunoperoxidase staining glass coverslips were inserted. Culture medium was Medium 199 (Flow Labs), supplemented with 4 mM L-glutamine, 50 μg/ml gentamicin, 2.5 μg/ml fungizone and 20 per cent fetal calf serum unless stated otherwise. Further additions were as stated. All cultures were incubated in humified 5 per cent CO_2/95 per cent air at 37° C for designated times. After 24 h non-adherent cells were gently washed off and the medium was replaced.

Chemicals

8-bromo-cAMP (sodium salt) was from Sigma; Human apotransferrin from Behring Diagnostics; Iodo-Gen from Pierce Chemical Co. Ammonium ferric citrate was from BDH.

Radioligand incubations

Iodination

Apotransferrin was fully saturated with iron. Di-

ferric transferrin was reacted for 10 min at room temperature in a glass vial coated with 100 μg Iodogen. Free ^{125}I was separated from the radio labelled transferrin on a disposable Sephadex G-10 column (Pharmacia) followed by extensive dialysis against Phosphate Buffered Saline (PBS).

Incubations

Culture dishes were washed twice with serum-free medium 199 prior to radiolabel incubations. Cells were incubated with ^{125}I-diferric transferrin at 4° C during indicated periods of time. Incubation was in 1.25 ml of Dulbecco's Modification of Eagle's medium with 20 mM HEPES with additions as indicated.

At the end of the incubation period cells were washed four times with 1 ml ice-cold medium and finally lysed in 1 ml of 0.1 M NaOH, at room temperature. Lysis was checked, using an inverted microscope. If necessary, cells were scraped from the dish with a rubber policeman. In all incubations non-specific binding was determined by adding 100-fold excess unlabelled diferric transferrin to a parallel series of dishes.

Radioactivities were determined with a Packard 500 C autogamma spectrometer.

Protein determination

Protein was determined according to Bradford [16], with bovine serum albumin as a standard.

Removal of cell surface ^{125}I-transferrin at low pH

Following incubation at 37° C with labelled transferrin, cells were cooled to 4° C and washed three times with ice-cold medium. The medium was removed and 600 μl ice-cold 0.25 M acetic acid/0.5 M NaCl (pH 2.3) was added. After 60 seconds 300 μl 1 M Na-acetate was added. The total volume was subsequently removed and the cells were washed three times with ice-cold medium. These washes were added to the acid-removable radio-activity. Cells were lysed in 0.1 M NaOH. Control experiments were done to check whether prolonged acid wash could not remove any more radioactivity and to check whether prolonged acid wash did not release cellular protein from the dish.

Immunoperoxidase staining

Stainings were done using a kit from DAKO Corporation (Santa Barbara, U.S.A.). Cells, cultured on glass coverslips were fixed for 20 min. in methanol containing 20 per cent (v/v) hydrogen peroxide (3 per cent) at room temperature.

In negative controls first (rabbit-raised) antibodies were replaced by normal swine serum.

After the incubations, according to standard kit procedures, specimens were rinsed with distilled water and covered with Mayer's hematoxylin for 8 min. After having been washed and dipped in 2 per cent (v/v) NH_4OH, they were mounted, using glycergel mounting medium.

Table 1. Dependence of ^{125}I-transferrin binding upon cellular density. Cells were 40 hours in culture at the time of radio-ligand incubation. Incubation was for 2 hours at 4° C. ^{125}I-transferrin concentration was 225 ng/ml. All densities were tested in triplicate in this experiment. Results are from one experiment from a serie of 3 highly similar experiments

Plating out density (.10^5 cells/ml)	Mean protein/dish (μg) mean (± S.D.)	Specific ^{125}I-Tf binding (pmol/mg protein) mean (± S.D.)	Percentage of control	Percentage non-specific binding
6.0	150 (± 12)	0.57 (± 0.00)	100	6
3.0	63 (± 13)	0.92 (± 0.12)	161	12
1.5	30 (± 6)	1.14 (± 0.15)	200	28

hCG determination

hCG, secreted into the culture medium, was determined by enzyme-immuno-assay on a ES 600 autoanalyzer (Boehringer Mannheim, FRG) at the laboratory for Clinical Chemistry, Academic Hospital Rotterdam/Dijkzigt.

Results

Cell density and proliferation

Initial experiments, using ^{125}I-transferrin binding, suggested that the number of specific binding sites per mg protein depended on the quantity of cellular protein in the dish. Experiments in which we varied the plating our cellular density (i.e. the number of cells per ml culture medium) confirmed this finding (Table 1).

Bromo-deoxyuridine take-up studies show that term cytotrophoblasts are not proliferative *in vitro* [2]. Low cellular density in the culture dish does not represent a proliferative stimulus for term cytotrophoblasts. High specific transferrin binding at low cellular occupancy of the culture dish is therefore independent of proliferation.

Differentiation

Differentiation can be induced in normal cytotrophoblasts by either cAMP-analogon treatment or prolonged culture periods.

cAMP-analogon treatment leads to a strong increase in both cellular hCG content and hCG-secretion into the culture medium within 24 hours. Immunoperoxidase staining for hCG of 8-bromo-cAMP treated cells shows a pronounced variation between cells in staining intensity, especially within syncytia. Whereas in untreated cells generally mononuclear cells react stronger than aggregates and syncytia, in 8-bromo-cAMP treated cells syncytia are stronger positive than mononuclear cells.

8-Bromo-cAMP treatment does not lead to an increase in syncytium formation. In these experiments, both in treated and untreated dishes after 40 h, approximately 50 per cent of nuclei was in mononuclear cells, the other half in aggregates and syncytia, containing up to 40 nuclei. Table 2 illustrates the increase in hCG secretion into the culture medium.

8-Bromo-cAMP induced biochemical differentiation of trophoblasts *in vitro* does not result in changes in TfR expression (Fig. 1). Neither changes in affinity of transferrin for its recetor, nor changes in the number of binding sites per mg protein occur. Scatchard analysis shows that the apparent K_D of the ligand for its receptor is 1.7 nM and the number of cell surface binding sites in these cells, cultured for 40 hours is approximately 7×10^{11} per mg protein (mean protein per dish: 176 μg (n = 26, S.D. = 14).

According to our experience (a series of a total of approximately 50 placental cultures) there is considerable variation between experiments in the survival of cells *in vitro*. Sometimes cellular protein tends to diminish in the course of several days, showing limited viability of cells in culture. In these experiments we always found an increase in specific binding as cellular protein decreases (Table 3B). This is similar to the phenomenon described in the previous paragraph. In other experiments cells were apparently thriving better, resulting in no decrease of mean (cellular) protein per dish. An example is shown in Table 3A. In these cases no changes were found in the number of TfR's per mg protein in the course of 40 to 65 hours in culture.

Table 2. HCG-secretion by cultured cytotrophoblasts. Influence of the presence of 1.5 mM 8-br-cAMP

± cAMP	Culture period (hours)	hCG (mIU/ml)
control (M199 + 20% FCS)	–	1
–	20	35
+	20	146
–	40	76
+	40	603

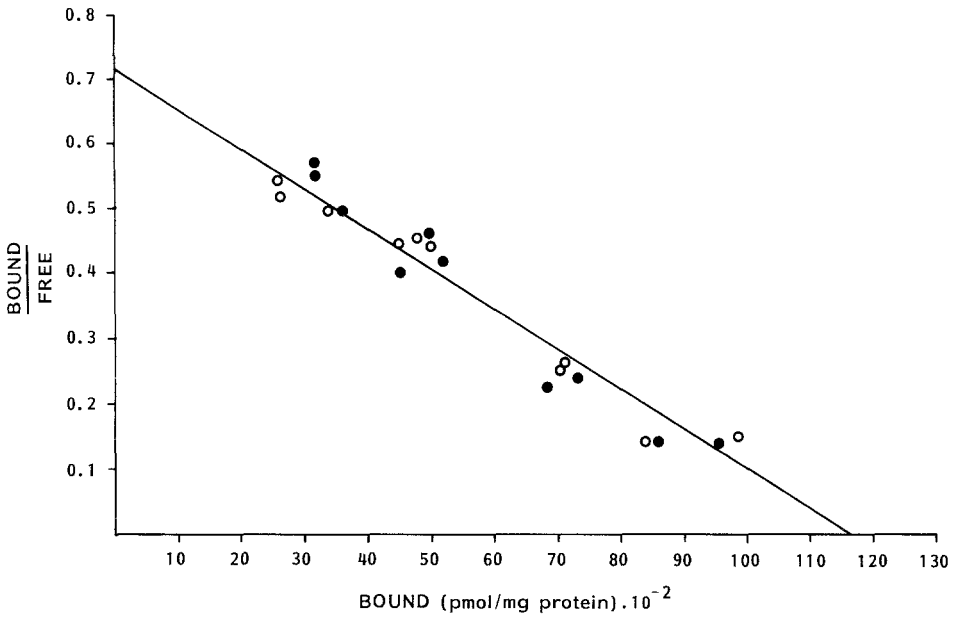

Fig. 1. Influence of 8-bromo-cAMP induced differentiation on transferrin receptor expression. Scatchard analysis of concentration-dependent ^{125}I-transferrin binding at 4° C to cultured cytotrophoblasts. Culture period: 40 hours.
●: cells treated during entire culture period with 1.5 mM 8-br-cAMP. Mean protein/dish: 0.18 mg (S.D. 0.01, n = 13).
○: controls (untreated). Mean protein/dish: 0.17 mg (S.D. 0.01, n = 13).

Iron availability

Table 4 shows that the addition of human diferric transferrin and, though to a lesser degree, ferric ammonium citrate reduces the level of TfR's on cultured cytotrophoblasts. This effect is not due to the presence of apotransferrin. Addition of transferrin reduces the number of receptors by approximately 65–75 per cent, ferric salts reduce the receptor levels by 25–35 per cent. Both control cultures and cells cultured in the presence of 0.1–0.8 mg/ml diferric transferrin were subjected to the acid wash procedure after incubation with ^{125}I-transferrin at 37° C. The ratio of surface to intracellular binding sites did not vary considerably between these samples.

Discussion

The level of TfR expression in cultured human cytotrophoblasts depends to a large extent on cellu-

Table 3. Influence of increased culture period on transferrin receptor number per mg protein, in two experiments; one without (A) and one with (B) changes in mean protein content per dish. Comparable results were obtained in several other experiments. Results of Scatchard analyses of concentration-dependent ^{125}I-transferrin binding at 4° C

		Culture period (hours)	Protein per dish (μg) mean (± S.D.)	n	Number of specific ^{125}I-transferrin binding sites per mg protein
A	I	45	96 (± 6.9)	10	$2.0\ 10^{11}$
	II	65	102 (± 11.8)	9	$1.9\ 10^{11}$
B	I	45	41 (± 8.1)	8	$1.2\ 10^{12}$
	II	65	25 (± 8.6)	8	$2.6\ 10^{12}$

lar density in the culture dishes. This is especially apparent when the plating out concentration is varied (Table 1). There is an inverse relation between the number of cells per dish and the level of TfR expression. In many proliferative cells it is difficult to separate the effects of proliferation and cellular density on TfR level. Low cellular density (through loss of contact inhibition) is a trigger for proliferation and results in a high level of TfR's. Since (term) cytotrophoblasts are not proliferative under *in vitro* conditions, this trigger for TfR expression is eliminated. In HeLa cells specific binding of ^{125}I-transferrin to cells decreases during exponential growth [7]. However, variation of plating out density does not influence specific transferrin binding in these cells, contrary to our findings.

An explanation for decrease in number of binding sites per cell with increasing occupancy of the tissue culture dish could be decreased exposure of cellular membrane to the incubation medium, possibly by formation of multilayer cells. However, microscopy-processed coverslips do not support substantial multilayer formation.

In general, the relationship between the degree of cellular differentiation and TfR level is more complex. Whereas erythroid differentiation leads to extinction of TfR expression [17] the number of receptors increases during the differentiation of monocytes to macrophages [18].

Differentiation of trophoblasts *in vitro* is known to be triggered by cAMP analogons [8–11]. It leads to an increase in hCG and progesterone secretion. No morphological differences can be demonstrated between cAMP treated and control cells. Especially the degree of syncytiumformation is not influenced. Apparently the multinuclear syncytial state is not a prerequisite for endocrinological differentiation. This has also been shown in strictly serum-free cultures, where no morphological differentiation occurs, cells remaining individual and spherical, but biochemical differentiation develops nonetheless [19]. cAMP treatment does not lead to any change in either the level of surface TfR expression or the affinity of ligand for its receptor. Thus, biochemical differentiation of cultured cytotrophoblasts does not affect the level of TfR expression.

In tumour cells like HL-60, dibutyryl-cAMP triggers inactivation of the TfR gene and monocytic differentiation of the cell [20]. cAMP analogons stimulate hCG expression in choriocarcinoma cells [21]. In this respect these tumour cells resemble normal trophoblast. Since TfR expression seems to be regulated differently in malignant cells com-

Table 4. High iron availability reduces transferrin receptor expression. After 24 hours in culture, dishes were washed and medium containing 5 per cent fetal calf serum and mentioned concentrations of human diferric transferrin or ferric ammonium citrate were added. 20 Hours later, specific ^{125}I-transferrin binding at 4° C was determined. All concentrations were tested in triplicate. Results from one experiment from a series of three similar ones. ^{125}I-transferrin concentration was 225 ng/ml. Incubation with radio-ligand during 2 hours

Addition (mg/ml)	Specific binding of ^{125}I-transferrin (pmol/mg protein) mean (± S.D.)	Percentage of control	Non-specific binding (percentage of total)
none (control)	0.52 (± 0.02)	100	5
0.8 apotransferrin	+		
50 μM DFO	0.50 (± 0.02)	96	5
0.1 transferrin	0.17 (± 0.01)	33	18
0.2 transferrin	0.17 (± 0.00)	33	21
0.8 transferrin	0.13 (± 0.01)	25	21
0.01 ferric ammonium citrate	0.35 (± 0.02)	67	10
0.05 ferric ammonium citrate	0.34 (± 0.03)	65	10
0.10 ferric ammonium citrate	0.38 (± 0.04)	73	10
0.20 ferric ammonium citrate	0.40 (± 0.03)	77	10

pared to normal cells, it would be of interest to know whether the level of TfR expression is influenced in cAMP treated choriocarcinoma cells.

As in K562 cells, treatment with diferric transferrin and ferric ammoniumcitrate reduces the level of TfR's in cultured cytotrophoblasts. Acid stripping of surface bound ^{125}I-transferrin shows that the ratio of surface to intracellular transferrin binding sites does not change under the influence of transferrin in the culture medium. This implicates that the total number of TfR's decreases, not the distribution of receptors over various pools of the endocytotic pathway. As in Rao's results [22] our experiments show a more pronounced effect of transferrin treatment, compared to treatment with iron salts. We conclude that Iron Responsive Elements seem to be operative in normal term trophoblast. Since IRE's tend to maintain cellular iron homeostasis it is difficult to explain persisting placental iron uptake and placental iron accumulation after fetectomy [23]. In the near future the protein involved in the communication between cytosolic iron and TfR's mRNA will probably be identified [24]. This protein might play an important role in placental iron metabolism.

We conclude that the level of TfR expression on the maternal-fetal exchange area is pre-programmed at a fairly constant level. A similar conclusion was reached by Van Dijk in a comparative study of the regulation of iron transfer during pregnancy [25]. Biochemical differentiation of cultured cytotrophoblasts does not influence placental TfR expression. *In vitro,* term human cytotrophoblasts are not proliferative [2].

Normal trophoblast differs remarkably from other cell types with respect to the relation between proliferation and TfR-expression. Proliferative cytotrophoblasts lack TfR's *in vivo* [26].

However, environmental iron availability, especially in the physiological diferric-transferring form does regulate TfR expression. This provides a feedback mechanism for placental iron uptake and makes it independent of maternal iron status. This protects transplacental iron transport against maternal iron deficiency, as well as against fetal iron overload in the case of high maternal iron availability.

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