PLACENTAL IRON UPTAKE
AND ITS REGULATION

REGULATIE VAN PLACENTAIRE IJZEROPNAME

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ABBREVIATIONS

BAEE	N α-benzoyl-L-arginine ethyl ester.
BrdU	5-Bromo-2'deoxyuridine.
(8-br)-cAMP	(8-Bromo)-adenosine 3':5'-cyclic monophosphate.
CD	Cluster of differentiation.
CMFS	Calcium- and magnesium-free solution of Earle's balanced salts.
DEAE	Diethylaminoethyl.
DFO	Desferrioxamine.
DMEM(-H)	Dulbecco's modified Eagle's medium (with HEPES).
DNase	Deoxyribonuclease.
EDTA	Ethylene diamine tetraacetic acid.
EGF	Epidermal growth factor.
FACS	Fluorescence activated cell sorter.
FCS	Fetal calf serum.
FITC	Fluorescein isothiocyanate.
hCG	Human chorionic gonadotropin.
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
hPL	Human placental lactogen.
IGF-1	Insulin-like growth factor 1.
IRE	Iron responsive element.
K_D	Dissociation constant.
LCA	Leucocyte common antigen.
M 199	Medium 199.
n	Number of observations.
NAD(H)	(Reduced) nicotinamide adenine dinucleotide.
PAGE	Polyacrylamidegel electrophoresis.
PBS	Phosphate buffered saline.

SD	Standard deviation.
SDS	Sodium dodecylsulphate.
SP-1	Schwangerschaftsprotein-1.
Tris	Tris(hydroxymethyl)-aminomethane.
TfR	Transferrin receptor.
UTR	Untranslated region.

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Chapter 1 General introduction.

1.1.1. General aspects of iron metabolism.

The ubiquitous element iron plays a key role in oxygen transport, in electron transport and in many enzymatic reactions.

There are two possible states of oxidation of iron: Fe(II) and Fe(III). Under aerobic conditions at neutral pH, Fe(III) is predominant. Since $Fe(OH)_3$ has a solubility product of only $4x10^{-38}$ M, the human body has devised several mechanisms to control this essential element.

The human body has no active iron excretion mechanism. Therefore, the iron balance can only be regulated by modulation of iron uptake in duodenum and jejunum. The daily loss of iron, under normal conditions is 1-2 mg.

Both iron deficiency and iron overload lead to disease, anemia and hemochromatosis respectively. Iron overload is especially dangerous since it catalyzes free radical formation. In the human body 70 per cent of the total amount of body iron of four grams is found in hemoglobin, 20 per cent is stored in ferritin, mainly in the liver and only 0.1 per cent is transported, bound to transferrin.

Three proteins: the iron transport protein transferrin, the cellular transferrin receptor and ferritin, the protein of iron storage play key roles in the regulation of iron metabolism.

1.1.2. Transferrin.

Human transferrin is a glycoprotein with a molecular mass of 79,550 daltons (1).

The single, folded polypeptide chain can be divided into two domains, so-called C- and N-terminal. Both domains contain one binding site for a Fe(III) atom.

The C-terminal domain also carries two complex-type asparagin N-linked carbohydrate chains. Each of these glycan chains has two, three or exceptionally four antennas (2-4). Normally, the terminal residues in the antennas are sialic acid. Differences in sialic acid content of the transferrin molecule are the cause of the so-called micro-heterogeneity (5,6).

Under physiologic conditions the binding of iron to transferrin is strong, with a K_D of approximately 10^{-20} M and (bi) carbonate serves

as a synergistic anion. Since transferrin is normally only 30 per cent saturated with iron, a mixed population of transferrin molecules occurs in the serum: transferrin without iron (apotransferrin), monoferric transferrin, iron being located on either of the two iron-binding sites (Fe_NTf and $TfFe_C$) and diferric transferrin. The two binding sites differ in their affinity for Fe(III), depending on the pH (7,8).
The presence of both iron-binding sites and their different iron-binding characteristics have led to hypotheses on functional differences (9,10). Though diferric transferrin is the preferred iron donor since it binds to the transferrin receptor with the highest affinity, both mono-ferric transferrins do not show separate donor functions (11,12).
The major site of transferrin synthesis is the liver, but synthesis also occurs in tissues like the mammary gland, placenta, Sertoli cells and the central nervous system (13-15).
In chicken liver, transcription of the transferrin gene and hence transferrin synthesis is stimulated by iron deficiency and estrogen (16-18).

1.1.3. Transferrin receptor (TfR).

The transferrin receptor is a transmembrane, homodimeric glycoprotein with a molecular mass of 180,000 daltons (19). Each identical 90,000 dalton subunit, covalently attached through a single disulfide bridge can bind one transferrin molecule.
The gene for the human TfR has been cloned and sequenced (20). Post-translational modifications of the TfR include fatty acid addition and phosphorylation.
The presence of TfR on the surface of cells is especially apparent in cell types that are proliferative and in cells requiring large amounts of iron for other purposes like erythroid precursors and the syncytiotrophoblast (21-23).
Apart from its important role in mediating iron delivery to cells, an alternative role in regulation of cellular growth has been suggested for the TfR (24).
In T-lymphocytes the TfR plays a permissive role in the activation of proliferation (25).

1.1.4. Ferritin.

Ferritin consists of 24 globular subunits, forming the empty near-spherical shell (apoferritin) with a molecular mass of 450,000 daltons. Each ferritin molecule is able to deposit up to 4000 iron atoms in its central cavity, essentially as inorganic ferric oxyhydroxyphosphate (26).

There are two kinds of subunits: the H or heart type and the L or liver type. Ferritin molecules are made up of both subunit types. H-subunits have a slightly lower pI and are able to incorporate iron more efficiently and can meet iron needs rapidly whereas the slightly more alkaline L subunit is metabollically stabler (27).

1.2. Pregnancy and iron metabolism.

1.2.1. Maternal iron metabolism.

Although the burden of fetal iron needs on maternal iron metabolism is relatively mild in humans, compared to other species, it is still considerable, 23 per cent of maternal iron stores is transported to the fetus (28). In guinea-pigs 200 per cent of maternal iron stores is needed for their off-spring (29).

In humans, the majority of trans-placental iron transport takes place in the third trimester (30).

Maternal transferrin is probably the sole iron donor for the human placenta (30).

Iron levels in maternal serum continuously drop in the course of pregnancy. Since transferrin concentration increases, the level of saturation of maternal transferrin with iron declines during pregnancy (30-32). It should be borne in mind that in late pregnancy blood volume increases with approximately 50 per cent, largely due to an expansion of plasma volume (33). This physiologic hemodilution of pregnancy makes the interpretation of hemoglobin and transferrin concentrations more difficult.

Maternal serum ferritin levels continue to decrease throughout pregnancy as well, even if the mothers are not anaemic (31,33,34).

This represents depletion of maternal iron stores. In humans, increased intestinal iron absorption in pregnancy is at least partly trigge-

red by depleted iron stores (35,36). It has been shown that there is a direct linkage between pregnancy and increased iron absorption in the gut in rats and guinea-pigs, without depletion of maternal iron stores (29, 37-39). The near-term situation of maternal iron state reflects iron deficiency, in many cases resulting in anaemia.
Iron suppletion can prevent maternal iron depletion. However, it does not seem to influence cord blood iron status parameters (32, 40-42). Therefore, even if the maternal iron state is precarious, the fetus is protected against iron deficiency.

1.2.2. General characteristics of transplacental iron transport.
At term, trans-placental iron transport is a fast process. Only a small portion of iron is retained in the placenta, the majority is passed on to the fetus (30).
Nonetheless, the placenta is autonomous in iron uptake (43, 44). If fetuses are removed in rats or guinea-pigs placental iron uptake continues for several days. This enables us to study placental iron uptake in vitro, in the absence of a fetus.
Transplacental iron transport is an one way (towards the fetus) active transport process: it operates against a concentration gradient and metabolic inhibitors, as well as hypoxia prevent iron transport (44, 45). The amount of iron transported to the fetus increases enormously: from 0.4 mg iron per day at 16-20 weeks pregnancy to 5 mg iron per day at term, in humans (30). The total amount of iron transported to the human fetus in the course of pregnancy is approximately 250-300 mg (46).

1.2.3. Fetal iron metabolism.
In the fetal circulation iron is bound to fetal transferrin. There are no functional differences with respect to iron binding characteristics between adult and fetal transferrin (47). Iron is directed towards ferritin in the fetal liver (48), and partly used in erythropoiesis. Up to a gestational age of 7 months, fetal erythropoiesis is mainly located in the liver (49). Fetal serum transferrin levels at term are low, with high levels of serum iron and thus very high saturation of transferrin with iron. Serum ferritin is high (31). Thus, these parameters reflect neonatal iron sufficiency.

1.3. Placenta and iron.

1.3.1. Human placentation.

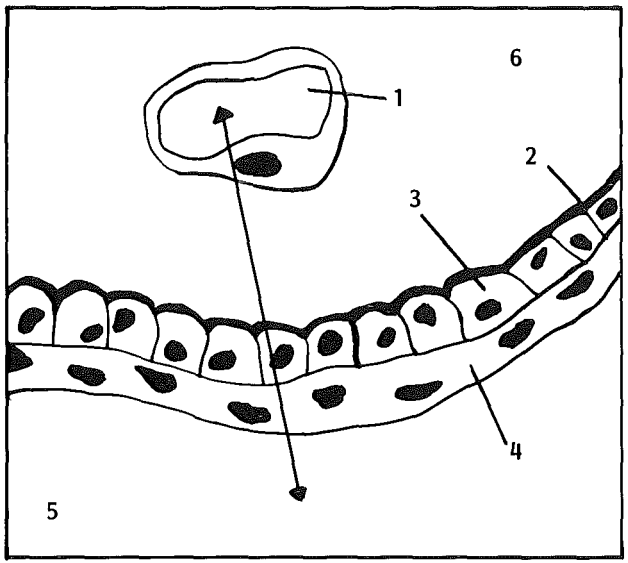
Like the guinea-pig placenta, the human placenta is hemomonochorial. This functional classification indicates that there is only one cell layer separating fetal and maternal circulations at term (50, 51).
Anatomically, whereas the guinea-pig placenta is labyrinthine, the human placenta has a villous structure: fetal trophoblast is arranged in tree-like villi, floating in spaces filled with maternal blood. The outer layer of a villus consists of syncytiotrophoblast. Numerous cells have fused, forming a layer with many nuclei and no intercellular membranes.
It has an apical side, facing maternal blood and a basal side which rests on cytotrophoblasts and a basement membrane. Early in pregnancy the cytotrophoblasts (mononuclear cells) form a continuous layer, the so-called Langhans' cell-layer, but at term only approximately 20 per cent of these cells are still present (51). Figure 1.1 illustrates some of these changes in placental histology in the course of pregnancy. The distance between the maternal and fetal circulation decreases as pregnancy progresses, facilitating diffusion between both circulations.
According to fetal needs, new exchange area can be formed by the fusion of cyto- with syncytiotrophoblast (52). Although both cell types are closely related, there are major differences. In vivo, only syncytiotrophoblast produces hormones like human chorion gonadotrophin (hCG), human placental lactogen (hPL) and proteins like the so-called Schwan-

Figure 1.1
Changes in human villous histology in the course of pregnancy.
First trimester (left) and term (right) human placental villus.
1. fetal capillary
2. basement membrane
3. cytotrophoblast
   (Langhans' cell layer)
4. syncytiotrophoblast
5. intervillous space, filled with maternal blood.
6. mesenchymal villous core (fibroblasts, Hofbauer cells).

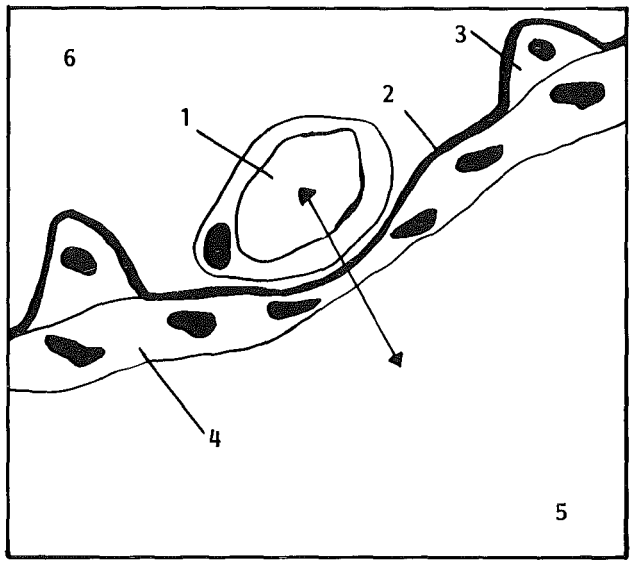
The length of the arrows indicates the distance between maternal and fetal circulations.

Figure 1.1

First trimester



Term



gerschaftsproteins (SP's) (53-55). Cytotrophoblast has many trophoblast stem cell characteristics. It remains proliferative throughout pregnancy and is undifferentiated (56).
Cyto- and syncytiotrophoblast are thought to form a paracrine system in which cytotrophoblast produces hCG-releasing hormone, as well as inhibin (57-59). Inhibin is a heterodimeric protein which selectively inhibits the release of follicle-stimulating hormone from the pituitary. It could also play a role in the regulation of hCG secretion (59).
As the placenta increases in size and weight, the exchange area is increased by increasing the number of villi as well as the degree of villous branching. It is obvious that these changes influence hemodynamics as well.
Trans-placental iron transport is receptor-mediated. The TfR-density on the syncytiotrophoblast apical membrane is likely to change in the course of pregnancy (60). Limited supply of normal first and second trimester placentas and large inter-species differences hamper the increase of experimental data on the ontogeny of placental transport (61, 62).

1.3.2. Placental iron uptake.

Maternal iron-loaded transferrin is taken up in the placenta through receptor-mediated endocytosis (45). Iron is subsequently selectively transported to fetal transferrin, the complex of transferrin receptor-maternal apotransferrin being recycled to the maternal circulation.
On its way to the fetus iron has to pass at least both the basal and basement membranes and fetal endothelium.
Transferrin receptors are located on the apical, as well as the basal side of syncytiotrophoblast (63-70), but are thought to be absent on cytotrophoblast (56,71,72). More details on placental transferrin receptors will be discussed in chapter 4.
Although there is a report on placental ferritin-receptors, there is not much experimental support for the hypothesis of ferritin-mediated placental iron uptake (73). Ferritin binding to purified villous membrane could only be demonstrated with horse spleen ferritin. Moreover, apoferritin had a higher affinity for the binding site than ferric ferritin.

1.3.3. Placental ferritin.

In early stages of pregnancy (4-5 weeks) electron-microscopy reveals the presence of abundant Golgi-derived granules in the syncytiotrophoblast (74). X-ray microanalysis demonstrates the presence of iron in electron-dense lamellae in the interior of these granules. They possibly represent lysosomal iron storage in early pregnancy to prevent fetal iron overload in a stage when fetal iron processing is immature. These granules have almost completely disappeared in the syncytiotrophoblast by 13 weeks of gestation.

Mössbauer spectroscopy indicates that iron, located near or in syncytiotrophoblast microvillous membrane, is in the same form as in ferritin (75).

The presence of ferritin in syncytiotrophoblast is also indicated by its presence in microvillous membrane vesicles (76).

The localization of ferritin, close to the plasma membrane suggests an active role in iron transport.

In guinea-pigs, total placental non-heme iron (mainly ferritin-iron) increases in the course of pregnancy but per gram placental wet weight or DNA there is an inverse relation between placental ferritin iron and trans-placental iron transport (77). It has been suggested that in this species ferritin not only serves as a buffer for iron, but is also involved in the regulation of the size of a low-molecular-weight iron pool that controls placental iron transport (77,78).

In humans placental ferritin concentrations (expressed per mg protein) increase in the course of pregnancy: first trimester 0.07 μg ferritin per mg protein, second trimester: 0.18; third: 0.25 (31). No data are available on placental ferritin-iron contents.

Mid-term placental ferritin has a more acidic pI than liver ferritin (L/H ratio placental ferritin 3:1; liver ferritin 4:1), whereas at term placental ferritin is supposed to consist mainly of L-subtypes (79,80).

Placental iron is not only located in tissue ferritin, but also in a low molecular weight pool, which is probably easier accesible for transport-purposes.

Inoue et al. have reported that lactate is the major ligand in this iron pool in the placenta (81). This hypothesis is especially attractive since large amounts of lactate are produced in the placenta, even

under aerobic conditions (82,83). Lactate is transported to the fetus to serve as a substrate in fetal oxidative metabolism (82). In vivo, the placental lactate/pyruvate ratio is approximately 50 (84). This ratio represents the redox state of cytoplasmic NADH/NAD^+ and is about five-fold higher in the placenta than in other tissues. The highly reduced cytoplasmic redox state in the placenta favors iron (II) to iron (III). Citrate, aminoacids and nucleotides have also been mentioned as possible intermediate ligands in iron transfer (85). The existence of a placental-tissue-specific-transferrin has been reported (13).

1.4. Cellular iron metabolism.

1.4.1. Transferrin-mediated iron uptake.

Transferrin provides the major if not only route for the delivery of iron to eukaryotic cells. After the binding of transferrin to its receptor, the occupied receptors are clustered in so-called coated pits.

Clathrin is a major constituent of the coating of these pits (86). The pits sink into the intracellular space, eventually losing their protein coat. Thus, a receptosome or endosome is formed (87).

Since receptosomes contain various ligands with different intracellular destinations, sorting has to take place. The pH inside of the endosome is lowered to 5.0-6.5 and consequently, iron is released from transferrin. The mechanism for the release of iron from transferrin is still a matter of debate. Two mechanisms have been proposed: protonation of the iron binding sites and reduction of iron (III) to iron (II).

The low endosomal pH is maintained by a membrane-located ATP-dependent proton pump. Proton ionophores like monensin and weak bases like methylamine inhibit iron-release from transferrin (88-90). At the endosomal low pH apo-transferrin has a high affinity for the transferrin-receptor and the intact entire complex is hence recycled, back to the cellular surface (91-92).

At environmental pH 7.4 apotransferrin is released from the receptor. The complete transferrin-cycle takes 3-7 minutes.

After its release from transferrin, iron is transported to ferritin

for storage or to the mitochondria for heme-synthesis or it is used in other iron-requiring processes. The nature of iron-ligands is discussed in the previous section. As an alternative for endocytosis reduction of diferric transferrin on the outside of cells and subsequent cellular uptake of ferrous iron has been proposed (93-96). A transferrin receptor associated NADH diferric transferrin reductase has been described in liver plasma membrane (96). Preliminary results have been reported suggesting the presence of this transferrin receptor associated enzyme in syncytiotrophoblast microvillous membrane as well (97).
In hepatocytes transferrin uptake can be mediated by both the transferrin receptor and the asialoglycoprotein receptor, depending on the terminal residues of transferrin's glycan chains (98). The intracellular route of these receptor-ligand complexes differs.

1.4.2. Cellular iron homeostasis.

The cell responds to changes in the amount of intracellular iron with changes in the levels of transferrin receptors and ferritin (99-102). Generally, when iron is limiting, the cell produces more transferrin receptors and less ferritin.
For ferritin, regulation takes place at a 26 base oligonucleotide centered around a predicted loop of the ferritin's mRNA 5'untranslated region (UTR). This oligonucleotide forms the so-called iron responsive element (IRE) (103). The IRE probably provides the recognition site for a protein (or protein containing complex) which, when bound, acts as a repressor of translation (104,105).
In the absence of iron, ferritin mRNA is found as part of messenger ribonucleoprotein particles. In the presence of iron the specific mRNA for ferritin is shifted to polysomes, reflecting active translation (106). Thus, a characteristic of the ferritin translational regulatory system is iron-dependent modulation of protein biosynthesis without corresponding alteration in the level of the mRNA.
Iron dependent regulation of the transferrin receptor gene correlates with transferrin receptor mRNA levels (99-101).
Whereas the IRE occurs as a single copy within the ferritin 5' UTR, there are five similar elements within the human transferrin receptor 3' UTR (107). The transferrin receptor 3' UTR sequence elements are capable of folding to form structures that are strikingly similar to

that predicted for the IRE located in the 5' UTR of ferritin. Contrary to ferritin-regulation IRE's probably regulate the degradation of the transferrin receptor message.
Thus, the IRE is a RNA element, capable of mediating two distinct regulatory events depending on its context within the transcript (107). This represents a model of the mechanisms involved in accomodating cellular iron needs and protecting the cell against toxic iron overload.
Recently fibroblasts from children suffering from neonatal hemochromatosis were used to study whether this disease represents a generalized inborn error of metabolism. It has been shown that these fibroblasts had normal IRE-mediated responses to cellular iron levels (108).

1.5. Scope of this thesis.

Iron transport in pregnancy is an active one-way process, from mother to fetus.
Early in gestation fetal iron needs are low, and so is trans-placental transport, but as erythropoiesis develops, rising fetal iron needs are met by trans-placental iron transport.
Apparently, the fetus is protected against iron toxicity as well as against iron deficiency, the latter even if maternal iron stores are depleted.
Fetectomy experiments in animals indicate that placental iron uptake is an autonomous process, independent of the presence of a fetus.
The narrow and, in the course of pregnancy, enormously changing difference between iron deficiency and iron toxicity strongly suggests that maternal-fetal iron transport needs to be regulated.
This thesis deals with some aspects of the regulation of placental iron uptake. Chapter 3 describes the development of a cell culture model to study placental iron uptake. Chapter 4 deals with characteristics of transferrin receptors in these cells and chapter 5 describes the regulation of transferrin receptor expression in normal human term trophoblast cells in vitro.
Finally, chapter 6 describes pregnancy-related changes of maternal transferrin, the iron donor for the placenta.
In this chapter an animal model is used, the guinea-pig.

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Chapter 2 Materials and Methods.

2.1. Materials.

2.1.1. Chemicals and Isotopes.

All chemicals used were of analytical grade. Bovine serum albumin; trypsin type III and 1:250, (t.c.), bromo-deoxyuridine and 8-bromo-cAMP (sodium salt) were from Sigma, U.S.A. Percoll, Density Marker Beads, Immobiline Dry Plates (pH 5-6), Ampholine (pH 4-6), Sephadex G-150, DEAE-Sephadex A-50, disposable Sephadex G-25 M (PD-10) columns, Coomassie Blue R250 and Ultrodex granulated gel were from Pharmacia/LKB, Sweden. Iodogen from Pierce Chemical Co., U.S.A. Human apotransferrin from Behring Diagnostics, FRG. Phosphate-buffered saline, pH 7.45 from Oxoid, U.K. Insulin (Velosulin) from Nordisk, Denmark. Ammonium ferric citrate from BDH, U.K. Glycopeptidase F, DNase I (grade II) from Boehringer-Mannheim, FRG.
HEPES and all other chemicals from Merck, FRG. $Na^{125}I$ from Radiochemical Centre Amersham, U.K.

2.1.2. Media and Sera.

Calcium- and Magnesium-free solution of Earle's balanced salts (CMFS), fungizone and gentamicin were from Gibco, U.K. Fetal Calf Serum, L-glutamin, Medium 199, and Dulbecco's modification of Eagle's medium (with 20 mM HEPES) (DMEM-H) were from Flow Labs., U.K.

2.2. Methods.

2.2.1. Cells.

2.2.1.1. Isolation of cells from human term placentas.

Normal term human placentas were obtained within half an hour of spontaneous vaginal delivery (Courtesy: Delivery Room, Department of Obstetrics, Academic Hospital Rotterdam/Dijkzigt, Rotterdam). A total of 30-35 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. Incubation was at 37°C in a shaking water-bath. The enzyme solution contained 1250 BAEE units trypsin (if not indicated otherwise 1:250, t.c.) and 475 Kunitz units DNase I, grade II per millilitre CMFS.

In all experiments described in chapter 5, 1 mM $CaCl_2$ and 0.8 mM $MgSO_4$ were added to the enzyme solution. These additions lead to improved cell yields and higher viability in vitro (1).
At the end of each trypsinization 6 fractions of 13.5 ml supernatant were each layered over 1.5 ml fetal calf serum (FCS).
Pellets were spun down at 1000 g for 5 min. They were resuspended in 1 ml DMEM-H 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 M 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. These fractions were washed once, using DMEM-H and checked under the microscope. Mononuclear cell fractions, virtually free of tissue fragments and cellular débris were used either directly or for cell culture.

2.2.1.2. Cell culture.

Cells were counted using a Burker counting chamber. They were diluted and plated out at a density of 6.10^5 cells/ml (unless indicated otherwise) in 35 x 10 mm Petri-dishes (Falcon, Becton Dickinson, USA). For microscopy purposes thin glass coverslips were inserted in the culture dishes.
Standard culture medium was M 199, supplemented with 4 mM L-glutamin, 20 per cent FCS, 50 µg/ml gentamicin and 2.5 µg/ml fungizone. All cultures were incubated in humified 5 per cent CO_2/95 per cent air at 37°C for designated time periods. After 24 h non-adherent cells were gently washed off and the medium was replaced. Further additions to the media as indicated in the descriptions of the various experiments.

2.2.2. Immunology.

2.2.2.1. Immunofluorescence.

2.2.2.1.1. Marker analysis.

Mononuclear cells were incubated with monoclonal antibodies. Detailed information about the monoclonal antibodies used is summarized in table

2.1.

Trophoblast-specific monoclonal antibodies ED 235 and ED 341 were used on cytospin preparations of freshly isolated cells and coverslip-grown cultures, fixed according to Contractor and Sooranna (2).

For ED 235, fixation was 5 min at room temperature in methanol: acetone (1:1 v/v) and for ED 341 20 min at 4°C with 2 per cent (w/v) paraformaldehyde in phosphate-buffered saline (PBS). Preparations were washed in PBS and appropriate dilutions of hybridoma culture supernatants were applied.

Contractor and Sooranna have shown that ED 341 reacts with both cyto- and syncytiotrophoblast whereas ED 235 recognizes cytotrophoblast (2).

For immunological cell surface marker analysis of cultured adherent cells non-fixed slides were incubated with indicated antibodies.

Table 2.1

Antibody	CD	Antigen recognized	Source
B1	CD 20	B cell antigen	Coulter Clone, USA
Leu-1	CD 5	T_1 antigen	Becton Dickinson, USA
Leu-4	CD 3	T_3 antigen	Becton Dickinson, USA
My4	CD 14	monocytic antigen	Coulter Clone, USA
VIM-D5	CD 15	myeloid antigen	Dr. W. Knapp, Vienna, Austria
HLe-1	CD 45	"leucocyte common antigen"	Becton Dickinson, USA
anti-HLA-DR	-	HLA-DR, non polymorphic antigen	Becton Dickinson, USA
661G10	CD 71	Transferrin receptor (T_9-antigen)	Dr. M. van de Rijn, A'dam, The Netherlands
ED 235	-	Trophoblast-specific (ref. 2)	Dr. S.F. Contractor, London, United Kingdom
ED 341	-	Trophoblast-specific (ref. 2)	Dr. S.F. Contractor, London, United Kingdom

MONOCLONAL ANTIBODIES

As a second-step reagent we used a fluorescein isothiocyanate-(FITC-) conjugated goat anti-mouse immunoglobulin antiserum (Central Laboratory

of the Blood Transfusion Service, Amsterdam, The Netherlands). The fluorescence stainings were evaluated using Zeiss (Carl Zeiss, FRG) and Leitz (Ernst Leitz, FRG) microscopes. The microscopes were equipped with phase-contrast facilities.

2.2.2.1.2. BrdU incubations, direct immunofluorescence.

These experiments were done with cells which had been cultured for approximately 40 h. Coverslips were washed twice and medium containing 10 μM bromodeoxyuridine (BrdU) was applied. After 2 h in the incubator coverslips were washed twice. For the visualization of BrdU incorporation coverslips were fixed in 70 per cent ethanol for 30 min. After air drying, the coverslips were immersed in 0.07 M NaOH for 2 min followed by immersion in 0.1 M Na_2 B_4 O_7, pH 8.5. Subsequently they were incubated with a FITC-conjugated mouse-anti-BrdU antibody (Becton Dickinson, USA). The coverslips were washed twice before being mounted for microscopic examination.

2.2.2.2. Immunoperoxidase.

All reagents were from DAKO Corporation (Santa Barbara, USA). Immunoperoxidase stainings were done on paraffin sections of term villous tissue, methanol-fixed smears of Percoll-purified mononuclear cell populations and fixed cells cultured on glass coverslips. Fixation was for 20 min in methanol with 20 per cent (v/v) hydrogen peroxide (3 per cent) at room temperature. Paraffin was removed from the sections before staining by placing them for 30 min at 56°C before transferring them to a toluene bath for 3 min. Then slides were placed in absolute methanol for 3 min and rehydrated. Sections were then covered for 5 min with 3 per cent hydrogen peroxide to remove any remaining endogenous peroxidase activity.

All incubations were followed by rinsing and washing in 0.05 M Tris, pH 7.6, for 30 min. Specimens were first covered with normal swine serum to suppress non-specific binding of immunoglobulins. After 20 min excess serum was tapped off and primary (rabbit-raised) antibodies against hPL,hCG and SP-1 were applied. In negative controls this was replaced by normal swine serum. After 20 min specimens were washed and incubated with swine anti-rabbit immunoglobulins for 20 min. The third step consisted of peroxidase/antiperoxidase (PAP). This was followed by

a 40 min incubation with a freshly prepared substrate solution of amino-ethylcarbazole in 0.1 M acetate buffer, pH 5.2, containing hydrogen peroxide. Specimens were rinsed with distilled water and covered with Mayer's haematoxylin for 8 min. After being washed and dipped in 2 per cent (v/v) NH_4OH, they were mounted, using glycergel mounting medium.

2.2.2.3. FACScan analysis.

Mononuclear cells were incubated with relevant monoclonal antibodies. For double marker analysis two monoclonal antibodies were used which differed in immunoglobulin subclass composition, e.g. an antibody of the IgG 1 subclass together with an antibody of the IgG 2 subclass.
Cells were first labelled with a monoclonal antibody which was visualized using a phycoerythrin conjugated goat anti-mouse immunoglobulin antiserum (Caltag Laboratories, USA) as a second step reagent. After blocking the free binding sites of the second step antiserum by use of normal mouse serum the cells were incubated with another monoclonal antibody. Subsequently, a FITC conjugated goat anti-mouse immunoglobulin subclass specific antiserum (Nordic Immunological Laboratories, The Netherlands) was added which recognized the last-added monoclonal antibody only. Cells were analyzed using a FACScan (Becton Dickinson, USA).
Ninethousand events were acquired and the results were depicted in a dot plot in which the x-axis represents the log fluorescence intensity for FITC and the y-axis represents the log fluorescence intensity for phycoerythrin. As a negative control cells were used which were labelled with normal mouse serum and the second step reagents respectively. Debris was excluded at analysis by drawing a gate in a dot plot which represents forward scatter and side scatter of the cells acquired.

2.2.3. Electron microscopy.

Mononuclear cells were fixed in suspension in 1.25 per cent glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.3 for 1 h at room temperature. Fixed cells were gently spun down at 400 g for 2 min. Fixative was removed and 1 ml of fresh human serum poured on top of the pellet. Cells were resuspended, then spun down at 1500 g for 2 min, the serum removed, and the pellet re-fixed for 2 h in 2.5 per cent gluta-

raldehyde in 0.1 M sodium cacodylate pH 7.3 to solidify the serum which subsequently held the cells together. Cultured cells were fixed in situ, before scraping them off the substrate with a rubber policeman and refixing them according to the above protocol.
After washing three times during 24 h in 0.1 M cacodylate buffer pH 7.3 containing 3 mM calcium chloride, the pellets were cubed and post-fixed in 1 per cent osmium tetroxide at 4°C for 1 h, rinsed in buffer, then dehydrated in a graded ethanol series followed by propylene oxide. Specimens were infiltrated with TAAB epoxy resin (Taab Laboratories Equipment, U.K.) and polymerised at 60°C for 72 h. Ultrathin sections were cut on a Reichert OMU III ultramicrotome and mounted on copper grids, double stained with uranyl acetate and lead citrate, and examined in a Philips 301 electron microscope at an accelerating voltage of 60 kV.

2.2.4. ^{125}I-transferrin binding and uptake.

2.2.4.1. Labelling procedure.

Prior to iodination human apotransferrin was fully saturated using iron (III) citrate and a tenfold molar excess of bicarbonate as the synergistic anion. 600 µg of diferric transferrin was reacted for 10 min at room temperature with 0.5 mCi ^{125}I in a glass vial coated with 100 µg Iodogen. Free ^{125}I was separated from the radiolabelled transferrin by passing the mixture down a disposable Sephadex G-25 M column, followed by extensive dialysis against PBS.

2.2.4.2. Binding assay.

Culture dishes, with cells grown on the plastic surface, were washed twice with serumfree DMEM-H prior to radiolabel incubations. Cells were incubated with ^{125}I-diferric transferrin at indicated concentrations at 4°C (i.e. on melting ice) during indicated periods of time. Incubation was in 1.00 or 1.25 ml of DMEM-H 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 (CK 2, Olympus, Japan). If necessary, cells were scraped from the dish with a rubber policeman.
In all incubations aspecific 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.

2.2.4.3. 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 radio-activity and to check whether prolonged acid wash did not release cellular protein from the dish.

2.2.4.4. DNA determination.

DNA was determined using a mithramycin binding assay (Sanbio, The Netherlands).

2.2.4.5. Protein determination.

Protein was determined according to Bradford (3) with bovine serum albumin as a standard.

2.2.5. hCG determination.

hCG, secreted in the culture medium, was determined by enzyme-immunoassay on a ES 600 auto-analyzer (Boehringer Mannheim, FRG) at the Laboratory for Clinical Chemistry, Academic Hospital Rotterdam/Dijkzigt.

2.2.6. Guinea pig-transferrin.

2.2.6.1. Sera and anti-sera.

Guinea-pig blood was obtained by heart puncture, under ether anaesthesia. Non-heparinized blood was centrifuged for 15 min at 1200 g. To obtain consecutive serum samples during pregnancy, guinea-pigs were purchased shortly after mating and heart punctures were performed regularly. Sera were stored at -20°C. Anti-guinea-pig-transferrin and anti-total-guinea-pig sera were raised in rabbits.

2.2.6.2. Isolation and purification of guinea-pig transferrin.

Guinea-pig serum was fully saturated with iron (III) citrate before ammoniumsulphate was added, up to 45 per cent saturation. After being kept overnight at 4°C, it was centrifuged for 20 min at 4000 g. The pellet was discarded and further ammoniumsulphate was added to the supernatant, up to 75 per cent saturation. The resultant was kept at 4°C for 24 h. The pellet, obtained after 20 min centrifugation at 4000 g, was dissolved in a small volume of distilled water and dialysed against distilled water for three times 24 h in the cold room.
This was followed by Sephadex G-150 chromatography (Elution buffer: 0.5 M NaCl, 0.1 M Tris, pH 8.1). Transferrin-containing fractions were dialysed against 0.05 M NaCl, 0.01 M Tris, pH 8.1 and brought on a DEAE-Sephadex A-50 column. (Elution buffer: 0.05-0.5 M NaCl in 0.01 M Tris, pH 8.1). Ion-exchange chromatography was then repeated.

2.2.6.3. Guinea-pig transferrin microheterogeneity.

2.2.6.3.1. Preparative isoelectric focusing.

Purified guinea-pig transferrin was dialysed against 0.01 M $NaHCO_3$ (pH 8.0), concentrated and saturated with iron (III) prior to focusing.
Preparative isoelectric focusing was carried out in Ultrodex granulated gel with Ampholine pH 4-6. Application Note 198 (LKB) was followed, with some modifications, as described by Van Eijk et al. (4).
Focusing conditions were: prefocusing during one hour at 10°C. Focusing at 25 mA, 1200 V and 30 W. This procedure was repeated once. Transferrin thus purified was homogeneous by immunodiffusion and SDS-PAGE (2.3.9).

2.2.6.3.2. Crossed immunoelectrofusing.

Both purified guinea-pig transferrin fractions and guinea-pig sera were characterized using crossed immunoelectrofocusing. To ensure complete iron saturation of transferrin, 100 μl serum was mixed with 5 μl 0.5 M $NaHCO_3$ and 3 μl 10 μM iron (III) citrate and then left at room temperature for 1 h. We employed the same procedures as described by De Jong and Van Eijk (5), using rabbit raised anti-guinea-pig-transferrin antiserum. Peak surface areas were calculated, multiplying the height of the peak and the width at medium height. Fast/slow ratio's represent

the proportion of respective peak surface areas.

2.2.6.4. Serum transferrin concentration determination.

Serum transferrin concentration was determined by single radial immunodiffusion according to Mancini (6), using rabbit raised anti-guinea-pig transferrin serum. Purified guinea-pig transferrin was used to make a calibration curve. Immunoprecipitates were coloured with 0.5 per cent Coomassie Blue R-250 in 50 per cent methanol, 7.5 per cent acetic acid.

2.2.6.5. Protein determination.

Protein determinations were done using E_{280}, E_{280} diferric transferrin (1 mg/ml) = 1.30, and according to Markwell (7), using bovine serum albumin as a standard.

2.2.6.6. Sialic acid determination.

Sialic acid was determined according to Horgan (8). Hydrolysis conditions were: 3 h at 70°C in 0.01 M H_2SO_4 (9).

2.3.7. Amino acid analyses.

Amino acid analyses were done as described by Van Eijk et al. (10), using a 4151 LKB Alpha plus amino-acid analyser (LKB, Sweden). All determinations were done in quadruplicate.

2.3.8. Carbohydrate analyses.

Carbohydrate analyses were done according to Van Eijk et al. (10, 11). All determinations were done in triplicate.

2.3.9. SDS-Polyacrylamide gel electrophoresis.

Molecular masses of native and deglycosylated slow and fast guinea-pig transferrin were estimated using sodium dodecylsulphate-polyacrylamide gelelectrophoresis (SDS-PAGE) according to Laemmli (12). A 5 per cent stacking gel, 12 per cent separating gel was used. Calibration proteins included albumin, catalase and lactate dehydrogenase. Deglycosylation was done using glycopeptidase F. Incubation buffer was 0.25 M sodium-phosphate, 10 mM EDTA, pH 8.6. Incubation for 18 h at 37°C. 0.05-2 U glycopeptidase F per 10 μg transferrin were used.

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Chapter 3 Some characteristics of purified human term cytotrophoblasts.

3.1. Introduction.

The presence of a fetus is not a prerequisite for placental iron uptake. This can be concluded from fetectomy experiments in animals (1,2). Thus, placental iron uptake can be studied under in vitro conditions.

Transferrin receptors are thought to be ubiquitous on proliferating cells (3). With regard to human trophoblast, it has been shown, using a monoclonal antibody against the human TfR on placental sections, that the TfR is present on syncytiotrophoblast, but not on cytotrophoblast (4).

Therefore, in order to study placental transferrin binding and iron uptake it is essential that homogeneous, well defined trophoblast cell populations can be prepared.

Hall et al. (5) introduced a trypsin/DNase enzyme-combination to isolate a mixture of mono- and multinuclear cells from human placentas. Comparing several enzyme combinations Morrish and Siy (6) concluded that a combination of trypsin and DNase provides highest cell viability and hormone production. Results were critically dependent on the trypsin lot, whereas parameters like medium type and various substrates had no influence. Using the same enzyme combination Kliman and co-workers (7) isolated mononuclear cell populations by adding a Percoll-gradient separation. Thus isolated mononuclear cells did not contain immunoreactivity for syncytiotrophoblast-products like SP-1, hCG or hPL. The presence of fibroblasts, endothelium and macrophages was excluded by negative reactions against vimentin and α_1-antichymotrypsin respectively. In vitro the mononuclear cells tend to aggregate and fuse and start producing SP-1, hCG and hPL. This strongly suggests that the mononuclear cells are cytotrophoblasts. This chapter describes a number of aspects of this in vitro model that have not been studied before. Several of these aspects are essential for experiments described in subsequent chapters.

3.2. Results.

3.2.1. General description.

Percoll gradient separation of the pellets from three consecutive trypsinizations leads to three distinct layers in the gradients: a bottom layer, consisting primarily of erythrocytes, a top layer, consisting of cellular debris and tissue fragments and a middle layer, sometimes composed of several closely related bands.

This middle layer consists of mononuclear cells with an average diameter of 10 μm.

The density of this Percoll-area, as determined by parallel-run gradients loaded with density marker beads is 1.048 to 1.062 g/ml. Normal cell yields, from 35 grams of villous tissue are $3\text{-}4x10^7$ cells, using calcium and magnesium supplemented enzyme solutions. After 24 h approximately 70 per cent of adherent cells were mononuclear, round, or with villus-like cytoplasmic protrusions, 20 per cent were bi-nucleated. Approximately 10 per cent of all cells contained 3,4 or 5 nuclei. Using phase-contrast microscopy it is practically impossible to judge whether cells are adjacent or truly syncytial. Immunoperoxidase-processed coverslips and especially electron-microscopy are somewhat clearer in this respect.

During prolonged culture periods the percentage of mononuclear cells slowly decreased (60 per cent at 40 h, 20 per cent at 72 h) and the relative amount of cellular aggregates and syncytia increased. After 72 h in culture 25 to 35 per cent of cells contained more than ten nuclei, sometimes up to 50.

3.2.2. Leucocytes.

Results of immunological marker analysis on cells isolated using either trypsin type III or trypsin 1:250 are presented in table 2.1. In 7 experiments cover-slip grown cells, isolated using trypsin 1:250, were tested for the presence of CD 14 positive cells. The percentage of positive cells ranged from 0-9 per cent (mean: 4, S.D.: 4.3).

Table 3.1

Experiment no.	1	2	3	4	5	6
Trypsin used to isolate cells (type III or 1:250)	III	III	III	1:250	1:250	1:250
CD*	Percentage of positive cells					
CD 20	1	0	0	0	1	0
CD 5	1	ND	ND	0	0	ND
CD 3	2	0.5	0	0	0	0
CD 14	40	16	17	2	4	1
CD 15	2	0	0	0	0	0
CD 45	ND	12	16	9	4	2
anti-HLA-DR	39	12	16	9	2	1

ND: not determined

*Cluster of differentiation as proposed by the Workshops on Human Leucocyte Differentiation Antigens.
(Paris, France, 1982; Boston, U.S.A., 1984; Oxford, U.K. 1986)
IMMUNOLOGICAL MARKER ANALYSIS ON MONONUCLEAR CELLS FROM PERCOLL GRADIENT CENTRIFUGATION.

3.2.3. Trophoblast antigens: ED 235 and ED 341.

Using cytospin preparations of freshly isolated cells, we found that 96 per cent of these cells reacted with trophoblast-specific monoclonal antibody ED 341. The antigen seemed to be located in the cytoplasm. 3.5 per cent of cells reacted with ED 235, which probably recognizes a membrane antigen and is also trophoblast specific. Coverslips with cultured cells showed enhanced reactivity with ED 235, compared to freshly isolated cells, the incidence of binding increasing from 3.5 to 22 per cent after 24 h, rising to 62 per cent after 40 h in culture. Reactivity with ED 341 decreased: 6 per cent of cells was positive after 24 h, diminishing further to 2 percent after 40 h.

3.2.4. Immunoperoxidase detection of hCG, hPL and SP-1.

Immunoperoxidase staining on fixed smears of Percoll-purified mononuclear cells was negative for all three antigens examined (SP-1, hCG and hPL). Staining for hCG, hPL and SP-1 on paraffin sections of term villi gave a pronounced red colour of similar intensity for all three antigens, strictly limited to syncytiotrophoblast. A negative control, replacing the first antibody with normal swine serum was always entirely negative. On coverslips grown cells, methanol-fixed, the following results were obtained. For SP-1, after 24 h, the red colour was strongly positive. Both mono- and multinucleated cells were positive. The reaction occurred mainly in the perinuclear region, and only slightly in cytoplasmic protrusions. Occasionally the colour reaction in mononuclear cells was only very slightly positive. After 40 h the colour was even more intense, and more pronouncedly perinuclear. At this time no negative-staining cells were seen on the coverslips studied. Expanding syncytial structures coloured less intensely. In some cases mononucleated cells apparently in the process of fusion with syncytial structures can be seen. These mononuclear cells react extraordinarily strong. After three days in culture, very large syncytia were seen with a reaction which varied within syncytia. The strongest reaction was again perinuclear. Mononuclear cells appear to react less intensely at this time. hCG peroxidase staining on coverslips resulted in a markedly less intense colour reaction, with a stronger progressive time course. Mainly binucleated cells and multinucleated syncytia gave a positive reaction. The same applies to apparently-fusing mononuclear cells. This colour intensified over the first 40 h. After 72 h expanding syncytia reacted less strongly. At this time point, mononuclear individual cells reacted only weakly.
The peroxidase reaction for hPL was less strong than that for hCG. Ater 24 h most cells reacted slightly positively. A stronger reaction was seen from small groups with two or three nuclei. Few mononuclear cells were entirely negative. After 72 h the intensity of the colour reaction was not markedly increased for this antigen. Syncytial structures were apparently homogeneous weakly positive.

3.2.5. Electron microscopy.

Figure 3.1. shows an example of a freshly isolated cell with the

typical ultrastructural features of cytotrophoblast. As in intact placental tissue, the nucleus contains dispersed chromatin and the cytoplasm is generally rather pale, with occasional profiles of rough endoplasmic reticulum and dispersed, small, rather electron-dense mitochondria. Occasional slender microvilli are present, similar to those found on the basal aspect of these cells in intact placental tissue. The nucleus contains dispersed chromatin and the cytoplasm is generally rather pale, with occasional profiles of rough endoplasmic reticulum and dispersed, small, rather electron-dense mitochondria. Occasional slender microvilli are present, similar to those found on the basal aspect of these cells in intact placenta. A nematosome is present on the plasma membrane and is considered to be specific to the cytotrophoblast cell (8). The nematosome is shown on an inset. Vacuoles of various shapes and sizes, some with amorphous or vesicular contents, can be seen throughout the cytoplasm. A majority of cells (up to 70 per cent) may represent the "intermediate" type of cell, which has a more convoluted nucleus with aggregated chromatin and a more electron-dense cytoplasm. Golgi bodies were often found in these cells, some with associated dilated vacuoles, but secretory droplets were rarely seen. A fine network of cytoplasmic filaments was apparent in most cells underneath the surface plasma membrane, probably associated with microvillous function. Occasional mitotic figures were seen.

Figure 3.1
Ultrastructural appearance of a mononuclear cell, from Percoll-gradient separation (density 1.048-1.060 g/ml) as described. This cell shows many cytotrophoblast-characteristics, as described in the results-section. x 13,650. The inset shows an enlargement of a nematosoma. x 36,400.

Figure 3.2
Electronmicroscopy of cultured cells. Culture period: 24 hours. Apparent syncytium, since no intercellular membranes are discernible. Some microvilli are visible. Further characteristics are described in the text. x 16,500.

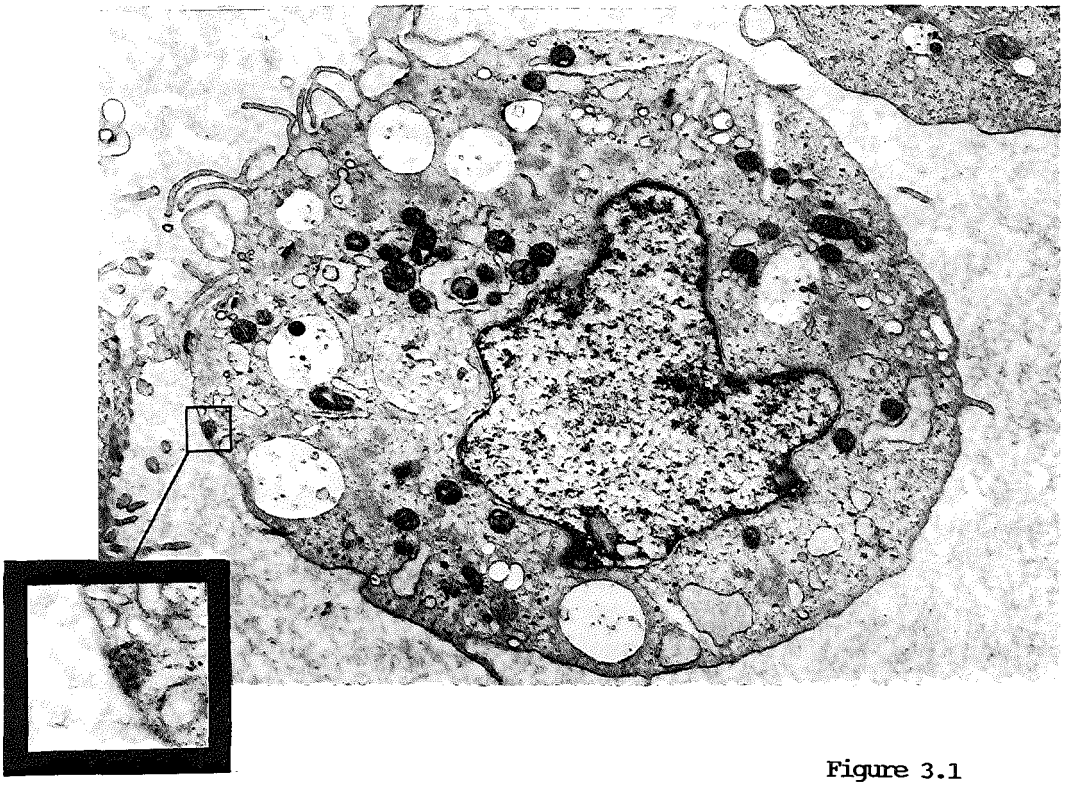

Figure 3.1

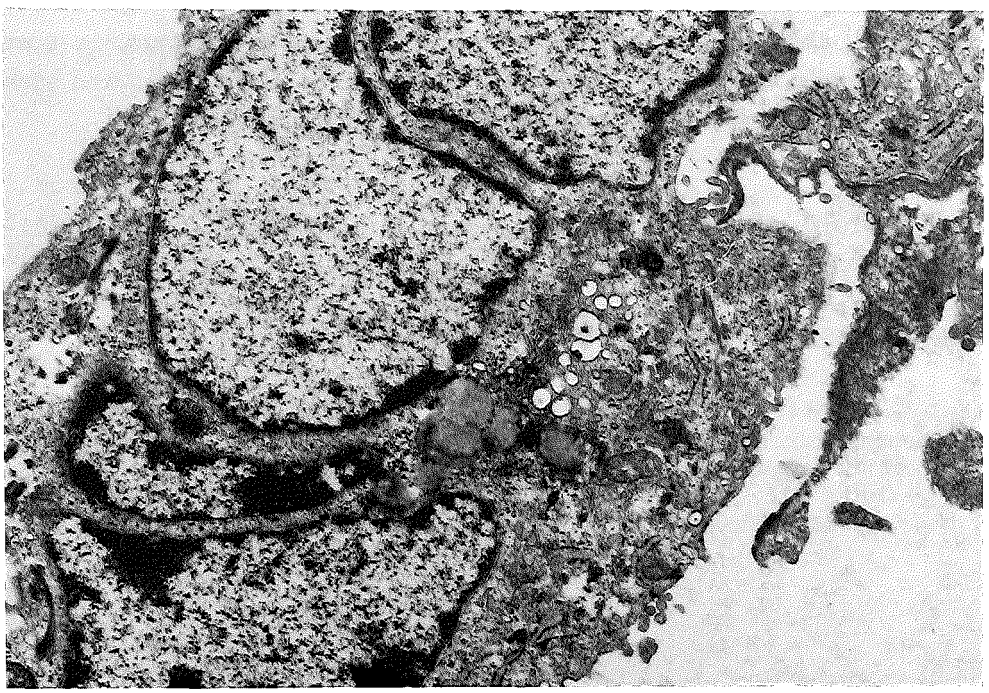

Figure 3.2

Electron microscopy of cultured specimens showed many cell clusters with incomplete fusion, as seen by the presence of intercellular membranes, often with desmosomal junctions. In many cases, cells with different inherent electron densities were associated so that delineation of the various components was quite striking. However, occasional multinucleate structures were found, suggestive of cell fusion and true syncytial formation. The nuclei were often somewhat convoluted in shape, with areas of heterochromatin subjacent to the nuclear membrane. In favourable sections, such cell masses appeared to be domed, with short, slender microvilli on the upper surface and a fairly smooth basal plasma membrane with only occasional, blunt processes. Narrow profiles of rough endoplasmic reticulum were present, and numerous clusters of free ribosomes gave the cytoplasm an electron-dense appearance. Mitochondria were scattered throughout, and occasional secondary lysosomes and clear vacuoles were present. Golgi cisternae and occasional vacuoles were found, although secretory droplets were rare; occasional fat globules were, however, evident. Cytoplasmic filaments were sometimes well developed, forming dense masses dispersed throughout the cytoplasm. An example is shown in figure 3.2.

3.2.6. Proliferation.

Incubation for 2 h with BrdU of cells cultured for 40 h revealed that less than 2 per cent of these cells were active in DNA synthesis.

3.3. Discussion.

Percoll-gradient separation of trypsinized human term placental villi results in mononuclear cell populations in the gradient-area with a density of 1.048-1.062 g/ml. These mononuclear cells are mainly cytotrophoblasts, with a varying amount of monocytes. The use of trypsin 1:250 results in low levels of monocyte-contamination, compared to trypsin type III.

Main et al. described that the level of contamination with monocytes also depends on the area in the gradient: 1.2-8.2 per cent in the area with a density of 1.048-1.055 g/ml, 13-23 per cent in fractions with densities from 1.055-1.075 g/ml (9). These authors used cell surface markers to study the presence of monocytes and lymphocytes. They

provided evidence that the use of this trypsinization protocol has no proteolytic effect on the membrane antigens studied.

Kliman et al., Kao et al. and Feinman et al. used the presence of the intracellular antigen α_1-antichymotrypsin to study the presence of macrophages (7,10,11).

This antigen is present in placental tissue macrophages (Hofbauer cells) (12), but not in monocyte-derived macrophages. Macrophages present in vitro can be both monocyte-derived (13) and Hofbauer cells. Using α_1-antichymotrypsin as a macrophage marker, less than 5 per cent of cells was positive (10,11). It has been shown that no endothelium or fibroblasts are present in these mononuclear cells (7).

The majority of mononuclear cells are cytotrophoblasts. This is demonstrated by a number of features. First their reactivity with trophoblast specific monoclonal antibody ED 341 and secondly by their in vitro behaviour. Mononuclear cells contain neither SP1, hCG or hPL. In vitro they rapidly start synthesizing these syncytiotrophoblast-products. SP-1 is rapidly and strongly expressed, hCG follows somewhat later and hPL is only weakly positive. This is in accordance with Kliman's findings (7). A remarkable change in reactivity with trophoblast-specific monoclonal antibodies ED 235 and ED 341 occurs in vitro. Reactivity with ED 341 decreases from 96 per cent on freshly isolated cells to 2 per cent on cells cultured for 40 h.

In contrast to this, reactivity with ED 235 increases from 3.5 per cent initially to 62 per cent after 40 hours. This could mean that the membrane antigen recognized by ED 235 is susceptible to our trypsinization procedure and is slowly synthesized de novo in vitro, even though this antigen has been shown on sections to be predominantly present on first-trimester cytotrophoblast (14).

In vitro cells aggregate and fuse. Fusion was elegantly demonstrated using both time lapse cinematography and microinjection of fluorescently labeled α-actinin, which diffused completely throughout syncytial cytoplasm within 30 minutes (7). We used electron microscopy to demonstrate fusion. In the course of the first two to three days in culture intercellular membranes disappear, demonstrating syncytiumformation. The pelleting procedure we employ permits cells to be examined in a variety of planes. Better assessement of syncytial formation may be possible if cells are processed in situ, permitting

sectioning to be strictly "en face". However, this presents various technical difficulties. This explanation might account for some of the incomplete fusion we observed in cell clusters.

It is generally recognised that the saccharides of cell surface play a vital role in cellular interactions and behaviour (15,16). We cooperated in a study of these cell surface carbohydrates, both on freshly isolated and cultured cytotrophoblasts. The results (17) are of limited interest to the subject described in this thesis and are therefore not discussed here.

Lack of bromo-deoxyuridine uptake shows that these cells are not active in DNA synthesis in vitro. This is in acccordance with the non-proliferative syncytiotrophoblast in vivo (18). This probably contributes to the limited life-span of our cultures, up to one week in vitro. Kliman and co-workers performed DNA content analysis of freshly isolated cells, 15 per cent of cells was actively dividing or ready to divide (7). This matches the proliferative nature of cytotrophoblast in situ (18).

We conclude that this cell culture model is suited for the study of placental iron uptake. Contamination with monocytes and monocyte-derived macrophages is low, using trypsin 1:250. This is important since macrophages have distinct characteristics in cellular iron metabolism (19). In vitro, mononuclear cells, cellular aggregates as well as syncytia acquire biochemical characteristics of syncytiotrophoblast. Thus homogeneous cell populations, expressing syncytiotrophoblast characteristics are obtained. The fact that syncytium-formation is not a prerequisite for biochemical differentiation is especially apparent in strictly serum-free cultures, where no morphological differentiation occurs, cells remaining individual and spherical, but biochemical differentiation develops nonetheless (10).

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Chapter 4 Transferrin receptor expression in human term trophoblast cells.

4.1. Introduction.

Placental iron uptake starts with the binding of iron-loaded maternal transferrin to its receptor on the apical side of the syncytiotrophoblast (1). The presence of TfR's at this microvillous apical membrane has been shown using immunofluorescence microscopy on cryostat sections (2-4). Ultrastructural studies showed transferrin (5,6) and its receptor (6), as did binding studies with ^{125}I-labelled transferrin on syncytiotrophoblast microvillous membrane vesicles (7,8). Placental TfR has also been isolated from placental homogenate (9) and microvillous membrane preparations (7, 10-12).

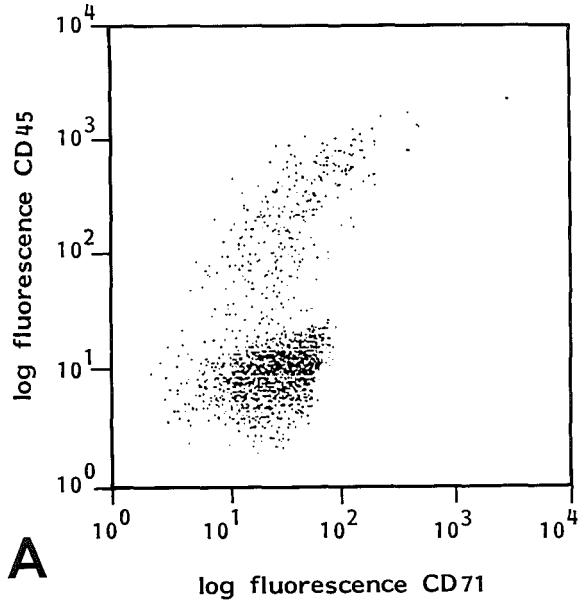
TfR's are also present in the basolateral membrane of the syncytiotrophoblast (14). In vivo, syncytiotrophoblast is formed through the fusion of underlying cytotrophoblast which is thought to lack TfR's (15,16). This lack of cytotrophoblastic TfR's is remarkable since cytotrophoblast is the proliferative cell type in human placenta, even at term. Generally, the presence of TfR's is associated with cellular proliferation and activation (17). This chapter discusses characteristics of TfR expression in freshly isolated and cultured human term cytotrophoblasts.

4.2. Results.

4.2.1. Level of TfR expression in freshly isolated cytotrophoblasts.

Immunological marker analysis reveals that the level of monocytes in Percoll-purified mononuclear cell suspensions is higher when trypsin type III is used to isolate cells, compared to trypsin 1:250 (chapter 3).

Figure 4.1
FACScan analysis of mononuclear cell suspensions from Percoll-gradient separation (density 1.048 - 1.062 g/ml).
On the x-axis log fluorescence for transferrin receptor (CD 71) is depicted, the y-axis represents log fluorescence for leucocyte common antigen (CD 45). Each dot represents one cell, tested for both antigens.
A : Cells prepared using trypsin 1:250.
B : Cells prepared using trypsin III.


log fluorescence CD45
10^4
10^3
10^2
10^1
10^0
10^0 10^1 10^2 10^3 10^4
log fluorescence CD71
A

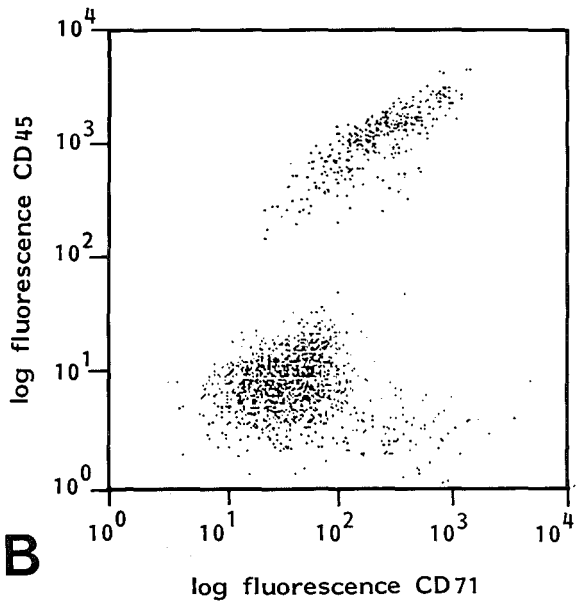

log fluorescence CD45
10^4
10^3
10^2
10^1
10^0
10^0 10^1 10^2 10^3 10^4
log fluorescence CD71
B

Figure 4.1

Table 4.1 shows that, using trypsin III, the amount of cells staining positive for a monocyte marker correlates highly with the percentage of cells containing TfR's in their surface membrane.
Fluorescence activated cell sorting shows a similar phenomenon. Fig. 4.1 illustrates that, using either trypsin III or trypsin 1:250 mononuclear cell populations consist of two main subpopulations. One population expresses Leucocyte Common Antigen (LCA) and transferrin receptors. The other population is LCA-negative and expresses TfR's at a lower level than LCA-positive cells. The distinction between both subpopulations with regard to TfR expression is somewhat clearer in cell suspensions prepared with trypsin III.

Table 4.1

Experiment number	Percentage of positive cells	
	CD 14	Transferrin receptor
1	40	31
2	9	8
3	3	4
4	1	2
5	17	16
6	16	12

IMMUNOLOGICAL MARKER ANALYSIS ON PERCOLL-PURIFIED MONONUCLEAR CELLS. Results from six different experiments, using trypsin type III.

4.2.2. TfR expression in cultured cytotrophoblasts.

4.2.2.1. Indirect immunofluorescence microscopy.

Table 4.2 gives the results of indirect immunofluorescence microscopy on cultured cells, using a monoclonal antibody directed against the human TfR.
Figure 4.2 shows an example of fluorescence microscopy for the TfR on cultured cells.

Table 4.2

Culture period (hours)	Percentage of cells positive for transferrin receptor
19	16
36	55
36	45
40	96
40	82
43	79
60	84

INDIRECT IMMUNOFLUORESCENCE ON CULTURED CELLS. Percentage of transferrin receptor positive cells. Results from two different experiments. Trypsin 1:250 was used to isolate cells.

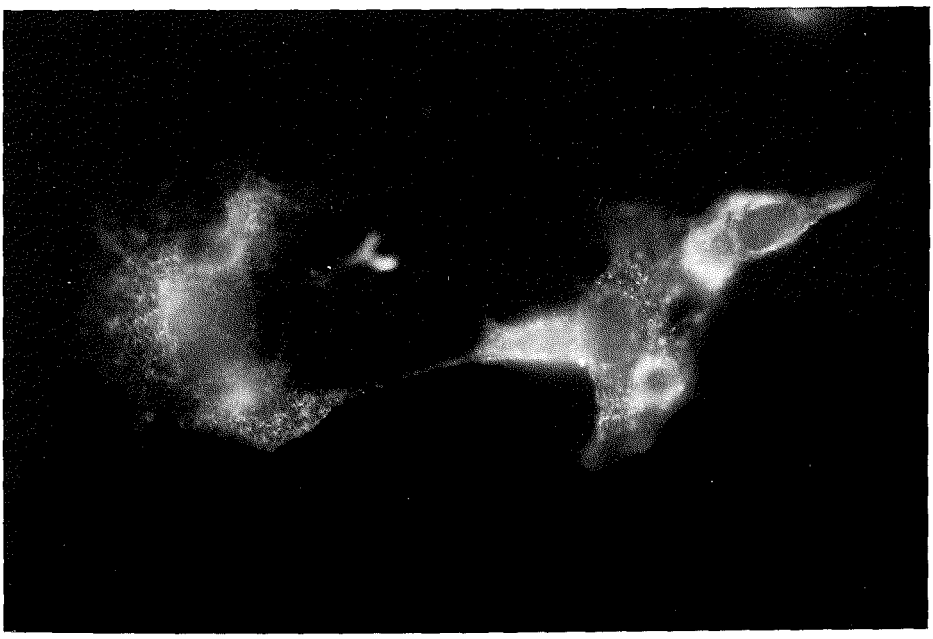


Figure 4.2
Fluorescence microscopy of cells cultured for 40 h, using indirect immunofluorescence to detect transferrin receptors as described. Apparently two groups of cells are making contact and may be fusing. Positive fluorescence can be seen on the whole outer cell membrane. x 630.

It is clear that the appearance of TfR's on cultured cells is time-dependent. Both mono- and multinuclear cells reacted positively. Since it is nearly impossible to distinguish with certainty between these forms, using uncoloured specimens, we have not tried to quantify this. It is noteworthy that no clearly syncytial structures were seen that did not stain positively for transferrin receptor.

4.2.2.2. ^{125}-I labelled transferrin binding and uptake.

Binding assays at 4°C, using ^{125}I-labelled transferrin showed specific, saturable binding. This binding was time and concentration dependent. Up to 90 per cent inhibition of radioligand binding by excess unlabelled transferrin shows specificity, since no inhibition of ^{125}I-transferrin binding can be achieved with excess bovine serum albumin. ^{125}I-transferrin binding was compared between freshly isolated cells and cells cultured for 40 h. Table 4.3 indicates that the number of cell surface transferrin binding sites increases approximately 5-10 fold.

Figure 4.3 shows concentration-dependence of ^{125}I-transferrin binding at 4°C, presented as binding per mg cellular protein. Scatchard analysis of these data from one experiment of a series of three highly comparable experiments indicates an apparent K_D of 1.2-1.9x10^{-9} M of transferrin for its receptor. The number of cell surface binding sites can be estimated to be approximately 1.2-2.0x10^{11} per mg protein. A Scatchard plot is shown in fig. 4.4. These data are for cells which have been cultured for 40 hours in Medium 199, supplemented with 20 per cent fetal calf serum. Prior to the binding assay, cultures were extensively rinsed with serum-free medium.

Specific concentration-dependent binding expressed per μg DNA in similar cultures and Scatchard analysis gives identical K_D-values (1.2-1.9x10^{-9} M). The number of transferrin binding sites is approximately 4.8-6.4x10^{9} per μg DNA. A Scatchard plot is shown in fig. 4.5.

Table 4.3

Experiment number		n	protein per sample (mg)	Specific ^{125}I-transferrin binding (ng/mg protein)
			mean (± SD)	mean (± SD)
A	1	10	0.64 (± 0.09)	6.9 (± 0.7)
	2	10	0.79 (± 0.07)	3.4 (± 0.2)
B	3	18	0.09 (± 0.02)	27 (± 6.4)
	4	2	0.204	62
			0.182	65
	5	2	0.068	81
			0.101	75

INCREASE IN NUMBER OF CELL-SURFACE TRANSFERRIN BINDING SITES IN HUMAN TERM CYTOTROPHOBLASTS IN VITRO. Specific ^{125}I-transferrin binding at 4°C. ^{125}I-Transferrin concentration: 250 ng/ml.
A. Freshly isolated cells, in suspension.
B. Cells cultured for 40 h (adherent).
Results from 5 experiments.

Figure 4.3
Concentration-dependence of ^{125}I-labelled transferrin binding at 4°C. Cells were incubated with different concentrations of ^{125}I-labelled transferrin for 2.5 h at 4°C. The dashed line represents the level of aspecific binding.

Figure 4.4
Scatchard plot of the specific binding data presented in figure 4.3.

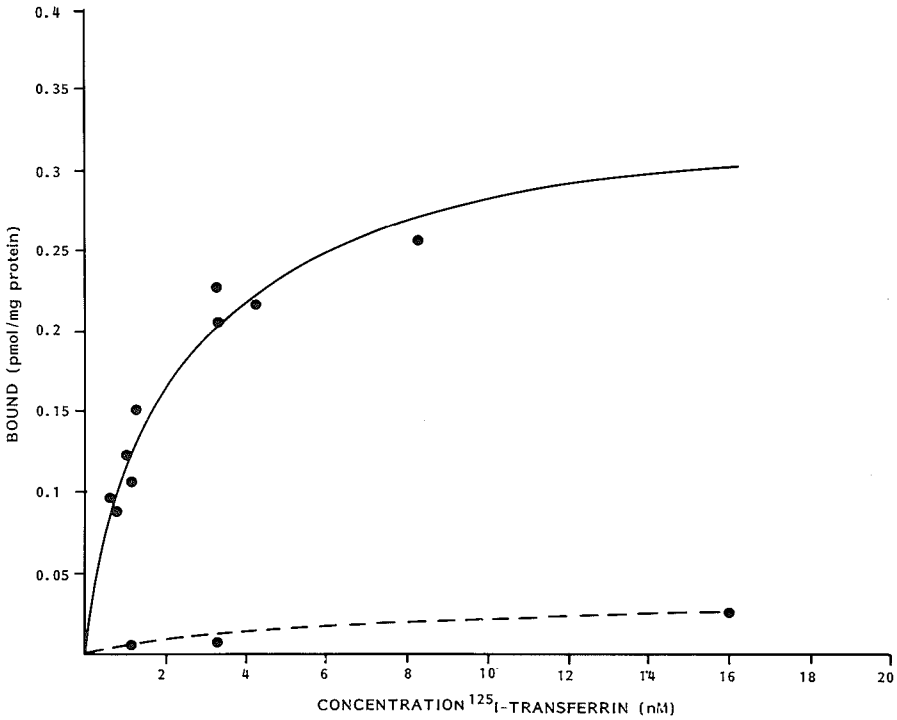


Figure 4.3

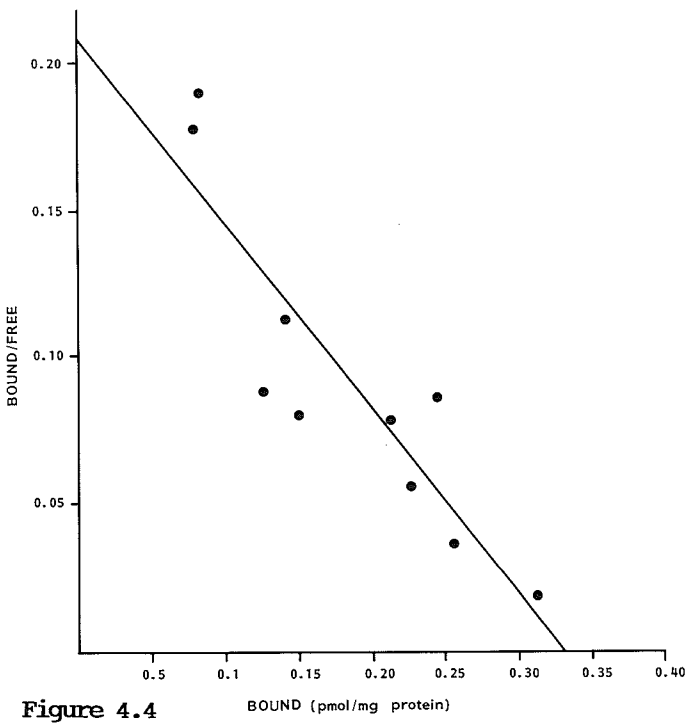


Figure 4.4

The acid wash procedure to distinguish intracellular ^{125}I-transferrin from surface bound ^{125}I-transferrin shows that in cells of both 20 and 40 hours cultures 50 per cent of the bound transferrin at 37°C under steady state conditions is surface bound, 50 per cent is intracellular. Single-cycle receptor mediated endocytosis curves (fig. 4.6) show that at least 30 per cent of initially surface-bound ligand is internalized within 5-10 minutes and is subsequently exocytosed into the medium.

Figure 4.5
Scatchard plot of specific concentration-dependent ^{125}I-transferrin binding, expressed per μg DNA.

Figure 4.6
Single cycle of receptor-mediated endocytosis of ^{125}I-labelled transferrin in cultured human term cytotrophoblasts. Cells were cultured for 40 hours in serum-containing medium. After being washed three times in serum-free medium they were cooled on melting ice and incubated at 4°C with ^{125}I-transferrin (225 ng/ml) for 2 hours. Then they were kept on ice, washed three times with ice-cold medium and incubated at 37°C in pre-warmed M199 containing 125 nM unlabelled diferric transferrin for indicated time periods. Medium was removed, cells were washed once, the wash being added to the medium fraction and the acid-wash procedure was performed. Surface radio-activity is acid-strippable. 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. Intracellular radioactivity is determined as the amount of radioactivity resistant to acid wash. Intracellular and surface values were corrected for the background of acid wash resistant radioactivity at 4°C.

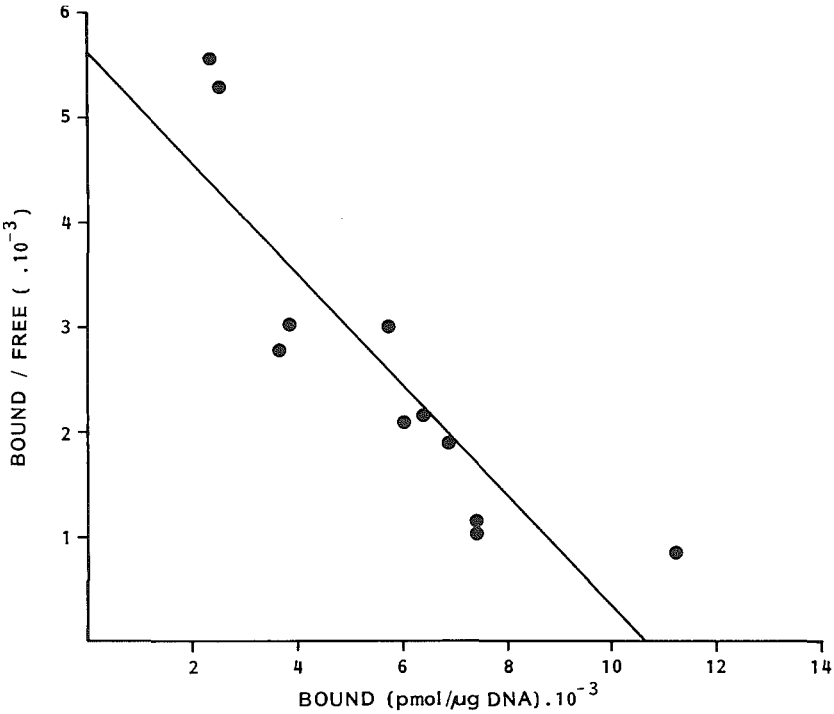


Figure 4.5

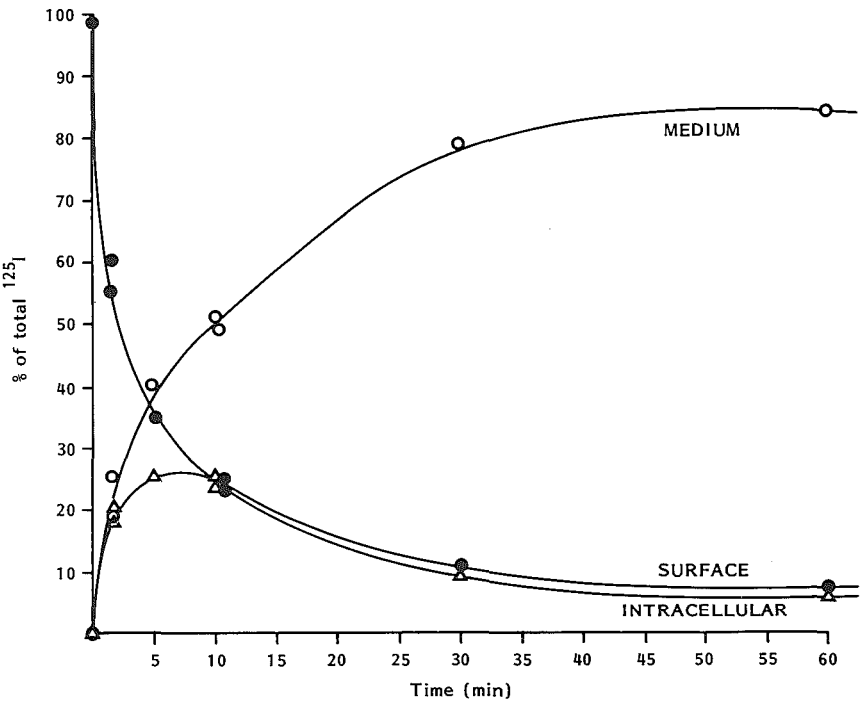


Figure 4.6

4.3. Discussion.

Results described in the previous chapter show that mononuclear cell suspensions prepared from human term placentas consist mainly of two cell types: a varying amount of monocytes and cytotrophoblasts. Both cell types in vivo have low levels of TfR expression. Differentiation leads to enhanced TfR levels in both cell types.

Trypsin, used to isolate cells, damages the TfR. There is however, a large time lag between trypsinization and marker analysis or radioligand incubation. It has been shown that tumour cells need 6-10 h to resynthesize 50 per cent of control levels of TfR's after trypsin treatment (18, 19).

FACScan analysis allows to compare the level of TfR expression by cytotrophoblasts with the TfR expression by LCA-positive cells, since both celltypes were subjected to the same trypsinization. FACScan analysis indicates that the level of TfR expression is lower in cytotrophoblasts than in LCA-positive cells. This difference is especially apparent if trypsin type III is used to isolate cells.

Immunological marker analysis on freshly prepared mononuclear cell suspensions from a number of experiments using trypsin III clearly shows that there is a good correlation between the percentage of cells positive for the monocytic marker CD 14 and the TfR respectively. This suggests that monocytes are the main, if not only, cell type having surface TfR's in these suspensions.

Radioligand incubations show that the level of TfR expression in freshly isolated cells is five to tenfold lower than in cultured cells. This increase can not only be attributed to resynthesis of trypsin-cleaved TfR's.

A very low level of TfR expression in cytotrophoblasts is in accordance with findings from cryostat sections.

Apparently cytotrophoblast is an exception to the rule that mainly proliferative cell types have high levels of TfR expression. In situ hybridization can probably show whether even actively proliferating individual cytotrophoblasts lack TfR's.

Our results also demonstrate that normal cytotrophoblast differs considerably from a mononuclear choriocarcinoma clone. These BeWo cells have high levels of TfR's (20).

In vitro, as is shown by us, the TfR appears on both cytotrophoblast

and (in vitro formed) syncytiotrophoblast. The latter is, in situ, a highly differentiated, non-proliferative terminal cell stage.

Using ^{125}I-labelled transferrin, the functionality of this receptor with respect to transferrin binding can be demonstrated. Transferrin binding to its receptor is concentration- and time dependent, saturable and specific. Scatchard analysis results in K_D-values of 1.2-1.9x10^{-9} M. This is in accordance with data found for choriocarcinoma and fat cells (K_D 1.4-4.1x10^{-9} M) (21,22). For choriocarcinoma cells however, somewhat lower affinities of transferrin for its receptor have also been reported: 1.3x10^{-8} M (20).

Using human term placental villi to prepare apical membrane vesicles, K_D values for transferrin-binding to its receptor have been obtained ranging from 2.8-4.5x10^{-8} M (7,8,10). VanderPuye et al. (14) found a K_D of 2.1x10^{-8} M in microvillous membranes and a ten-fold higher affinity of transferrin for its receptor at the basolateral side (K_D: 2.5x10^{-9} M). Basal membranes had about half the binding capacity of microvillous membranes.

The number of surface binding sites per cell (assuming a mean equivalent of 6 pg DNA per cell) is 2.9-3.8x10^4 after 40 h in vitro.

Nestler found that cellular protein content is 130-170 μg/10^6 cytotrophoblasts (23). Using this, the number of transferrin binding sites can be calculated to be approximately 3x10^4 per cell surface. Thus, there is good agreement between data based on quantitative DNA and protein analyses.

The level of TfR expression in normal trophoblast is considerably lower than in choriocarcinoma cells: 1.5-1.6x10^6 surface binding sites per malignant cell (20, 21). This difference can at least be partially explained by the fact that choriocarcinoma cells are highly proliferative whereas normal trophoblast cells are inactive in DNA-synthesis under in vitro conditions (chapter 3).

Kinetic studies on transferrin endocytosis show two major differences between normal trophoblast cells and the tumour cell lines studied before. The proportion of TfR's at the cell surface is high in normal trophoblast: 50 per cent. In choriocarcinoma (BeWo/JAR) as well as in HeLa cells this percentage is 30-40. Secondly, although endocytosis is as rapid in normal as in malignant trophoblast, the level of endocytosis is considerably lower in normal trophoblast. Approximately 25-30

per cent of surface bound transferrin is internalized in 5-7.5 min. In K562, as in BeWo this percentage is approximately 40-45 per cent (20). Thus, choriocarcinoma cell lines, previously proposed as models to study placental iron uptake, differ in several important aspects with regard to TfR expression from normal trophoblast cells.

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Chapter 5 Regulation of transferrin receptor expression in cultured human cytotrophoblasts.

5.1. Introduction.

Placental iron uptake is TfR mediated. Maternal iron-loaded transferrin is taken up by the syncytiotrophoblast through receptor-mediated endocytosis. Iron is subsequently released from transferrin, probably in an acidic environment and the transferrin-transferrin receptor complex is recycled to the cellular surface.
Cellular iron metabolism has mainly been studied in various tumour cell lines. TfR numbers are an important regulating factor in cellular iron uptake. The level of cellular TfR expression is influenced by several mechanisms, which will be discussed subsequently.

5.1.1. Cellular proliferation and transferrin receptor expression.

In general, proliferation is a trigger for TfR expression (1). This is especially apparent in mitogen-stimulated T-lymphocytes (2).
Highly proliferative cells, like malignant cells, have high levels of TfR expression.
Under in vitro conditions, low cellular occupancy of the tissue culture dish generally leads to proliferation and therefore, high TfR levels in adherent cells. Exponential growth leads to a reduction of TfR levels (3). However, varying cellular plating out density does not influence TfR expression in HeLa cells (3).

5.1.2. Cellular differentiation and transferrin receptor expression.

Differentiation has several effects on the level of cellular TfR's, depending on the function of the mature cell. In the course of erythroid development cellular TfR levels decrease (4). On the other hand, the level of TfR expression increases during the maturation of monocytes to macrophages (5).
In developing myoblasts the receptor number is related to proliferative state. Terminally differentiated, non-proliferative myotubes have a higher level of expression than non-proliferative myoblasts (6). Undifferentiated, highly proliferative tumour cell lines have high levels of TfR expression. Differentiation of malignant cells can be induced by dimethylsulfoxide or dibutyryl-cAMP. This results in a sharp

decrease of the TfR level (7,8). Dibutyryl-cAMP treatment inactivates transcription of the TfR gene in malignant HL-60 cells and induces monocytic differentiation. After 72 h dibutyryl-cAMP treatment of these cells, surface TfR's were no longer detectable (8).
It has been shown that biochemical differentiation of normal term cytotrophoblasts is also triggered by cAMP (9-12). This differentiation leads to increased secretion of hCG and progesterone. mRNA levels for both hCG subunits as well as cytochrome P450 cholesterol side-chain cleaving system increase. mRNA's for actin and fibronectin are down-regulated by cAMP.
cAMP treatment also leads to higher levels of hCG secretion in choriocarcinoma cells (13).
Cytotrophoblasts in vitro also differentiate in the course of time: both morphologically (increased syncytium formation) and biochemically (enhanced immunoperoxidase reactivity for hCG and hPL, chapter 3).
Thus there are two ways to stimulate differentiation of cultured cytotrophoblasts, addition of cAMP to their culture medium and prolonged cell culture. The effect of both ways on TfR-expression will be studied.

5.1.3. Cellular iron content and transferrin receptor expression.
TfR and ferritin synthesis in cells is, at least partially, regulated by iron (14). If iron is abundant the level of TfR's is low and cellular ferritin content is high. The 3' untranslated region of the mRNA for the human TfR is necessary and sufficient for iron-dependent control of TfR mRNA levels. The level of TfR's in K 562 cells, a human erythroleukemic cell line, can be manipulated by treating these cells with either desferrioxamine, a well-known iron (III)chelator or diferric transferrin (15). The same applies to normal rat hepatocytes (16). Whereas changes in TfR-levels are accompanied by corresponding changes in TfR mRNA, ferritin biosynthesis is altered by redistribution of mRNA between polysome and nonpolysome pools (17).

5.1.4. The effect of growth factors on transferrin receptor expression.
Whereas cellular iron content influences the total number of TfR's, growth factors cause a redistribution of TfR's from intracellular

pools to the cell surface (18-20). Therefore, contrary to the effects seen when cellular iron status is influenced, the total cellular receptor number does not change due to these growth factors.
The redistribution-effect varies between growth factors but generally it is rapid and more or less transient. Moreover, it is thought to be related to the mitogen action of growth-factors (19-21).
Castagnola et al. showed (21) that in KB carcinoma cells, epithelial cells in which EGF stimulates growth, EGF causes a rapid, transitory increase in the number of surface TfR's, returning to basal levels in 15 min. On the other hand EGF inhibits growth in cloned A431 malignant epithelial cells. In these cells EGF causes a 50 per cent decrease in surface TfR levels after 30 minutes. Ward and Kaplan (19) demonstrated that hormones like insulin and IGF cause a redistribution of TfR's to the surface in some cell types, but not in all. They suggest that a large internal receptor pool is a prerequisite. Cell types showing the TfR-redistribution phenomenon react similarly to the addition of serum if cultured in serum-free medium. Addition of fetal bovine serum results in a rapid and concentration-dependent increase in cell-surface binding. On the other hand, withdrawal of serum from the media of HeLa cells grown in medium with 10 per cent fetal bovine serum leads to a 50 per cent decrease in cell surface TfR levels. This effect is seen for at least 30 minutes after withdrawal of serum and is therefore not very transient. As with insulin and IGF, TfR redistribution due to serum addition or withdrawal was not seen in all cell types. Surface TfR levels in K562 and Epstein-Barr virus transformed human lymphoblasts were again unaffected.
Whereas EGF acts solely through an increase in the rate of TfR exocytosis, IGF-1 decreases the rate of endocytosis as well (22). Both mechanisms result in redistribution to the cellular surface, but the effect of IGF-1 is less transient that the EGF-effect (23). Growth factors have a number of influences on human term cytotrophoblasts in vitro. These cells have EGF-receptors (24,25) and EGF induces trophoblast-differentiation (25,26). Insulin modulates cytotrophoblastic aromatase protein mass and hence steroidogenesis (27) as well as placental lactogen synthesis (28).

5.2. Results.

5.2.1. 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 out cellular density (i.e. the number of cells per ml culture medium) confirmed this finding (Table 5.1).

Table 5.1

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

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.

Bromo-deoxyuridine take-up studies show that term cytotrophoblasts are not proliferative in vitro (chapter 3). 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.

5.2.2. 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 5.2 illustrates the increase in hCG secretion into the culture medium.

Table 5.2

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

HCG-SECRETION BY CULTURED CYTOTROPHOBLASTS.
Influence of the presence of 1.5 mM 8-br-cAMP.

8-bromo-cAMP induced biochemical differentiation of trophoblasts in vitro does not result in changes in TfR expression (Fig. 5.1). Neither changes in affinity of transferrin for its receptor, 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 $7x10^{11}$ per mg protein (mean protein per dish: 176 μg (n = 26, S.D.= 14).

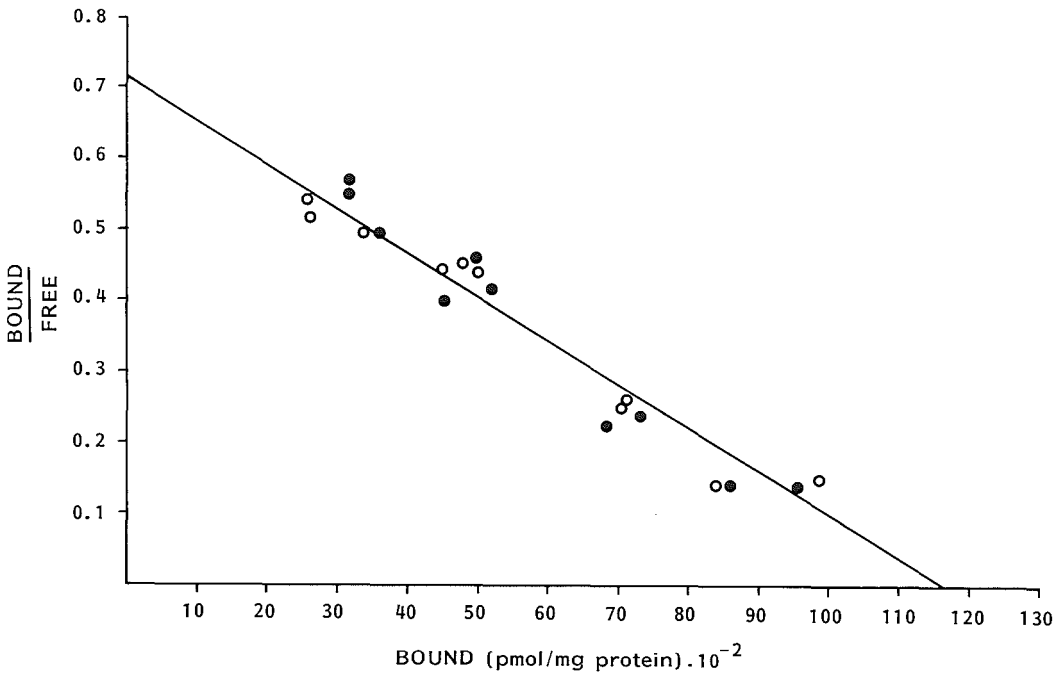

Figure 5.1
Influence of 8-bromo-cAMP induced differentiation on transferrin receptor expression.
Scatchard analysis of concentration-dependent ^{125}I-transferrin binding at 4°C to cultured cytotrophoblasts. Culture period: 40 hours.
● : cells treated during entire culture period with 1.5 mM 8-br-cAMP. 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).

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 5.3, A). This is similar to the phenomenon described in the previous section. In other experiments cells were apparently thriving better, resulting in no decrease of mean (cellular) protein per dish. An example is shown in

table 5.3, B. 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 5.3

		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	$2.6\ 10^{12}$

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.

5.2.3. Iron availability.

Table 5.4 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 diferrric 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.

Table 5.4

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 " "	0.17 (± 0.00)	33	21
0.8 " "	0.13 (± 0.01)	25	21
0.01 ferric ammonium citrate	0.35 (± 0.02)	67	10
0.05 " "	0.34 (± 0.03)	65	10
0.10 " "	0.38 (± 0.04)	73	10
0.20 " "	0.40 (± 0.03)	77	10

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.

5.2.4. Growth factors and serum.

Table 5.5 shows that the removal of serum in the medium of serum-grown cytotrophoblasts has little or no effect on the level of surface TfR's. If cells are kept for longer periods of time in serum-free media (24 to 48 hours) the cellular yield decreases most of the time compared to a parallel set of dishes with serum-containing medium. This results in higher receptor levels. However, in occasional experiments where the cellular yield was not influenced by the absence of serum in the culture medium, there was no difference in receptor levels. The effect of insulin on surface TfR levels is illustrated in table 5.6.

Table 5.5

Time in serum-free medium at 37°C	n	Specific surface binding of ^{125}I-transferrin (pmol/mg protein) mean (± S.D.)	Percentage of control
0 (control)	3	1.53 (± 0.08)	100
1 min	2	1.66 (± 0.00)	108
2 "	3	1.45 (± 0.04)	95
5 "	3	1.27 (± 0.01)	83
10 "	3	1.36 (± 0.02)	89
15 "	3	1.41 (± 0.07)	92
20 "	2	1.43 (± 0.09)	93

INFLUENCE OF SERUM-WITHDRAWAL ON CELL SURFACE ^{125}I-TRANSFERRIN BINDING. Cytotrophoblasts were cultured for 40 hours in serum containing 20% FCS. They were put on ice, without being washed, for 30 minutes. Medium was subsequently removed and displaced with cold (4°C) DMEM-H without serum. During indicated time periods the dishes were incubated in pre-warmed M199 without serum at 37°C in 5% CO_2. Cells were then cooled on ice and specific binding of ^{125}I-transferrin at 4°C was determined. ^{125}I-transferrin concentration: 1 μg/ml.
Results from one experiment from a series of three highly similar ones.
Mean protein per dish: 240 μg (S.D. = 18, n = 21)

Table 5.6

Insulin concentration (nM)	Cellular protein (mg/dish)	Specific binding of ^{125}I-transferrin (pmol/mg protein)
0 (control)	0.13	1.8
duplicate	0.11	1.8
1	0.13	1.9
duplicate	0.13	1.9
10	0.10	1.8
50	0.12	1.8
duplicate	0.14	2.0
100	0.12	1.9
duplicate	0.13	1.8
250	0.13	1.9
500	0.13	1.9

EFFECT OF INSULIN ON ^{125}I-TRANSFERRIN BINDING AT 4°C TO CULTURED TERM CYTOTROPHOBLASTS.
Cells were cultured for 30 hours in medium with 20 per cent fetal calf serum. They were then changed to serum-free medium and kept overnight in this medium in the incubator.
The next day they were washed extensively and incubated with a concentration series of insulin. Since maximum insulin binding is at 4°C (28) cells were incubated on melting ice in DMEM-H with 0.25 per cent BSA (w/v) and 0-500 nM insulin. Dishes were subsequently warmed to 37°C in this medium and kept at 37°C for 30 minutes to allow the redistribution of TfR, put on ice again and incubated with ^{125}I-transferrin for 90 minutes.
^{125}I-transferrin concentration: 750 ng/ml. Non-specific binding was determined to be 9 per cent.
One typical experiment from a series of three.

5.3. Discussion.

The level of TfR expression in cultured human cytotrophoblasts depends to a large extent on cellular density in the culture dishes. This is

especially apparent when the plating out concentration is varied (table 5.1). 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 (3). 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. 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 (4) the number of receptors increases during the differentiation of monocytes to macrophages (5).

Differentiation of trophoblasts in vitro is known to be triggered by cAMP analogons (9-12). 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. 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.

Many receptors for ligands such as growth factors elicit their transmembrane signals by manipulation of the intracellular level of the cyclic nucleotides, polyphosphoinositides and free cytosolic calcium. Sharma et al. recently showed that receptor-mediated endocytosis of diferric transferrin by both hepatocytes and HL 60 cells is independent

of second messengers (29). Therefore, if TfR phosphorylation has a regulatory role during endocytosis, it is likely to be mediated by a second messenger-independent protein kinase.

In tumour cells like HL-60, dibutyryl-cAMP triggers inactivation of the TfR gene and monocytic differentiation of the cell (8). cAMP analogons stimulate hCG expression in choriocarcinoma cells (13). In this respect these tumour cells resemble normal trophoblast. Since TfR expression seems to be regulated differently in malignant cells compared 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 (15) 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 (30). In the near future the protein involved in the communication between cytosolic iron and TfR's mRNA will be identified (31). This protein might play an important role in placental iron metabolism.

In vitro, endocrinological and biochemical characteristics of cytotrophoblasts are influenced by growth factors like EGF, IGF as well as insulin (24-27). These hormones also have an effect on iron metabolism in a number of cell types, though not all (19). Cell types in which TfR distribution over various pools of the endocytotic pathway is affected by these hormones react rapidly ($t_{\frac{1}{2}}$ 3 minutes) and considerably (30-50%) to the addition or withdrawal of serum to their culture medium. The decrease in cell surface TfR levels due to serumwithdrawal is maintained for at least 30 minutes in these cells. The effect of serumwithdrawal or insulin-addition on surface TfR levels is negligible in term cultured cytotrophoblasts. In rat fat cells 1-1000 nM insulin

leads, within 3 minutes to a threefold increase in surface TfR levels. This increase is maintained for at least 20 minutes (18). The absence of this redistribution effect in term cytotrophoblasts may at least partially be explained by the high proportion of cellular TfR's already at the surface (chapter 4). Ward and Kaplan (19) described that the redistribution effect is also not seen in K562 and Epstein-Barr virus transformed lymphoblasts.

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 (32). Biochemical differentiation of cultured cytotrophoblasts does not influence placental TfR expression. No influence of growth factors, present in maternal serum surrounding the syncytiotrophoblast outer membrane, on TfR distribution could be demonstrated. However, environmental iron availability, especially in the physiological diferric-transferrin form does regulate TfR expression. This provides a feed-back mechanism for placental iron uptake and makes it independent of maternal iron status. Trans-placental iron transport is thus protected against maternal iron-deficiency.

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Chapter 6 Pregnancy-related changes in maternal serum transferrin.

6.1. Introduction.

Maternal iron-loaded transferrin is the sole iron donor for the human placenta (1). Pregnancy influences maternal serum transferrin in several ways. In humans, maternal serum transferrin concentrations increase, both in iron-supplemented and non-supplemented women (2). The total circulating amount of transferrin increases even more, due to the increase in circulating volume.

Serum iron concentration remains constant or decreases (in non-iron-supplemented women) and thus transferrin-iron saturations fall in the course of pregnancy. In chickens, transferrin synthesis in the liver is stimulated by iron deficiency as well as by estrogens (3).

Human transferrin microheterogeneity is not only determined by iron-saturation but also by transferrin's sialic acid contents (4). The share of highly sialylated transferrin fractions increases in the course of pregnancy (5). Both pregnancy and estrogen-treatment lead to an increase of the branching of carbohydrate chains in several glycoproteins (6).

Changes, both qualitatively as well as quantitatively, in maternal transferrin could have important physiological consequences for its iron-donating function to the placenta and other tissues.

This chapter is devoted to changes, especially qualitative, in maternal (and fetal) transferrin. For reasons of convenience we chose an animal model, the guinea-pig. Guinea-pig pregnancy is short (2 months), its placentation resembles human placentation and much research has been aimed at unraveling iron metabolism during its pregnancy (7-9).

6.2. Results.

6.2.1. Slow and fast isotransferrins.

Both in rats and in guinea-pigs, the existence of so-called slow and fast isotransferrins has been noted (7,10). These isotransferrins differ in electrophoretic mobility. Whereas rat isotransferrins can be separated on DEAE-Sephadex A-50 (10) this is not the case in guinea-pigs. Ion exchange chromatography of guinea-pig transferrin gives one single, symmetrical peak.

In preparative iso-electric focusing of guinea-pig transferrin a distinct discontinuity between slow and fast transferrin was seen on flat-bed preparative gels, after several hours of focusing. The yield of fast transferrin was ten times as low as that of slow transferrin. Figure 6.1 shows crossed immunofocusing patterns of purified isotransferrins.

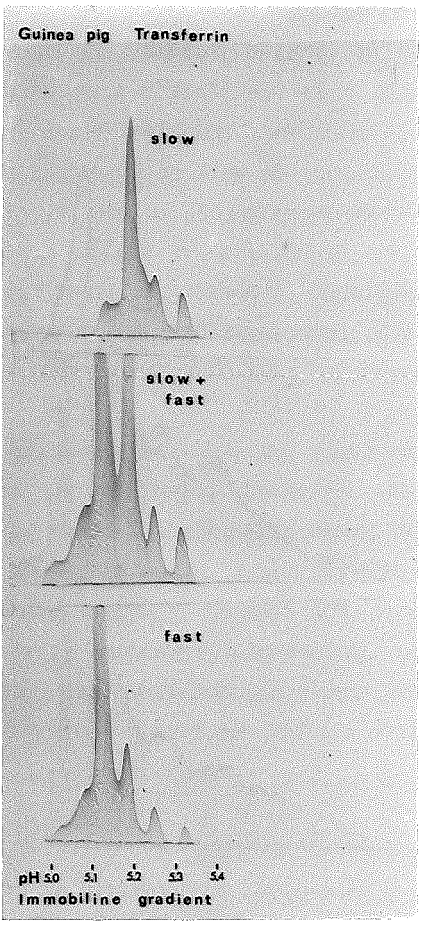

Figure 6.1
Crossed immunofocusing patterns of guinea-pig isotransferrins.

Fast isotransferrin has a slightly lower isoelectric point. Both isotransferrins were pure by immunodiffusion against anti-total guinea-pig serum and on SDS-PAGE. SDS-PAGE of isotransferrins, under unreduced conditions, showed a small but significant difference in electrophoretic mobility between both transferrins, in accordance with a molecular mass difference of 3000-5000 daltons. SDS-PAGE of neonatal guinea-pig serum showed the presence of both isotransferrins as discrete bands, supporting the existence of two proteins of different molecular mass. Incubation with glycopeptidase F abolished this difference on SDS-PAGE, indicating that the carbohydrate part of the glycoprotein is responsible for the difference in electrophoretic mobilities (11).
Table 6.1 presents the results of aminoacid and carbohydrate analyses. Some amino acids show significant differences between slow and fast, notably threonine, glycine, alanine and valine. Most striking, however, are the differences in carbohydrate composition.

6.2.2. Isotransferrins and pregnancy.

Table 6.2 shows serum transferrin concentrations for pregnant and non-pregnant female guinea-pigs, as well as the ratio of fast to slow isotransferrins. Although the number of data is limited, it is apparent that, whereas serum transferrin concentrations slightly drop in the course of pregnancy, the fast/slow ratio increases from non-pregnant values of approximately 0.2 to values of 0.5-0.8 at term. Therefore the relative amount of fast isotransferrin increases from approximately 15 to approximately 35 per cent in the course of pregnancy. The changes in transferrin composition are even more pronounced in neonatal guinea-pigs. Table 6.3 shows that F/S ratio's in new born guinea-pigs fall dramatically in the first few weeks of post-natal life.

6.3. Discussion.

In many species, changes in transferrin molecular mass - within one genetic variant - have been shown to be solely due to differences in the carbohydrate chain(s). This also applies to rat slow and fast isotransferrins. There is no difference in amino-acid composition between these two isotransferrins (10).

Table 6.1

Protein (mol/mol)	Slow	Fast
Asp	57.2	57.5
Thr	27.9	37.7
Ser	58.5	60.2
Glu	77.1	79.8
Gly	49.5	41.0
Ala	65.2	57.5
½Cys	13.2	11.6
Val	44.0	41.0
Met	6.5	8.8
Ile	24.5	22.3
Leu	69.2	68.7
Tyr	24.1	22.1
Phe	33.3	32.8
Lys	49.6	48.5
His	19.3	18.8
Arg	29.7	29.1
Glucosamine	6.2	10.4
Mannose	4.7	6.9
Galactose	2.9	4.3
Sialic acid	2.7	5.9

Transferrin molecular mass: 80,000

AMINO-ACID AND CARBOHYDRATE ANALYSES OF GUINEA-PIG ISOTRANSFERRINS

Rat slow transferrin (pI 5.65) has 3 sialic acid residues per transferrin molecule. Fast isotransferrin (pI 5.85) has 2 sialic acid residues per transferrin molecule (12,14). Both rabbit and rat transferrins have one glycan chain per molecule (13,15).

To our knowledge, only in cattle, transferrin molecular mass heterogeneity within one genetic variant must be due to differences in the polypeptide, since deglycosylated transferrin also showed molecular mass heterogeneity (15). In this species large transferrin probably differs from small transferrin only in the presence of an additional carboxyl-terminal peptide.

Table 6.2

Guinea-pig number	Gestational age (days)	Serum transferrin concentration (mg/ml)	Fast/Slow Ratio
1	11	4.8	0.30
	32	4.7	0.42
	42	3.5	0.42
2	18	4.1	0.14
	42	3.8	0.42
	50	3.5	0.55
3	10	4.9	0.15
	21	4.8	0.29
	31	4.1	0.32
4	25	3.9	0.37
	45	3.5	0.83
	55	3.5	N.D.
5	55	N.D.*	0.43
6	55	N.D.	0.60
7	non-pregnant, female	N.D.	0.18
8	non-pregnant, female	N.D.	0.21

*N.D.: Not determined

SERUM TRANSFERRIN IN FEMALE GUINEA-PIGS

Table 6.3

Age (days)	Serum transferrin concentration (mg/ml)	Fast/Slow Ratio
2	N.D.	1.43
6	4.4	0.71
11	4.1	0.48
20	4.4	0.43

*N.D.: Not determined.

SERUM TRANSFERRIN IN NEONATAL GUINEA-PIGS.

The difference in molecular mass between small and large cattle-transferrin is approximately 6400 daltons.

Guinea-pigs would be highly exceptional among rodents if slow and fast transferrin differ in amino-acid composition. It is however possible that a peptide-fragment is removed from fast transferrin to convert it to slow transferrin. This seems to be the most likely explanation for the "one gene, two polypeptides"-hypothesis. In newly synthesized human transferrin, the N-terminal aminoacid valine is preceded by 19 amino-acids. This peptide is released before secretion, and it probably has a signal function in transferrin secretion (16). An alternative explanation could be the presence of trace amounts of heme or heme-hemopexin in our preparations. These are notoriously difficult to remove from transferrin. It has been claimed that only additional chromatographic steps, utilizing carboxymethylcellulose or phenylboronate are able to remove these contaminations (17).

The difference in carbohydrate content between both guinea-pig isotransferrins is very pronounced, showing that the main difference between both isotransferrins is located in the glycan chain(s). Slow guinea-pig isotransferrin carbohydrate analysis resembles human biantennary transferrin (18,19). Fast isotransferrin's heteropolysaccharide chain has a different composition.

The relative amount of fast isotransferrin, having a highly sialylated carbohydrate chain which is therefore probably highly branched as well, increases in the course of pregnancy. This phenomenon is similar,

though more pronounced, to the situation in human pregnancy. Recently, de Jong and van Eijk described that already early in human pregnancy the proportion of the highly sialylated transferrins increases, mostly at the cost of the tetrasialotransferrin. It reaches a maximum level at about the 28th week of pregnancy, which is maintained (20). The changes are reversed rapidly after delivery. Oral contraceptives bring about a qualitatively similar effect.

Thus there is a striking correlation between the increment in the production of the highly sialylated transferrins and increasing transplacental iron transport, in humans as well as in guinea-pigs. The observation that the composition of transferrin from healthy individuals is being maintained within narrow margins with regard to prosthetic allomerism (17) suggests that more than mere synchronism is at stake during pregnancy. A change in glycosylation of transferrin is also encountered in various diseases (5,21).

Contrary to the situation in humans, we found that the concentration of transferrin in guinea-pigs decreases slightly in the course of pregnancy. This has been described by others as well (8).

Avvakumov and Strel'chyonok found that a pregnancy-related human transcortin variant differs from transcortin only with regard to carbohydrate structure (22). The pregnancy related variant has five tri-antennary sugar chains whereas transcortin normally has two tri- and three biantennary carbohydrate chains. First trimester syncytiotrophoblast microvillous membrane vesicles have two classes of specific binding sites for both transcortins. One of these displays a relatively high binding capacity and a significantly higher affinity for transcortin than for the pregnancy-associated variant. The other class of binding sites occurs in the membranes at a far lower concentration, but it has a higher affinity for the pregnancy-associated transcortin variant. This illustrates that the physiological consequences of differences in carbohydrate chains of glycoproteins can be complex.

Differences in glycoproteins' carbohydrate chains can be recognized by lectins. For instance, the hepatic binding protein specific for galactose is involved in the clearance of glycoproteins from the circulation. Therefore, terminal carbohydrate chain residues can either provide specificity or mask underlying sugar residues. Lectins provide specific

carbohydrate binding, both at the cell surface and at intracellular levels, thus sorting glycoproteins for different intracellular fates. Isotransferrins, differing in carbohydrate chains, can therefore have different affinities for various cell systems (bone marrow, placenta, liver) and thus direct iron to various destinations, both in the fetus as well as in the mother.
Furthermore, intracellular iron processing can also differ between isotransferrins.
Not only transferrin, but its receptor as well are glycoproteins. It has recently been recognized that TfR's are a heterogeneous population, probably differing in glycosylation (23). A difference in TfR-glycosylation might influence transferrin-binding, since the absence of carbohydrate residues in the TfR decreases its affinity for tranferrin considerably (24).

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Chapter 7 General discussion.

Trans-placental iron transport increases enormously in the course of pregnancy (1). Early in pregnancy iron is stored in the placenta (2,3) and small amounts of iron are transported to the fetus. In the last trimester large amounts of iron are transported across the placenta and only small amounts are retained in the placenta (1).

Little is known about adaptive changes in the course of pregnancy. Apparently the fetus is protected against toxic iron overload early in pregnancy. Clinical observations in thalassemia patients suggest that ferric iron is even more toxic to fetal liver cells than it is in post-natal life (4). This makes protection against iron overload even more important. Iron retained in the placenta, early in pregnancy, is probably located in ferritin. Thus, iron overload in the fetus can be prevented. Ferritin has been attributed a role in regulating placental iron transfer (5,6). Qualitative and quantitative changes in placental ferritin in the course of pregnancy, and its localization, close to the syncytiotrophoblast apical membrane, are very suggestive in this respect.

Ferritin is possibly involved in short-term regulation of placental iron uptake as well. We have shown in chapter 5 that cultured term cytotrophoblasts respond to high iron availability in the culture medium rapidly by decreasing TfR numbers. If Iron Responsive Elements are operative in maintaining iron homeostasis in term cytotrophoblasts, it would be expected that cellular ferritin levels are modulated according to iron availability as well. However, in vivo experiments in animals, employing operative removal of the fetus, indicate that placental iron uptake is not regulated rapidly by trophoblast iron content. Iron uptake continues in the absence of the fetus (7,8).

It seems to be worthwhile to investigate the role of ferritin, both in the short-term and long-term regulation of placental iron uptake more closely. The protein-connection between cytosolic iron and IRE's in both ferritin- and TfR-mRNA's is likely to be clarified soon, shedding more light on the regulation of cellular iron homeostasis (9-11).

Long-term regulation of iron transport is particularly difficult to unravel. It is unclear what role iron plays in changes influencing placental iron uptake. For instance: the surface of the maternal-fetal

exchange area increases in the course of pregnancy. It remains to be clarified what regulates placental growth and where iron fits in. It is unclear whether TfR density on the syncytiotrophoblast apical plasma membrane changes in the course of pregnancy. We found that TfR-expression in this cell type is at a constant level, not influenced by biochemical changes accompanying differentiation.

Whereas the number of EGF-receptors has been reported to increase in the course of pregnancy, based on EGF-binding to syncytiotrophoblast membrane-vesicles from placentas of various gestational ages (12), opposite conclusions have been reported as well (13). The conclusion that the EGF-receptor density decreases in the course of pregnancy was based on quantitative immuno-peroxidase techniques. The difference may, at least partially, be due to the fact that no methods are available to isolate equally pure syncytiotrophoblast apical membrane-vesicles from placentas of various ages.

It has been suggested that growth-factors play an important role in enhancing the proliferative phase of feto-placental growth (gestational age 14-24 weeks) (14), thus influencing long-term adaptation of transplacental transport. Results described in chapter 5 suggest that growth factors do not play an important role in short-term regulation of TfR distribution between intracellular and cellsurface pools in cultured term cytotrophoblasts. Hence growth factors do not influence placental iron uptake in cultured term trophoblast cells. This might be related to the fact that term cultured cytotrophoblasts are not proliferative, as was shown in chapter 3. Thus, our results suggest that effects of growth factors on TfR distribution are closely related to proliferation.

Sun et al. proposed an intriguing hypothesis to explain insulin's stimulation of growth (15). Insulin-induced increased exposure of TfR's at the cell surface is thought to increase transferrin-bound iron (III) reduction, since a transplasmamembrane electron transport system is coupled to the TfR. Hence electron transport across the membrane increases. Coupled to this is proton release from the cell through the Na^+/H^+ antiport. Subsequent alkalinization of the cytoplasm is related to the mitogenic action of growth factors (16).

Recently a method has been described to isolate pure cytotrophoblasts from first trimester human placentas (17). These cells are proliferati-

ve in vitro, with doubling times of 48-96 h. Some of these first trimester cytotrophoblast cell lines were propagated continuously for 8 months. This is an attractive model to study the relation between proliferation, differentiation and TfR-expression. Also, the effect of serum-withdrawal from the culture medium and insulin on TfR distribution in these cells is of interest. Comparison of data thus obtained with results described in this thesis for term cytotrophoblasts could shed more light on the relation between growth-factor induced TfR-redistribution and the mitogenic role of growth factors.

Clinical situations provide little further evidence with regard to the regulation of trans-placental iron transport. Case stories have been reported of women receiving high doses of iron intra-venously at the beginning of the last trimester (18). This resulted in increased fetal hepatic iron stores, suggesting a lack of short-term regulation of trans-placental iron transport. There are two conditions in which chronic fetal iron overload can occur: neonatal hemochromatosis and rhesus-antagonism, treated with repeated blood transfusion in utero. It is unknown whether thus induced chronic fetal iron overload leads to down-regulation of placental iron uptake. An extra difficulty lies in the uncertainty whether neonatal hemochromatosis represents total body iron overload or merely a redistribution with iron overload in the liver and low levels of iron located in hemoglobin (4). Both placental iron concentration and total placental iron content are not affected in neonatal hemochromatosis.

A major factor influencing iron metabolism in pregnancy may be protein glycosylation. Both transferrin and its receptor are glycoproteins. Parallel changes in transferrin glycosylation and increasing transplacental iron transport, as described in chapter 6, suggest an adaptation in iron metabolism. Avvakumov and Strel'chyonok's results, discussed in chapter 6, in transcortin research (19) illustrate that various isotransferrins should be used in as many testsystems as possible, in order to investigate the physiological consequences of changes in glycosylation. An animal model will be particularly helpful in this respect, enabling both the use of many readily available cell systems as well as experiments directed at unraveling the mechanisms regulating transferrin biosynthesis.

Furthermore, TfR's are no homogeneous population. Cells requiring

large amounts of iron synthesize an additional TfR, differing qualitatively from the conventional one (20).

Post-translational modification, like glycosylation of the TfR is a likely explanation for this phenomenon. Glycosylation is not necessary for specific binding of transferrin to its receptor, but the affinity of this binding can be influenced greatly by the presence or absence of carbohydrate residues (21).

Regulation may be involved in placental iron release to the fetal circulation. Both the amount and the form in which iron is released may be influenced. An interesting, though highly speculative, way in which the placenta might regulate iron processing at the fetal side is through a recently recognized humoral regulatory mechanism controlling erythropoiesis. In the human placenta inhibin is produced (22). Both inhibin and its antagonist, activin regulate the proliferation of erythroid progenitor cells, thus modulating erythro-differentiation (23).

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SUMMARY

Iron plays a key role in many biochemical reactions. It is thus also essential for the developing fetus. The fetus depends for its iron demands on the mother. Maternal serum transferrin serves as the iron donor. It delivers its iron to the placenta and hence it is transported to the fetus.

Experiments in animals show that placental iron uptake continues if the fetus is removed.

Fetal iron needs increase in the course of pregnancy. This is partly due to the onset of fetal hemoglobin synthesis. Fetal iron needs are met, even if the mother has a (mild) iron deficiency. Widely used iron suppletion in pregnancy improves maternal iron status, whereas neonatal iron status is hardly influenced.

The observation that the fetus, on the one hand, does not suffer from iron overload, when its iron processing capacity is low, whereas, on the other hand, it is protected against iron deficiency even if iron supply is limited, strongly suggests that maternal-fetal iron transport is regulated.

This thesis is a study on mechanisms regulating placental iron uptake.

Chapter 1 gives a survey of iron metabolism. Emphasis is laid on the impact of pregnancy on iron metabolism and on the proteins and biochemical mechanisms involved in controlling iron.

Chapter 2 describes all the materials and methods used in the subsequent chapters.

Chapter 3 to 5 are devoted to a study of the regulation of transferrin-receptor-expression on the maternal-fetal exchange area. The transferrinreceptor (TfR), located on the apical side of syncytiotrophoblast, is primarily the molecule that mediates placental iron uptake. Within the syncytiotrophoblast, iron is released from maternal transferrin. Iron continues its way to the fetal circulation and maternal apotransferrin is recycled to maternal blood.

In the first part of this study a method is employed that isolates homogeneous, well defined cells from the human term placenta. These cells are cytotrophoblasts. Under in vitro conditions they fuse, thus forming a syncytium. All cells, whether mononuclear or fused start expressing biochemical characteristics of syncytiotrophoblast. They

synthesize a number of hormones that are in vivo strictly limited to syncytiotrophoblast. Some general characteristics of this cell culture model, relevant to this study, are described in chapter 3. Cytotrophoblasts are, for instance, not proliferative under in vitro conditions.
Chapter 4 takes a closer look at TfR-expression on freshly isolated cytotrophoblasts and the changes in this respect in culture. Freshly isolated cytotrophoblasts have a very low level of TfR-expression. After approximately 40 h in vitro all cells have TfR's, at an average of approximately 80,000 per cell. These receptors are present in equal quantities on the cell surface and intracellularly. This shows that the level of TfR's in normal trophoblast is approximately 50-fold lower than in their malignant counterpart, choriocarcinoma.
Chapter 5 describes the effect of a number of mechanisms, that are known to influence TfR-expression in other cell types, on cytotrophoblasts. Changes in TfR-levels on the maternal-fetal exchange area will immediately affect placental iron uptake. It is shown that the number of TfR's per cell is influenced by the number of cells in the culture dish. Low cellular occupancy of the culture dish leads to high TfR levels. In other cell types, low cellular density in vitro leads to proliferation and hence high levels of TfR's. This is not the case in human term cytotrophoblasts, since they are not proliferative in vitro. Differentiation of cytotrophoblasts in vitro can be induced in two ways, either by adding cAMP to their culture medium or by expanding the culture period. Both ways do not lead to any change in TfR-expression in these cells.
Growth factors induce a redistribution of TfR's in many cell types. TfR's are mobilized from intracellular receptor pools to the cell surface. Withdrawal of serum from the culture medium generally has an opposite effect. The number of TfR's at the cell surface is not affected in either way in term cytotrophoblasts.
The addition of extra iron to the culture medium, especially if bound to transferrin, leads to lower levels of TfR's in trophoblast cells. This suggests a feed-back mechanism between maternal iron-availability and placental iron uptake capacity. Hence, placental iron uptake per cellular unit can be maintained at a constant level. Since the maternal-fetal exchange area increases in the course of pregnancy, placental iron uptake can still be adjusted to increasing fetal needs.

In chapter 6 the effects of pregnancy on maternal transferrin, both qualitatively as well as quantitatively are discussed. An animal model (the guinea-pig) is used in this chapter. It is shown that the main change involves transferrin's carbohydrate chain. Both maternal and fetal transferrin are affected.
Finally, in chapter 7 the results obtained in this study are placed in the broader perspective of the knowledge on iron metabolism in pregnancy.

SAMENVATTING

IJzer speelt een sleutelrol bij veel belangrijke biochemische processen. Ook voor de zich ontwikkelende foetus is ijzer onontbeerlijk. Hij is voor zijn ijzerbehoefte afhankelijk van de moeder. Het in het moederlijk bloed circulerende transferrine fungeert als ijzerdonor: via de placenta vindt ijzertransport plaats naar de foetus.
In proefdierexperimenten is gebleken dat de placenta ijzer aan de moeder blijft onttrekken indien de foetus operatief verwijderd wordt.
In de loop van de zwangerschap stijgt de foetale behoefte aan ijzer. Dit komt onder andere door de op gang komende hemoglobinesynthese. Ook indien de moeder (mild) ijzergebrek heeft wordt aan deze foetale behoefte tegemoet gekomen. De algemeen gebruikelijke ijzersuppletie in de zwangerschap is dan ook primair van belang voor de moeder, de neonatale ijzerstatus wordt er nauwelijks door beïnvloed.
Het feit dat de foetus enerzijds niet met ijzer wordt overladen, zolang de verwerkingscapaciteit voor ijzer laag is, anderzijds ook in situaties van geringe ijzerbeschikbaarheid beschermd is tegen ijzergebrek maakt het aannemelijk dat transport van moeder naar kind in de zwangerschap gereguleerd wordt.
In dit proefschrift is een onderzoek beschreven naar regulerende factoren van de placentaire ijzeropname.
In hoofdstuk 1 wordt een overzicht gegeven van het ijzermetabolisme. Daarbij wordt in het bijzonder ingegaan op de invloed van de zwangerschap op het ijzermetabolisme en op de eiwitten en biochemische mechanismen die betrokken zijn bij de beheersing van de ijzerstatus.
Hoofdstuk 2 beschrijft alle materialen en methoden die in dit onderzoek gebruikt zijn.
In de hoofdstukken 3 tot en met 5 wordt het onderzoek beschreven naar de regulatie van transferrinereceptor-expressie op het uitwisselingsoppervlak tussen moeder en kind. De transferrinereceptor (TfR), gelokaliseerd op de apicale zijde van de syncytiotrofoblast, is primair het molekuul waarmee de placenta ijzer (gebonden aan moederlijk transferrine) opneemt. Binnen deze cellaag wordt ijzer van transferrine afgesplitst, het ijzer vervolgt zijn weg naar foetaal transferrine in de foetale circulatie. Moederlijk apotransferrine keert terug naar het moederlijk bloed.

Voor dit deel van het onderzoek is gebruik gemaakt van een methode waarbij één celtype uit de placenta gezuiverd wordt en in kweek wordt gebracht. Dat celtype is de cytotrofoblast. In kweek versmelten deze cytotrofoblasten met elkaar, aldus een syncytium vormend. Alle cellen, zowel gefuseerde als niet-gefuseerde krijgen in vitro de biochemische kenmerken van de syncytiotrofoblast: zij gaan een aantal hormonen produceren die in vivo alleen in de syncytiotrofoblast voorkomen. Een aantal algemene kenmerken van dit model, die van belang zijn voor dit onderzoek, worden beschreven in hoofdstuk 3. Zo blijken de cellen in vitro niet proliferatief te zijn.

In hoofdstuk 4 wordt gekeken naar het voorkomen van TfR's op vers geïsoleerde cytotrofoblasten, en de veranderingen die in de celkweek optreden. Vers geïsoleerde cytotrofoblasten blijken een bijzonder laag niveau van TfR-expressie te hebben. Na 40 uur kweken hebben alle cellen TfR's, gemiddeld ongeveer 80.000 per cel. Deze receptoren zijn gelijk verdeeld over het oppervlak en het inwendige van de cellen. Daarmee blijkt dat normale trofoblast cellen ongeveer 50 maal minder TfR's hebben dan hun kwaadaardige tegenhanger, het choriocarcinoom.

In hoofdstuk 5 wordt het effect onderzocht van een aantal factoren waarvan bekend is dat zij bij andere celtypes de TfR-expressie beinvloeden. Immers, veranderingen in TfR-bezetting op het uitwisselingsoppervlak tussen moeder en kind zullen onmiddellijk gevolgen hebben voor de placentaire ijzeropname. Het blijkt dat het aantal TfR's per cel in vitro in hoge mate afhankelijk is van de hoeveelheid cellen per kweekbakje. Bij een geringe bezetting van het oppervlak van het bakje zijn er veel TfR's per cel aanwezig. In het algemeen is het zo dat onvolledige begroeiing van het kweekoppervlak een stimulans is voor cellen om te gaan prolifereren, hetgeen leidt tot hoge TfR-expressie. Bij cytotrofoblasten uit de terme placenta is dat niet het geval: zij zijn niet proliferatief in vitro.

Cytotrofoblasten zijn in vitro op twee manieren aan te zetten tot differentiatie: enerzijds door cAMP aan te bieden in het kweekmedium, anderzijds door langer te kweken. Beide manieren blijken niet te leiden tot veranderingen in TfR-expressie in deze cellen.

In veel celtypes hebben groeifactoren een herverdelingseffect op TfR's: er worden TfR's gemobiliseerd vanuit het inwendige van de cel naar het celoppervlak. Onttrekking van serum in het kweekmedium heeft veelal een

tegengesteld effect. In de terme cytotrofoblast blijken beide factoren geen invloed te hebben op het aantal TfR's op de plasmamembraan.
De toevoeging van extra ijzer aan het kweekmedium, met name indien gebonden aan transferrine blijkt te leiden tot lagere TfR-expressie in de trofoblast. Dit duidt erop dat er een terugkoppeling bestaat tussen ijzerbeschikbaarheid in het moederlijk serum en opnamecapacteit in de placenta. Een dergelijk mechanisme houdt de placentaire ijzeropname per cellulaire eenheid redelijk constant. Aangezien het uitwisselingsoppervlak tussen moeder en kind sterk toeneemt in de loop van de zwangerschap, kan de placentaire ijzeropname aangepast worden aan de toenemende foetale ijzerbehoefte.
In hoofdstuk 6 wordt beschreven wat de effecten zijn van zwangerschap op de aard en de hoeveelheid moederlijk transferrine, de ijzerdonor voor de placenta. Hiertoe wordt een proefdiermodel, de cavia, gebruikt. Het blijkt dat met name de koolhydraatketen van het transferrinemolekuul sterk verandert in de loop van de zwangerschap, zowel bij de moeder als bij de foetus.
In hoofdstuk 7 tenslotte wordt getracht de in dit proefschrift beschreven resultaten in te passen in de bestaande kennis van het ijzermetabolisme in de zwangerschap.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 16 maart 1959 te Tilburg geboren. In 1977 begon hij, na het behalen van het Gymnasium B diploma aan het Theresialyceum te Tilburg, aan de studie Farmacie aan de Rijksuniversiteit te Utrecht. In 1978 stapte hij over naar de studie Geneeskunde aan dezelfde Universiteit. In 1984 werd het doctoraalexamen afgelegd en in december 1985 het arts-examen. Van 1 januari 1986 tot december 1989 was hij werkzaam op de afdeling Chemische Pathologie (Vakgroep Biochemie) van de Erasmus Universiteit te Rotterdam. Daar werd het hier beschreven onderzoek verricht. Op 1 januari 1990 begint hij aan de opleiding tot kinderarts in het cluster St. Elisabeth-ziekenhuis Tilburg, Wilhelmina-kinderziekenhuis Utrecht.

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