# IRON METABOLISM IN CULTURED CYTOTROPHOBLASTS

## (A model of the maternal-fetal interphase)

IJzermetabolisme in gekweekte cytotrophoblasten

(Een model voor de moederlijke foetale tussenlaag)

PROEFSCHRIFT

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**Chapter IV. (cont.)**

## ABBREVIATIONS

| | |
|---|---|
| cAMP: | 3'-5' cyclic Adenosine monophosphate |
| CPM: | Counts per minute |
| DABCO: | 1,4-Diazabicyclo[2,2,2]octan |
| DEAE: | Diethylaminoethyl |
| Df: | Desferrioxamine |
| DMEM-H: | Dulbecco's modified Eagle's medium (with HEPES) |
| DMEM-H-G: | Dulbeco's modified Eagle's medium (with HEPES and glucose) |
| DNA: | Desoxyribonucleic acid |
| DNase: | Desoxyribonuclease |
| DPM: | Desintegrations per minute |
| EBSS: | Earle's balanced salts solution (calcium and magnesium free) |
| EDTA: | Ethylenediaminotetraacetate-disodium |
| FAAS: | Flameless atomabsorption spectrophotometer |
| FAC: | Ferricammoniumcitrate |
| FCS: | Fetal calf serum |
| FITC: | Fluorescein isothiocyanate |
| FITC-RAM: | Fluorescein isothiocyanate coupled to rabbit anti mouse IgG |
| Fe-NTA: | Iron-nitrilotriacetate |
| HEPES: | 2-[4-(2-Hydroxyethyl)-1 piperazinyl]-ethanesulfonic acid |
| HBSS: | Hanks balanced salt solution |
| hCG: | Human choriogonadotropin |
| hPL: | Human placental lactogen |
| hTf-2Fe: | Human diferric transferrin |
| IgG: | Immunoglobulin-G |
| $^{125}$I-hTf-(2Fe): | $^{125}$Iodine labelled diferric human transferrin |
| IRE: | Iron responsive element |
| IRE-BP: | Iron responsive element binding protein |
| kD: | Kilo dalton |
| $K_d$: | Dissociation constant |
| $k_{int}$: | Internalization rate constant |
| $k_{ext}$: | Externalization rate constant |
| $k_{bind}$: | Ligand-receptor binding rate constant |
| $k_{diss}$: | Ligand-receptor dissociation rate constant |
| KGM: | Keratinocyte growth medium |
| MEM: | Modified Eagles medium without L-methionin and L-glutamin |
| M199: | Medium-199 |
| NTA: | Nitrilotriacetate |
| PAGE: | Polyacrylamidegel electrophoresis |
| PBS: | Phosphate buffered saline |
| PMSF: | Phenyl-methyl-sulfon-fluoride |
| mRNA: | messenger Ribonucleic acid |
| RNase: | Ribonuclease |
| SD: | Standard deviation |
| SDS: | Sodium dodecylsulphate |
| SP-1: | Schwangerschaftsprotein-1 |
| TfR(s): | Transferrin receptor(s) |
| TCA: | Trichloric acid |
| Teric: | Polyoxyethyleine 9-laurylester (Polidocanol) |
| $T_{cycle}$: | Total cycle time |
| Tris: | Tris(hydroxymethyl)-aminomethaan |

## Chapter I. IRON AND THE PLACENTA.

### I-1. General Introduction.

On earth, iron has presumably played a role in the creation of circumstances suitable for life and finely man.[196,224] Not until approximately 2000 BC however, mankind became aware of the existence of the metal.

The earliest manuscript iron was mentioned in, originates from Egypt, 1500 BC. Iron was used as component of remedies for baldness and pterygium.[114]

Also in Greece iron was introduced which can be deduced from its role in the legend of Iphyclus (1200 BC).

Via weapons and tools iron became familiar in all layers of society and therefore not unlogically, it became associated with war and the god Mars.

The symptoms of severe (iron deficiency) anaemia were noticed by Lange in 1554.[255] The proposed therapy was: purging, bathing and even bleeding.[323] Also pregnancy was recommended ("if legally permissable").

Although the ancient physicians already suggested that iron was responsible for the colour of blood, it lasted until 1713 before Lemmery and Geoffry proved its presence in living tissues.[240]

Since then knowledge on iron metabolism has increased more rapidly with a climax in the 20$^{th}$ century. In 1832 a relation was shown between reduced iron contents in blood and what was called 'Chlorosis' (iron deficiency anaemia).[122] In 1925 it was discovered that in blood not all iron was haemoglobin related.[123]

The possibilities in iron research were extended significantly when $^{59}$Fe became available in 1938.

In 1946 an iron binding serum protein was discovered, which generally became known as 'transferrin'.[165,333] Many aspects of this protein have been investigated. Iron binding characteristics were explored (reviewed by: Aisen, 1980) and transferrin microheterogeneity was discovered.[6,103,257]

Other iron binding proteins (like the iron storage protein 'ferritin') were found and details of cellular iron uptake, via transferrin receptors and receptor mediated endocytosis were elucidated.[184,233,274]

The major discovery of the last decade was the relation between transferrin receptor

and ferritin synthesis via structures on their mRNA's, the so called 'Iron Responsive Elements'.[155]

Because of the rapid improvements in the field it is likely that in the (near) future major attention will be paid to the molecular biological and genetic aspects of transferrin, transferrin receptor and ferritin synthesis despite the fact that many questions at the biochemical level (e.g. cellular iron release) are still unanswered.

**I-2. Iron.**

Iron with its molar mass of 56 D plays a key role in a large number of biochemical processes and is therefore essential to almost every form of life. In humans iron has many functions.[77] Apart from its function in the transport of oxygen (via haemoglobin and myoglobin) iron is essential in electron transport (cytochromes, aconitase), catalytic enzyme functions (hydroxylases, mono-oxygenases) and DNA synthesis (ribonucleotide reductase).[290]

Of iron two main valence states occur, the divalent ferrous form ($Fe^{2+}$) and the trivalent ferric form ($Fe^{3+}$). In solutions and in the presence of oxygen (e.g. the human circulation) ferrous iron is rapidly oxidized to the ferric form, which, under physiological conditions (pH 7.4), is rapidly hydrolysed to insoluble polymeric hydroxides. The equilibrium concentration of free $Fe^{3+}$ is approximately $10^{-18}$ mol/l and the solubility product of $Fe(OH)_3$ only $4x10^{-38}$ mol/l.[364] Therefore in body fluids $Fe^{2+}$ or $Fe^{3+}$ can hardly exist as free ions, they have to be chelated by other molecules like proteins.

The total amount of iron in the human body is 3-5 grams. The majority is found in haemoglobin (70 %). Twenty percent is stored in ferritin and only 0.1 % is transported bound to transferrin. Intracellularly a small portion is bound by structures of low molecular weight.[19,265]

Due to the minimal loss of only 0.5-1.5 mg of iron daily, an avarage uptake of 1 mg iron per day is required. The iron stores are balanced by the uptake of iron in the duodenum, which can be stimulated by the supplementation of vitamin C.[152,271,331,367]

Iron is involved in a wide range of diseases. Deficiency of iron leads to anaemia and iron overload to haemochromatosis. In neonatal haemochromatosis a surplus of iron can be found in the liver and several other organs, although total body iron

does not seem to be increased.[1,213] Iron is held responsible for the inflammation in rheumatoid arthritis,[33,407] via its role in the production of hydroxyl radicals (Haber-Weiss reaction) which can damage DNA, proteins and membranes.[431] It has been suggested that iron, via the same mechanism, is involved in Multiple Sclerosis, and other diseases of the central nervous system.[185,244] Furthermore it reduces fertility,[391] and in the case of iron deficiency, it may cause preterm deliveries.[339] Iron shortage affects mental development (a process which is reversible).[32,40,177,253,287,362,414,418] It also decreases immune responses, of which the implications may be small because a lot of pathogens do need iron too.[78,232,425] Moreover, therapeutically enhanced serum iron levels increase the number of infectious diseases.[261]

**I-3. Transferrin.**

The major function of human transferrin (hTf) is the binding and transport of iron in the circulation. For the fetus it is the major, if not the only, source of iron.[121] Human transferrin is a serum ß-globulin of 80 kD. Studies on its structure and function have been reviewed many times.[e.g. 5,190] It is a monomeric glycoprotein with two domains (an N- and a C-site) both capable of binding one $Fe^{3+}$ atom (affinity constant: 1 to 6 x $10^{22}$ $M^{-1}$, for the N- and C-site respectively).[111] Other metal ions like $Zn^{2+}$, $Cu^{2+}$ and $Al^{3+}$ may be bound as well, for which, as for the binding of $Fe^{3+}$, the concomitant binding of an anion ((bi)carbonate) is required.[4] The binding of iron is pH dependent. At pH 5-6 transferrin rapidly loses its iron, which is of major importance in the cellular uptake of iron (see further).

The hTf gene is located on chromosome 3.[437] It is mainly synthesized in the liver. Production of transferrin, however, has also been shown in adult brains, Sertoli cells and fetal muscles cells.[243,341,356] mRNA for transferrin has been detected in placental homogenates suggesting the synthesis of hTf in the placenta.[341]

Transferrin blood concentration is 50-110 $\mu$mol/l which is increased by hormones, like estrogen, iron deficiency, and inflammation, for it is one of the acute-phase proteins.[146,178,237,268] About 24 hours after injection of turpentine hTf is increasingly synthesized.[283,340,392] Because the hTf-transferrin receptor interaction (see further) is affected by other acute phase proteins, a secundary effect cannot be ruled out.[136] The transferrin plasma pool is thought to be replaced every 12 hours.[197]

Transferrin iron saturation is approximately 30 %. Under pathological conditions, however, this can raise up to 100 % (haemochromatosis). In a growing fetus the hTf iron saturation is approximately 60 %, indicating an 'uphill transport' of iron through the placenta. Iron saturation declines to adult levels in 6 months.[261] Under these circumstances, four types of transferrin occur in the circulation: iron free 'apo-transferrin', two types of monoferric transferrin (iron bound to either one of the binding sites) and diferric transferrin.

The binding of transferrin to its receptor is highly specific but can be affected by ferritin in very high concentrations.[399] Diferric transferrin binds to the transferrin receptor with the highest affinity,[17,399,439] presumably because of the conformational change induced by iron binding.[139] This accounts for the binding to placental TfRs as well. Compared to eachother, the two types of monoferric transferrins show no difference in affinity.[159,161] Others could not detect any difference in affinity of apo, monoferric and diferric transferrin for the TfR.[51] This study was done on syncytiotrophoblast microvillous membranes, but its implications are unclear.

The C-terminal domain of hTf is glycosylated.[363] Most frequently, four glycan branches are present, but from four up to eight branches can be found (Figure I-1).[103,219] The terminal residue of these N-linked carbohydrate chains is sialic acid. From zero (asialo-transferrin) up to eight (octasialo-transferrin) sialic residues are found. Due to the number of branches and specially the number of sialic acids microheterogeneity can be seen when the protein is studied by iso-electric focussing.[189] Special patterns seem to be related to specific diseases.[118,190,191]

During pregnancy, the number of glycan branches on transferrin increases.[34,190] This is in contrast with the glycosylation of the total pool of placental surface proteins.[10] The relevance of this phenomenon is not yet understood, for, in guinea pig, it does not affect iron uptake by erythroid cells in the mother nor in the fetus.[89] In humans and in the guinea pig, the affinity of transferrin for its receptor even decreases with an increasing number of glycans.[90,238] It has been suggested that the increase in glycosylation of placental proteins may have a funtion in the inhibition of interactions between fetal trophoblasts and maternal leukocytes.[10]

A protein closely related to transferrin is lactoferrin, which also binds two iron atoms. Compared to transferrin the affinity of lactoferrin for iron is much higher and

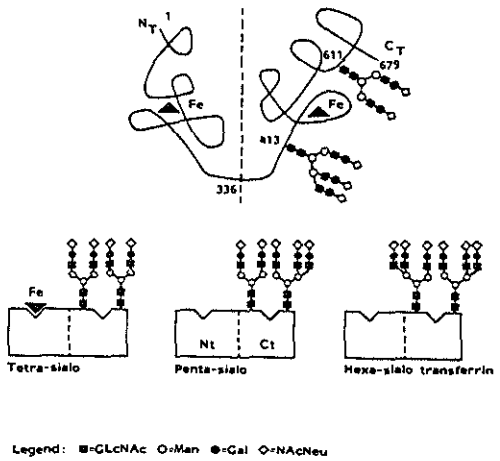


**Figure I-1. Transferrin**
Schematic drawing of the structure of human transferrin, with its C- and N-terminal domains. Three examples of the microheterogeneous forms of transferrin are shown. Most of the transferrin present is in the tetra-sialic form. (Drawing from: de Jong, van Dijk and van Eijk, 1990.[190])

is not pH dependent. This results in an antimicrobial activity even if lactic acid has been released by microbial cells and/or stimulated leukocytes.[150] Lactoferrin does not seem to play any role in transplacental iron transport.[21,289,388]

Other iron binding serum proteins are haptoglobin and haemopexin. Haptoglobin binds free haemoglobin to enable the liver to take up and reuse the haem bound iron before it is lost into the urine. Serum haptoglobin concentration can be used as a standard of intravascular haemolysis. Free plasma haem is bound by haemopexin, also to prevent it from being secreted. Like haptoglobin.[205] haemopexin does not seem to play a role in the regular iron transport. Nevertheless, haemopexin receptors can be isolated from human placentae,[380] and the expression of transferrin receptors is down-regulated by haemopexin.[381]

### I-4. Ferritin.

Ferritin is the major iron storage protein.[282] The protein is assembled of 24 polypeptide subunits which form a symmetrical shell (Figure I-2). In this shell iron is stored as a complex: $(FeOOH)_8(FeO\text{-}OPO_3H_2)$. Maximally 4500, but normally 2000-2500 atoms of iron are stored. The storage of iron in ferritin is very efficient, and, it

seems likely that for that reason the protein is widely distributed in nature.[9,389]

Two types of subunits exist: heavy (H) and light (L) with molar masses of 21 and 19 kD respectively.[11,98] Unclear is the report of a third, glycosylated, 'G'-subunit.[76]

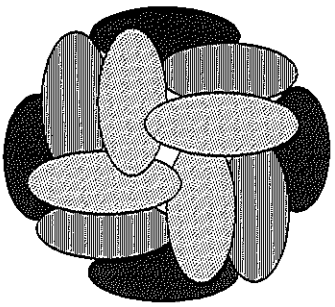

**Figure I-2. Ferritin.**
The ferritin molecule consists of 24 subunits which form a hollow shell. Iron enters the molecule via specific channels in between these subunits.

The combination of these ferritin subunits varies per organ, which causes microheterogeneity and immunological differences.[153,267] H-subunit-rich ferritins are found in the heart, HeLa-cells and the placenta, whereas liver and spleen ferritins consist predominantly of L-subunits. There may be functional differences between the H- and L-subunits.[98] L rich ferritins are more stable because of a salt bridge between amino acid Lys[62] and Glu[107].[329] The H rich ferritins usually have lower iron contents but can handle iron more rapidly.[41,98] Nevertheless, L-subunits have a co-operative role in the uptake of iron.[242] Waldo et al. (1991) showed that H-isoferritins take up iron more easily because of the formation of Fe(III)-tyrosinate complexes.[411] Wada et al. (1991) suggested that an appropriate spatial charge density across the cavity surface rather than specific amino acids are required for the uptake of iron by ferritin.[410]

The differences between the subunits are reflected in their intended functions.[12] L rich ferritins are thought to be long term storage proteins where H rich ferritins may have a function in cell-protection.[151] H rich ferritins also have a cytokine function inducing downregulation of cell proliferation.[52] In the light of these differences it is not surprising that H- and L-subunits are preferentially synthesized in response to different stimuli like inflammation and iron overload.[41,239,313]

The majority of ferritin is located intracellularly.[330] Approximately 30 % of the liver ferritin, however, is specially synthesized to be secreted into the circulation,[254] but not all evidence for this hypothesis is conclusive.[247] Ferritin blood concentrations are

67-533 pmol/l for males and 22-311 pmol/l for females (Dijkzigt, University Hospital Rotterdam). Its iron saturation is rather low (400-450 atoms of iron per ferritin molecule).[434] Ferritin was also detected intra-articularly, and in seminal plasma.[33,225]
The cellular uptake of circulating ferritin might take place via receptors, for specific receptors have been detected on placental brush-border membranes, and T and B lymphoid cells.[7,379]
Ferritin synthesis is controlled at the level of translation (see further). It is digested in secondary lysosomes and its half life is 31-58 h depending on sex and age.[246] In iron overload it is 'degraded' to haemosiderin.[31,318,320,426,435]

### I-5. Transferrin receptor

The first step in the delivery of iron to the cell is the binding of transferrin by the transferrin receptor (TfR).[108,184] The TfR is a transmembrane glycoprotein composed of two identical disulfide bonded isomeres of 95 kD (Figure I-3).[108,169,266,297,396,397]
Transferrin receptors can be detected on almost every cell but are mainly expressed by proliferating cells and cells requiring iron for specific purposes, like reticulocytes and syncytiotrophoblasts.[125,128,248,409] There is one report of a second type of transferrin receptor on hepatocytes with a low affinity for hTf but non-saturable.[18] Remarkable is the fact that no TfRs are found on balicytotrophoblasts, the proliferating precursors of the syncytiotrophoblasts.[168]
The number of surface TfRs are affected by cell-differentiation,[64,162,279] cell-activation,[145] and a large group of different substances like iron, aluminium, haemopexin, and vitamin D.[35,266,381,382,386]
Transferrin receptors are located on the cell surface as well as intracellularly. This intracellular receptor-pool consists of TfRs being synthesized,[295] TfRs taking part in the endocytic cycle (see further) and possibly non-functional TfRs in storage-pools.[417] Distribution of receptors among these pools can be influenced by, for instance, insulin, cell-differentiation, and anti-TfR antibodies.[106,383,427]
Non-membrane bound TfRs are present in serum.[27,74,75,172] The plasma TfR is most likely a monomeric truncated form, lacking the cytoplasmic and transmembrane domains of the intact receptor.[347,387] Serum TfR concentration is a measure of erythropoiesis,[27,172,215,355] and can be used as standard for tissue iron stores.[75,355,394]

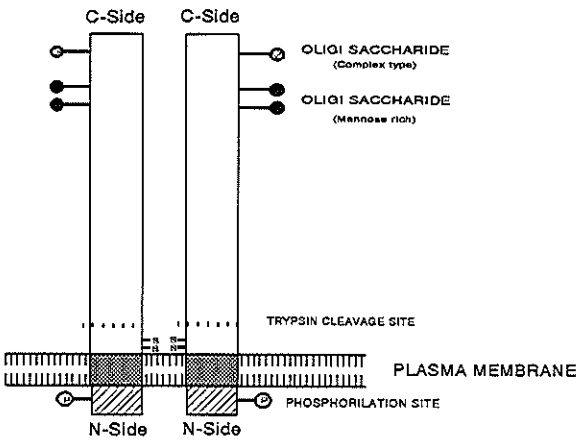


**Figure I-3. Transferrin receptor.**
The transferrin receptor consists of two identical polipeptide structures of 95 kD, both capable of binding one transferrin molecule. Oligo saccharides are present at the C-terminal domain, and at the N-terminal domain a phosphorilation site is present. (The drawing was adapted from Testa, Pelosi and Peschle)[387]

It can also be used as an indicator of iron deficiency.[120,172,353] Serum TfR concentration increases during pregnancy, especially in the third trimester.[28,217]
Finally, TfRs may play a role in the protection of syncytiotrophoblasts from maternal immune responses.[116]

### I-6. Cellular iron uptake.

In humans the major iron source of cells is transferrin. Small amounts of iron are delivered to the cell via ferritin, haemopexin and haptoglobin. The latter proteins, however, do not seem to have a structural role in cellular iron-supply.[205,401] In vitro inorganic iron compounds can also deliver iron to cells.[179,229,374]
The uptake of iron starts with the binding of transferrin by its receptor.[170,183,184] From there on two mechanisms are suggested for iron to enter the cell. Firstly, iron is released at the cell-surface and enters the cell via a 'membrane binder',[161,275,404] and, secondly, iron enters the cell via a process generally known as 'receptor mediated endocytosis'.[69,193,274,317]
During the first steps in this process the hTf-TfR complexes are clustered in clathrin

coated pits.[147,304] These pits function as molecular filters.[46] For this highly efficient uptake, TfRs require specific structures in the cytoplasmatic tail.[398] Second messengers do not seem to be functional.[344] Next, these clathrin coated pits are internalized by formation of endosomes.[317] Of the surface TfRs, 10-15 % is internalized each minute.[147,422]

Controversy exists over the necessity of occupation of the TfR for internalization.[209,366,421]

Because different receptors with different destinations are endocytosed via the same endosome, sorting must take place intracellularly.[296,368,369] It has been shown that even TfRs follow different routes.[202,360]

Intracellularly, endosomes become acidic, a condition that is maintained by an ATP-dependent proton pump.[124,314,317,337] At pH 5.0-6.5, Fe is released and subsequently transported to the cytoplasm by a process unknown so far. Nevertheless, we know that the TfR plays an important role in the intracellular release of iron, and minimizes nonspecific iron loss at the cell surface.[20]

The apotransferrin remaines tightly bound to the receptor and the entire complex is recycled back to the cell-surface.[79] Due to the environmental pH of 7.4, hTf dissociates from the TfR leaving both ready to be reused.

The TfR recycle time varies. Recycle times of 3 min have been found,[176] 6 min,[68,208] 14-20 min[106] up to 32 min[148] depending on cell type and culture conditions.[106]

Receptor-mediated endocytosis is not specific for TfRs. For instance asialoglycoprotein, haemopexin and insulin receptors recycle as well.[68,357,358,384]

In the cytoplasm iron is bound by ligands of a low molecular weight pool. Most likely this pool consists of a mixture of small iron chelating molecules.[19,180,182,423] It has been claimed that the low molecular weight pool does not participate in the cytoplasmic transport of iron.[408] Their results seem compatible with a steady state situation, which leaves open the possibility of a functional low molecular weight pool.

Depending on the function of the cell, iron is transported via the low molecular weight pool to cellular organels, ferritin or - in polarized cells - to the 'opposite' side.[296,343]

### I-7. Cellular iron homeostasis.

Cellular iron uptake and storage should be regulated very carefully. Cells do need iron but a surplus of free iron could be dangerous. To solve this problem cells possess a highly specific regulation mechanism.

Both the synthesis of TfRs (iron uptake) and ferritin (iron storage) are controlled at translational level via so called "Iron Responsive Elements (IRE's).[155,156] IRE's are 28 nucleotide stemloops in the 3' and 5' region of TfR and ferritin mRNA respectively (Figure I-4);[280] structures with a characteristic six-membered loop (CAGUGX).[61] These structures are also present on mRNA's of other proteins, not primarily involved in iron metabolism.[71,203]

TfR mRNA contains multiple IRE's,[280] whereas only one IRE mediates the iron dependent control of ferritin mRNA. The effectiveness of the latter IRE depends on the spacing between the 5' terminus of the ferritin mRNA and the IRE.[133,134]

An "Iron Responsive Element-Binding Protein" (IRE-BP) can bind to the IRE.[23,325,377] This IRE-BP is also known as "Ferritin Repressor Protein" (FRP) and "Iron Regulating Factor" (IRF). The IRE-BP, identified as a ≈90-kD protein, shows

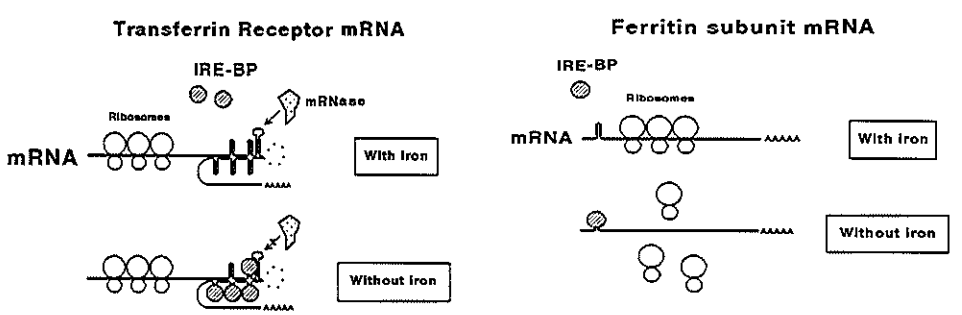


**Figure I-4. The Iron-Responsive-Element hypothesis.**
In the left graph is depicted the effect of the IRE-BP on transferrin receptor mRNA translation. Without iron, also the affinity of the IRE-BP for the IREs, located at the 3' side, increases, which makes the mRNA less assessable for degradation enzymes (polygonal structure).
The right graph shows the effect of the binding of the Iron-Responsive-Element-Binding Protein (IRE-BP) (filled circle) on ferritin subunit mRNA translation. In case the intracellular iron concentration is low, the affinity of the IRE-BP for the IRE located at the 5' side, increases and ribosomes are hampered to start mRNA translation. With iron, ribosomes can approach the ferritin subunit mRNA without difficulties.

similarities with aconitase, a protein of the citric acid cycle.[140,199,210,327] This connection between iron uptake and energy production might contain a clue for the growth stimulating effects of iron. Aconitase mRNA is transcribed from a single gene on chromosome 9.[327] By oxidation sufficient or high intracellular iron levels alter the conformation of the IRE-BP,[157] which changes the Fe-S cluster of the protein.[140,141] This change reduces the affinity of the IRE-BP for the IRE followed by enhanced degradation of the IRE-BP. Also hemin is thought to have a specific effect on the IRE-BP via a specific binding site on the protein,[377] and causes Friend erythroleukemia cells to preferentially synthesize H-subunits.[70]

Depending on the location of the IRE, dissociation of the IRE-BP has different effects. Ferritin subunit mRNA, with its IRE's at the 5' side, becomes available for translation. TfR mRNA, with its IRE's at the 3' side, is degraded more rapidly.[281] By this mechanism high intracellular iron levels will lead to reduced TfR synthesis and elevated ferritin synthesis. The reduction in TfR numbers and the increase in ferritin will protect the cell from free iron.[223] Iron shortage has the opposite effect.[259] One of the consequences of this regulation mechanism is that the amount of cellular mRNA is not directly related to the amount of protein synthesized.[239,325]

The control of iron homeostasis via IRE's is present in several cell-types, but has not yet been demonstrated in syncytiotrophoblasts. In most of the studies on iron homeostasis, iron was supplied in inorganic compounds (like nitrilotriacetate iron or ferric ammonium citrate) in stead of a more physiological form (like transferrin). This is of special importance, because the cellular handling of iron, applied in different compounds varies.[70,91,92,181]

**I-8. Placenta characteristics.**

In mammals, fetuses are supplied with nutrients via the placenta. The morphology of the placenta differs per species and can be classified on basis of shape or the number of tissue layers separating the maternal and fetal circulation.[137,138,204,278,365,412] According to the latter classification four types of placentae occur: the epitheliochorial placenta (six (cell-)layers separate the maternal and fetal circulation; horse, pig, cattle), the syndesmochorial placenta (five (cell-)layers; sloth), the endothelial placenta (four (cell-)layers; some bats, cat, dog) and the haemochorial

placenta (three layers; man, rodents, rabbit, guinea pig, bat). The human placenta (placenta discoïdalis) has a tree-like structure (Figure I-5). Villi are surrounded by maternal blood. The exchange area is enlarged via an increase in the number of villi during pregnancy,[351] while microvilli on the apical cell-membrane of the syncytium further enlarge the surface area (up to 14 $m^2$).[230]

The human placenta is haemochorial, which implicates that in the mature placenta only three (cell-)layers separate the maternal and fetal circulations (Figure I-5): the syncytium, formed by syncytiotrophoblasts, the fetal endothelial cells and in between the connective tissue.

The syncytium, a structure with many nuclei and no intercellular membranes, is in direct contact with the maternal blood. It is formed by differentiation and fusion of

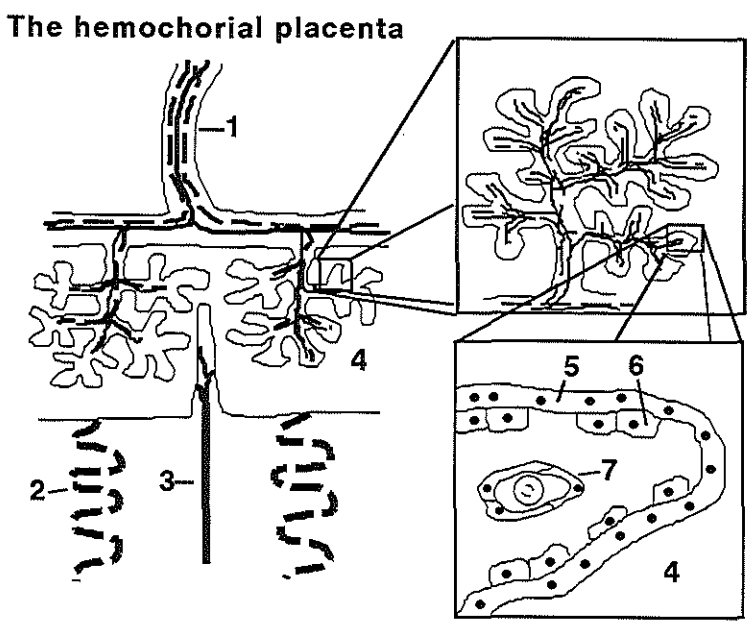


**Figure I-5. The haemochorial placenta.**
Schematic drawing of the tree like structure and the histology of the human haemochorial placenta. The figures represent 1: Umbilical cord, 2: Spiral artery, 3: Vein, 4: Intervillous space, 5: Syncytium formed by syncytiotrophoblasts, 6: Cytotrophoblast, 7: Fetal capillary with one erythrocyte.

the underlying cytotrophoblasts.[107,351] Both syncytio- and cytotrophoblasts together represent approximately 13 % of the placental weight.[227]

Despite the close relationship between cytotrophoblasts and syncytiotrophoblasts, many differences exist between these cells. In contrast to syncytiotrophoblasts, cytotrophoblasts are proliferative throughout pregnancy.[53,351] It has been suggested that cytotrophoblasts undergo up to four amplification divisions before they fuse with the syncytium.[351] The cytotrophoblast-syncytiotrophoblast ratio does not change during pregnancy,[351] which could be explained in part by the loss of syncytiotrophoblasts into the maternal circulation.[312]

Syncytiotrophoblasts produce specific hormones: human chorion gonadotropin (hCG), human placental lactogen (hPL), progesterone, human pregnancy-specific $\beta_1$-glycoprotein and schwangerschaftsproteins (SP's),[67,97,119,166,207,291,419,420] whereas cytotrophoblasts produce hCG-releasing hormone and inhibin.[201,307,309] cAMP affects hormone production in general,[292,308,321,400] and hCG specifically stimulates the production of progesterone by syncytiotrophoblasts.[63]

For this thesis, one of the major differences is the expression of transferrin receptors (TfRs) by syncytiotrophoblasts.[35,96,117,126,127,302] Though proliferative throughout pregnancy, both first and third trimester villous cytotrophoblasts lack TfRs.[53,54,168] Only on the proximal portion of cytotrophoblast columns, an area of high proliferative activity, TfRs were detected.[53] In the mouse labyrinthine placenta, TfRs are expressed primarily on the differentiated trophoblast cells.[96] In the normal human placenta, TfRs are located on the apical cell-membrane,[117] which is indirectly suggested by the fact that transplacental iron transport is saturable from the maternal side.[85]

Vanderpuye Kelly and Smith showed the presence of TfRs in isolated basal membranes of the syncytial layer.[403] The function of these receptors is puzzling. The transport of iron is clearly one-directional, from mother to fetus. Perfusion experiments with the guinea pig placenta showed an equally low retention in the placenta of albumin and diferric transferrin after bolus application at the fetal side, suggesting the absence of hTf TfR binding.[85] There is some evidence for transferrin synthesis in the placenta.[341] Maybe this hTf is involved in intracellular iron transfer. In that case basal TfRs might mediate iron transport across the basal membrane.

**I-9. Placental transport.**

In all mammalian species the placenta exercises many functions, not in the least the transfer of nutrients and gases from mother to fetus.

The transfer of nutrients can be mediated by specific membrane components like carriers and channels, but can also occur without specific membrane components. In the latter case transfer is purely diffusional, downhill a concentration gradient. In the former case transport can be both active as well as inactive.[220] Examples of the non-mediated diffusional transfer are water, respiratory gases, urea, uric acid, hypoxanthine and in general small lipophilic metabolites. Transport mediated by carriers or channels is called passive (facilitated) if the driving force is a downhill electrochemical gradient, and active if it is directed against an electrochemical gradient. Examples of actively transferred metabolites using carriers or channels are $Ca^{2+}$, $Mg^{2+}$, $Fe^{3+}$ and nearly all aminoacids.[83,87,121,221,373]

When the diffusional capacity of the interface is the limiting factor for transplacental transport the transfer is called: diffusion (membrane) limited (uric acid, hypoxanthine). When a transport process is flow limited, it is the blood flow which is the limiting factor.[112,113,269,338] Examples of the latter type of transport are water, ureum, and antipyrine. Active transport of nutrients is usually rapid, so that flux rates become flow dependent.

Nutrients are transferred transcellularly (crossing the cellular membrane) or paracellularly. Evidence for paracellular transport has been obtained in the rabbit,[372,373] and the guinea pig,[198,392] although continuity of these structures with both sides of the syncytiotrophopblast has not been prooved yet.[198] These paracellular channels are believed to play a role in the transfer of small inert hydrophilic molecules.[372]

Factors affecting placental transport processes are maternal and fetal blood flow rates, the size of the exchange area, the type of transport mechanism, diseases and toxic agents. In the third trimester placental transport capacity rises exponentially. This is most likely caused by the maturation of transport processes because the placental growth curve flattens earlier than the fetal.[256,328] Examples of diseases and toxic agents affecting placental transport functions are diabetes mellitus and cadmium.[299,310,395,402]

**I-10. Placental iron transport.**

The mechanism of maternal-fetal iron transfer depends on the structure of the placenta (see Placenta characteristics).[204,342] Mechanisms involved are phagocytosis of maternal erythrocytes in endotheliochorial placentae (sheep, some bats), absorption of iron by the yolk sac (rat and other rodents), absorption of iron rich uterine secretions by accessory placental structures (pig) and absorption of maternal transferrin-bound iron in haemochorial placentae (human, guinea pig, rabbit).[204]

Already in 1937 a relationship between pregnancy and serum iron concentration was assumed.[154] In man, the major, if not the only, source of iron for the fetus is maternal transferrin.[121] Also in rat, rabbit and guinea pig, Tf is involved.[84,85,378] There are reports on the existence of a ferritin receptor in the placenta as well,[379] though this could be the TfR for this receptor also binds ferritin.[17] The guinea-pig placenta takes up ferritin via a process compatible with receptor-mediated endocytosis.[228]

In human pregnancy, 250-300 mg of iron is transported,[57] which is approximately 25 % of the maternal iron stores.[305] The majority of this transport takes place in the third trimester.[121] In rats iron is incorporated mainly in the fetal liver.[102] In man, at term, transplacental iron transport is a fast process and, by that time, the placenta hardly stores any iron.[311]

The enormous amount of iron transferred to the fetus has its implications for the mother.[143] Maternal iron deficiency does not seem to affect the iron status of the fetus, although it increases the ratio of placental weight to fetal weight.[132] Furthermore, the lowest haemoglobin levels are associated with the highest birthweights and the largest placentae.

In man there is clearly an optimal maternal hematocrit for pregnancy outcome.[129] Other parameters like serum ferritin and transferrin concentrations may be used as a standard of maternal iron status.[60] However, because of the day-to-day variation, at least 3 to 10 measurements are required to accurately determine these parameters.[3,42]

The guinea pig placenta is autonomous in iron uptake; if fetuses are removed the placenta continous with the uptake of iron.[131,256,432]

In the guinea pig, rat, rabbit and human placenta, transplacental iron transport is a

one way active process.[16,43,234,273,293,432] Transferrin iron saturation is higher in the fetal circulation.[352] In rabbit, guinea pig and human maternal transferrin is endocytosed via receptor mediated endocytosis.[17,72,88,94] In man, iron dissociates from transferrin before it is transported to the fetus.[100] After iron is released from transferrin into the cytosol, it is most likely bound by elements of the low-molecular weight fraction. Although arguments in favour of the presence of this iron-pool have been obtained in human, guinea pig and rat,[72,85,256,432] its composition is still unclear. Many candidates have been mentioned, among which lactate.[180] Lactate is produced in large quantities by the endotheliochorial sheep placenta,[55] but not by the haemochorial human placenta.[334] Most likely, in human, lactate does not participate in transplacental iron transport, because it is mainly transferred from the fetus to the mother.[334]

Brown et al. (1979) showed that human placental ferritin has tissue specific antigenicity, and most likely consists of several isoferritin populations.[50] These isoferritins differ in subunit composition.[235] Five isoferritins have been separated by DEAE-Sephadex A-25 chromatography.[218] Because of the tissue specific antigenicity, anti-placental ferritin antibodies can be obtained which could be helpful in the assessment of toxaemia of pregnancy.[262]

Syncytiotrophoblast ferritin might play a specific role in transplacental iron transport.[86] It is present in all layers of the trophoblast, especially near the surface.[30,50,294] In the haemophagous badger placenta the maternal-fetal iron transport is even regulated via ferritin.[99]

The publications on the consequences of maternal iron status for fetal iron stores are contradictory. Singla, Chand and Agarwal reported reduced fetal iron levels in infants of anaemic mothers.[352] Unfortunately, their publication does not explain in detail the procedure used to obtain umbilical blood samples, leaving open the possibility of contamination of cord blood with maternal blood. Others did not find any differences between infants born from anaemic or healthy mothers.[322,413] In rats, seriously iron-deficient mothers gave birth to iron deficient pups.[231] Except for the nervonic acid to lignoceric acid ratio in the brain sphingomyelin, the effects were rapidly reversable by iron-supplementation. Experiments in rabbit and rat suggest the absence of an efficient short-term control mechanism for the reactions on both

induced iron overload as well as iron deficiency.[43,258,272] (Lane, 1968) A long-term regulation mechanism could be present because alteration of maternal iron stores before mating did not change the transfer of iron to the fetus.[272]

The influence of the fetus on placental iron transport is unknown. Fetectomy experiments in rats suggested the absence of a short-term regulation mechanism,[43,263] but similar to the effects of maternal iron supplies, long-term control is not excluded[87]

In human, transplacental iron transport control seems to guarantee sufficient iron supplies to the fetus. It has been suggested that maternal iron deficiency anaemia could increase the risk of preterm delivery.[339] On the other hand, specially preterm infants often have insufficient iron stores.

Protection of the fetus from iron overload appears less well developed.[24,39,225,316,371] Pathological processes affecting the placenta, like diabetes mellitus, may have their impact on the distribution of iron in the fetus.[310] However, these results were obtained from neonates who died within 7 days postnatally.

**I-11. Fetal iron metabolism.**

Among others, the fetus requires considerable amounts of iron for cell growth and haem synthesis. Under normal conditions, and even if maternal iron stores are depleting, the fetus acquires adequate amounts of iron. In the fetal circulation iron is bound by fetal hTf with the same characteristics as adult hTf.[405] Fetal hTf transports iron to the liver where it is stored in ferritin,[121] or used in erythropoiesis.[82,83]

In human pregnancy, fetal ferritin levels increase from 17.7 $\mu$g/l (18-20 weeks) to 56.8 $\mu$g/l (32-35 weeks).[59] At term fetal serum hTf concentration is low. In combination with high hTf saturations, these parameters reflect sufficient iron stores. Postnatally serum ferritin levels increase, most likely because of the pause in erythropoiesis.[350]

The effects of marginal iron stores at birth (preterm infants) have been subject of investigation. Despite lower total body iron stores, compared to term neonates, preterm infants are at risk to develop iron overload shortly after birth. Not only do they often receive blood transfusions, but iron absorption is, regardless of the needs, proportional to the dietary iron contents as well.[345] High blood iron levels are

associated with oxygen radical injury in preterm infants.[376] and infections occur frequently if iron is artificially supplied.[22,261] Inside the uterus the child is loaded with iron, but shortly after birth it seemes to be protected from it. Due to rapid growth, haem synthesis, and inadequate iron uptake in the gut, iron stores could be depleted again in several months.[253,287,345,414] The influence of the fetal iron status on transplacental iron transport is unknown (see: Placental iron transport).

In neonatal haemochromatosis fetal iron metabolism is disturbed. Livers of these patients are overloaded with iron, which might be present in organs not primarily involved in iron storage or erythropoiesis as well,[213] but total body iron contents are not increased, suggesting that the primary pathogenesis is not an excess of transplacental iron transport.

**I-12. Scope of the thesis.**

For many years transplacental iron transport is subject of investigation. The results obtained strongly suggest that in the placenta some kind of transport regulation mechanism is present, but the location of this mechanism is obscure. We do know that transferrin receptors are expressed by syncytiotrophoblasts and that receptors densities are affected by iron. But, does this also mean that the amount of transported iron is influenced as well?

In syncytiotrophoblasts ferritin is available in large quantities, but to what purpose? Does it only store iron for direct cellular needs? Does it prevent iron from being transferred to the fetus? And what is the role of IRE's in syncytiotrophoblasts, if they are present?

These are the subjects this thesis will deal with. After a general introduction (Chapter I) and an explanation of the materials and methods generally used (Chapter II), the in vitro culture of cytotrophoblasts will be discussed (Chapter III). TfR-expression and its regulation are the subjects dealt with in Chapter IV. In Chapter V iron uptake will be described, followed by the role of ferritin (Chapter VI). Finally the place of the IRE's in the transplacental iron transport process will be discussed (Chapter VII).

## Chapter II. MATERIALS AND METHODS.

### II-1. Chemicals.

Calcium and magnesium free solution of Earle's Balanced Salts (EBSS), Fetal Calf Serum (FCS), Dulbecco's Modification of Eagle's Medium with 20 mM HEPES (DMEM-H), Medium-199 (M199), Hanks Balanced Salt Solution, translabelled $^{35}S$-Methionin, penicillin, streptomycin and amphotericin were obtained from ICN Biomedicals, Zoetermeer, NI. Keratinocyte Growth Medium (KGM) was obtained from Clonetics USA, via Instruchemie Hilversum, NL. 2-[4-(2-Hydroxyethyl)-1 piperazinyl]-ethanesulfonacid (HEPES) and 1,4-Diazabicyclo[2,2,2]octan (DABCO) from Merck Nederland BV (Amsterdam, NL). Ethylenediaminotetraacetate-disodium (EDTA) from Siegfried SA, Zofingen, CH. Modified Eagles Medium without L-methionin and L-glutamin (MEM) from Gibco, Live Technology, UK. Gentamicin from Schering Corp., USA. Trypsin (1:250 t.c.) and propidiumiodine from Sigma Chemical Company, St. Louis, USA. DNase grade II, dispase and collagenase from Boehringer Mannheim (D). Apo-transferrin from Behringwerke, Marburg, Germany. $^{59}FeCl_3$ and $Na^{125}I$ from Radiochemical Centre Amersham, UK. Iodo-Gen and Pierce Micro BCA Protein Assay Reagent from Pierce Europe BV, Oud Beijerland, NI. Percoll, Agarose EF, Sepharose 4B, Protein A-Sepharose CL4B were obtained from Pharmacia (Uppsala, S). Centricon-10 microconcentrators from Amicon (Grace BV, Rotterdam, NL). All chemicals were of the highest purity available.

### II-2. Monoclonal antibodies.

Anti-desmoplakin I/II antibody was obtained from ICN-immunobiologicals (Zoetermeer, NL). Fluoresceine-isothiocyanate (FITC) labelled rabbit-anti mouse IgG from DAKO Corporation (Santa Barbara, USA).

### II-3. Trophoblast-cell isolation.

Normal human placentae were obtained from the Department of Obstetrics, University Hospital Rotterdam/Dijkzigt, Rotterdam, within half an hour after spontaneous delivery. These placentae were processed according to either the procedure as described by Bierings et al. (Procedure A),[36] or by a procedure based

on that of Karl et al. (Procedure B).[194,195]

In procedure A the cell-isolation technique of Hall et al., (1977) and Kliman et al. (1986) was modified by the addition of $CaCl_2.2H_2O$ (1 mM) and $MgSO_4.7H_2O$ (0.8 mM) to the enzyme solution.[36,142,211]

Procedure B was performed as follows. A thin layer of endometrial tissue was discarded and a total of 60-70 gram villous tissue was cut out from the maternal side of the placenta. These villi were washed in 0.15 M NaCl (4°C), minced and exposed to an enzyme solution consisting of 100 ml $Ca^{2+}$, $Mg^{2+}$ free Hanks solution, 2.5 U/ml dispase, 25 mM HEPES, pH 7.4 for 40 min at 37°C. Collagenase (12 mg) was added directly to the villi/enzyme solution mixture, followed by prolonged incubation for 15 min at 37°C. Digestion was stopped by the addition of 100 ml $Ca^{2+}$, $Mg^{2+}$ free Hanks solution supplemented with 25 mM HEPES and 2 mM EDTA. The obtained cell-suspension was serially filtered through wire mesh (pore size from 1000 $\mu$m to 90 $\mu$m) and centrifuged (10 min, 750 g). The pellet was resuspended in approximately 4 ml $Ca^{2+}$, $Mg^{2+}$ free Hanks solution with 25 mM HEPES, and layered on top of Percoll gradient. This Percoll gradient was made from 70 % to 5 % Percoll ($^v/_v$) in 5 % steps. Highly purified cytotrophoblasts were obtained from the middle of the Percoll gradient (density, 1.048-1.062 g/ml).[35,212] The gradient was centrifuged for 20 min (900 g). The middle section, in between the erythrocytes (bottom) and the cell-debris (top), was roughly removed and washed with $Ca^{2+}$, $Mg^{2+}$ free Hanks solution containing 25 mM HEPES. DNase (approximately 10 mg) was added to the cell-suspension followed by incubation for 5-10 min at 37°C. Subsequently the cell-suspension was layered on top of a second identical Percoll gradient and centrifuged for 20 min (900 g). Cytotrophoblasts were carefully removed by fractionation of the gradient. Finally the were washed twice with $Ca^{2+}$, $Mg^{2+}$ free Hanks solution, containing 25 mM HEPES.

By these procedures a cell-population is isolated consisting of cytotrophoblasts for at least 95 %.[194,211] This has been reconfirmed in our laboratory by immunocytochemical staining with a panel of monoclonal antibodies (CD3, CD5, CD14, CD15, CD20, leucocyte common antigen CD45, and the trophoblast specific antigens ED 235 and ED 341 both from S.F. Contractor, London UK permitting positive as well as negative identification.[35,73] Furthermore the percentage of hCG,

hPL and SP-1 producing cells was revealed by immunocytochemical staining.[35,37] Cells obtained were counted (using a Bürker counting-chamber) and, depending on the type of experiment either directly used or diluted to 6 x $10^5$ cells/ml in culture medium. Of this cell-suspension 2.5 and 7.5 ml was plated out in 35 and 60 mm Falcon culture dishes, respectively (Greiner and Söhne, FRG). Except where otherwise specified culture medium consisted of 80 volume % M199 (Flow Labs); 20 volume % FCS; 4 mM L-glutamine; 0.3 mg/ml gentamicin; 50.0 IU/ml penicillin; 50.0 $\mu$g/ml streptomycin and 2.50 $\mu$g/ml amphotericin. Osmolality was 280-300 mOsmol/kg, pH 7.4. The medium was sterilized by filtration on a 0.22 $\mu$m Millipore-GS filter (Millipore SA Molsheim, F). Cell-cultures were incubated at 37 °C in humidified 5 % $CO_2$/95 % air.

**II-4. Cell-culture conditions.**

In general the cells were allowed to recover from the isolation procedure for 18-24 hours. Subsequently, to remove non-adherent cells, the culture dishes were washed twice with M199. In a few experiments the cells were allowed to recover for only 2½ hours but otherwise identically handled. Cell culture was continued at 37°C in humidified 5 % $CO_2$/95 % air in fresh, identical culture-medium or fresh medium supplemented with 1.25 $\mu$M human diferric transferrin (hTf-(2Fe)), 10 $\mu$g/ml ferric ammoniumcitrate (FAC) or 50 $\mu$M desferrioxamine (Df).

**II-5. Transferrin iodination.**

Diferric transferrin was obtained by full saturation of human apotransferrin, using iron-nitrilotriacetate (Fe-NTA) (ratio 1 mol Fe to 2 mol NTA) and a tenfold molar excess of bicarbonate as the synergistic anion (buffered in 0.1 M Tris-HCl, pH 8.2). The excess of Fe-NTA was removed on a PD-10 Sepadex column (Pharmacia, Uppsala, Sweden) and by extensive dialysis against Tris-HCl buffer (pH 8.2). Transferrin iron saturation was checked by measuring the E470/E280 ratio which was always close to 0.045, indicating full saturation. One mg diferric transferrin was incubated with $^{125}$I (0.5 mCi) for 20 min at room temperature in a glass vial coated with 100 $\mu$g Iodo-Gen. Free $^{125}$I was separated from radiolabelled transferrin on a PD-10 Sephadex column, followed by extensive dialysis against PBS (pH 8.2). The

final specific activity of the diferric $^{125}$I-labelled transferrin varied between 230 and 535 x $10^6$ CPM/mg protein.

**II-6. Saturation of transferrin with $^{59}$Fe.**

To saturate transferrin with $^{59}$Fe the same procedure was used as described for non-radioactive iron. $^{59}FeCl_3$ was added to nitrilotriacetate (NTA) in a molar ratio of 1 to 2. This solution was together with a tenfold molar excess of bicarbonate as the synergistic anion added to a solution of apo-transferrin (iron to transferrin molar ratio 1 to 2). The reaction was buffered in 0.1 M Tris-HCl (pH 8.2). After 20 min free NTA-iron was removed on a PD-10 Sepadex column and by extensive dialysis against Tris-HCl buffer (pH 8.2). Transferrin iron saturation was checked by measuring the E470/E280 ratio.

**II-7. Protein determination.**

A homogenous sample was obtained by sonication for 10 seconds on melting ice. Protein concentration in the samples containing distilled water was determined according to Bradford.[45]

The samples containing Triton X-100 were analysed according to Lowry et al.[252] with the modification of Wang and Smith,[415] or, if protein concentrations were expected to be low (< 0.050 $\mu$g/ml), with the Pierce Micro BCA Protein Assay Reagent. Bovine serum albumin was used as standard.

**II-8. DNA-determination.**

DNA-contents of the samples were determined using the Nuclesan-100 kit (Sanbio BV, The Netherlands). This test is based on mitramycin.[173]

**II-9. ß-hCG determination.**

Culture medium ß-hCG concentrations were measured using an ES-600 auto-analyzer (Boehringer Mannheim, D). The medium was removed at indicated times, and replaced by identical culture medium. Prior to analysis the medium was centrifuged for 5 min at 1200 g. Supernatants were used for ß-hCG determination. The origin of the hCG was determined by immuno-cytochemical staining (all

reagents from DAKO Corporation, Santa Barbara, USA).[35]

### II-10. Statistics.

To estimate the significance of the results, the Student's t-test was used in experiments with only two groups. In experiments with more than two groups the Student-Newman-Keuls test was used. In this test the outcomes are corrected for multiple comparisons. The Chi-square test was used for the experiments on receptor distribution.

## Chapter III. CYTOTROPHOBLAST DIFFERENTIATION IN CULTURE.

### III-1. INTRODUCTION.

Several models can be used to investigate transplacental transport processes. For instance, for general studies on the transfer of nutrients across the placenta the in vitro perfusion of placentae.[43,85,88,222,270,336] Detailed studies at sub-cellular level are not possible with this model.

A model highly suitable for detailed studies on the cellular processes involved in transplacental transport, is the in vitro culture of either malignant choriocarcinoma cell-lines,[164,302] or nonmalignant trophoblast cells.[130,147,211] The major disadvantage of this model is the loss of the tissue connections which might affect the processes studied.

Both in vitro culture of malignant cell-lines and of non-malignant cells have their (dis-)advantages. BeWo, JEG-3 and JAR choriocarcinoma cell-lines, are very usefull in studies on a wide range of cellular processes; because of their proliferative behaviour, many experiments can be performed with large numbers of cells.[93] In studies on cellular mechanisms involved in transplacental iron transport, these cell lines are less suitable. Malignant cell-lines, as it happens, do need large amounts of iron for cellular growth and cell-division. Much of the iron taken up by the cell, is therefore used for these processes, rather than being transferred to the "fetal side". In this respect, nonmalignant cytotrophoblast cells, freshly isolated from nonpathological placentae, presumably approach the in vivo behaviour of the syncytium more closely.

Nevertheless, also the in vitro culture of cytotrophoblasts is not the ideal research model, because, once isolated from term (human) placentae, cytotrophoblasts do not proliferate anymore. For every single experiment freshly isolated cells are required and since the preterm intrauterine conditions and the vitality of the isolated cells differ, the outcomes of similar experiments vary appreciably.

Still, in vitro culture of cytotrophoblasts was chosen as study model. Although interexperimental outcomes vary, the results within each experiment are highly comparable to each other.

To isolate cytotrophoblasts from chorionic villi, several protocols exist.[142,194,211,250,438]

The cytotrophoblasts used in the experiments described in this thesis were isolated from term human placentae using two different procedures based on those described by Kliman et al. and Karl et al.[194,211] These isolation procedures differ in the type of enzymes used, though both have a final Percoll gradient centrifugation as decribed by Kliman et al. in commen.[211]

Numerous studies have contributed to our present knowledge on the closely related characteristics and behaviour of cultured cytotrophoblast.[285,346,436] As mentioned above, cultured cytotrophoblasts do not proliferate though differentiate into syncytiotrophoblastlike structures.[211,212,285] In culture many cytotrophoblast characteristics are lost and syncytiotrophoblast specific proteins are expressed.[110]

To monitor cytotrophoblast differentiation, morphological and biochemical criteria are used. Morphological criteria are the aggregation and fusion of the cells,[95,211,212,285] which are cell-density and hCG dependent.[15,346] The moment of fusion can widely differ with time.[207] Examples of the biochemical differentiation are the expression of specific membrane antigens,[26,35,110] the production of syncytiotrophoblast specific hormones (hPL, hCG, SP-1),[35,207,211,285] and the expression of glycoproteins, like the transferrin receptor.[35,94] The biochemical rather than the morphological criteria are sensitive to culture medium composition, and additives. Not surprisingly, many biochemical functions are affected by specific growth factors and hormones.[63,277,288] Cyclic AMP also plays an important but selective role in trophoblast cell-metabolism.[35,67,119,292,321,370,400] Adenylate cyclase stimulators cause similar effects.[291] The cAMP induced effects are DNA mediated.[214] BeWo choriocarcinoma cells start to differentiate in reaction to treatment with cyclic AMP metabolism affectors.[106,429] Although BeWo choriocarcinoma cells seem useful in experiments on cAMP related processes, their in vitro culture is at best an imperfect model of trophoblast specific gene expression, because germ cell specific genes are expressed as well.[214]

Biochemical and morphological differentiation do not necessarily parallel each other.[36,95] Even the criteria for biochemical differentiation do not always occur simultaneously. Cyclic AMP for instance, strongly enhances hCG production but does not change transferrin receptor expression.[36] In the literature controversy exists about the value of the different criteria for biochemical differentiation.

Since in studies on cellular iron metabolism iron poor medium has to be used, and because in our experiments differentiated cytotrophoblasts are required, syncytium formation by cytotrophoblasts cultured under iron poor conditions (which induce biochemical differentiation was investigated.[35] The results were compared with the effects of Kerotinocyte Growth Medium (KGM) in which both biochemical differentiation and syncytium formation have been shown to occur.[95]

## III-2. MATERIALS AND METHODS.

### III-2.1. Cell isolation and culture conditions.

Cells were isolated according to procedure A as described in Chapter II (see Trophoblast-cell isolation), and cultured in either a medium based on M199 or KGM. The composition of the M199 based medium is described in Chapter II (see Trophoblast-cell isolation). The medium based on KGM consisted of 90 % ($^{V}/_{V}$) Keratinocyte Growth Medium; 10 % ($^{V}/_{V}$) FCS; 0.3 mg/ml gentamycin; 50.0 IU/ml penicillin; 50.0 $\mu$g/ml streptomycin and 2.50 $\mu$g/ml amphotericin. Cell culture conditions and cell densities were as described in Chapter II for the control series (see Cell-culture conditions), except that the cells were cultured on 15 mm round cover slips in 35 mm culture dishes. At each time that cells were immunolabelled, the culture medium in the remaining dishes was renewed.

### III-2.2. Protein and DNA determination.

Dish protein and DNA concentrations were measured as described in chapter II.

### III-2.3. Immunolabelling.

Desmosomes were visualized by double immunolabelling. After indicated culture periods the culture medium was removed. The cells were washed three times with phosphate buffered saline (PBS, pH 7.4) and fixed by incubation (30 min) with precooled methanol 4°C. After one wash (PBS) the cells were stored at 4°C in PBS containing 0.5 % ($^{W}/_{V}$) bovine serum albumin and 20 mM $NaN_3$ until all samples were available.

The coverslips were transferred to 12 wells-plates and incubated, for 1 h at room

temperature, with an optimal concentration of anti-desmoplakin I/II in PBS. Then they were washed 5 times in PBS + 0.5 % ($^{v}/_{v}$) Tween-20 and incubated, for 1 h at room temperature, in PBS with fluoresceine-isothiocyanate labelled rabbit anti mouse IgG antiserum (FITC-RAM, in an optimal concentration) and 1:50 diluted human heat inactivated serum. After 5 washes with PBS + 0.5 % ($^{v}/_{v}$) Tween-20, the cover slips were treated with propidiumiodine (5$\mu$g/ml in PBS) for 10 s and washed once with PBS + 0,5 % ($^{v}/_{v}$) Tween-20. Finally the cover slips were placed up side down on a drop of a DABCO solution (100 mg DABCO in 1 ml of a 2/1 mixture of Glycerol and PBS), on microscope slides.

### III-2.4. Scoring of syncytium formation.

The microscope slides were randomly coded by one assistant and examined by another, using a Zeiss fluorescence microscope. The first 100 nuclei seen, were scored for their presence in single cells, cell aggregates and syncytia as shown in Figure III-1. Syncytium formation was checked using a confocal microscope (Nikon, Optiphot; Silvius Laboratory, Leiden, NL). With this equipment and soft-ware support, cell 'slices' of 1 $\mu$m thick can be studied for the binding of FITC-RAM and propidiumiodine, and

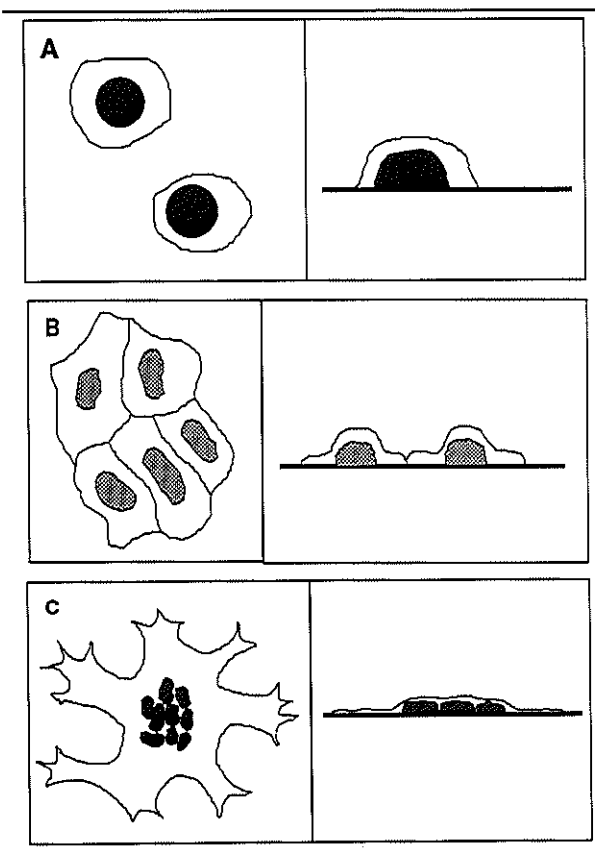


**Figure III-1. Syncytium formation.**
Schematic drawing of the various stages in syncytium formation of cytotrophoblasts in culture. (A: Single cells, B: Cell aggregate, C: Syncytium).

internuclear membranes can be detected and checked on continuity.

The number of nuclei per syncytium was scored in the first 30 syncytia seen and the average number was calculated. The average syncytium size was estimated by measuring the surface area size of the first 10 syncytia seen, using the Videoplan Image Processing System.

## III-3. RESULTS.

### III-3.1. Dish protein and DNA contents.

Culture-dish protein content varied (from 50 up to 150 $\mu$g per dish) between samples isolated from different placentae. The variation in protein concentration between samples prepared from cells isolated from one placenta was small and therefore acceptable (SD maximal 10 % of mean dish protein content; constant with time).

Up to 72-90 h after isolation, protein concentration remained stable but then fell significantly ($\alpha_T < 0.01$). Dish protein contents were independent of culture medium supplementation with hTf-(2Fe) (Figure III-2).

DNA concentration also declined (from 2.0 to 1.5 $\mu$g/dish) after 72 h of culture ($\alpha_T$ <0.05) indicating cell-loss. Nevertheless, DNA concentration was more stable than dish-protein content which is depicted in Table III-1 showing protein/DNA-ratios. It can be concluded that cultured trophoblasts are viable for at least a period up to

| Culture Time | Experiment 1 | | | Experiment 2 | | | Experiment 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Day | Mean | SD | n | Mean | SD | n | Mean | SD | n |
| 1 | 23.2 | 2.6 | 10 | 30.7 | 0.5 | 5 | 29.6 | 1.1 | 6 |
| 2 | 22.3 | 5.0 | 9 | 31.9 | 2.7 | 9 | 30.2 | 4.0 | 6 |
| 3 | 23.7 | 2.6 | 10 | . | . | . | 28.3 | 2.5 | 6 |
| 4 | . | . | . | 22.3 | 1.9 | 13 | . | . | . |
| 5 | . | . | . | 14.0 | 1.7 | 17 | 15.4 | 2.1 | 6 |
| 6 | 15.0 | 1.9 | 10 | . | . | . | . | . | . |
| 7 | . | . | . | 14.1 | 1.9 | 20 | . | . | . |

**Table III-1. Mean Protein/DNA ratios. Influence of culture time and hTf-(2Fe).** Reproduced are the mean protein/DNA ratios ($\pm$ SD, n) of three highly similar experiments.

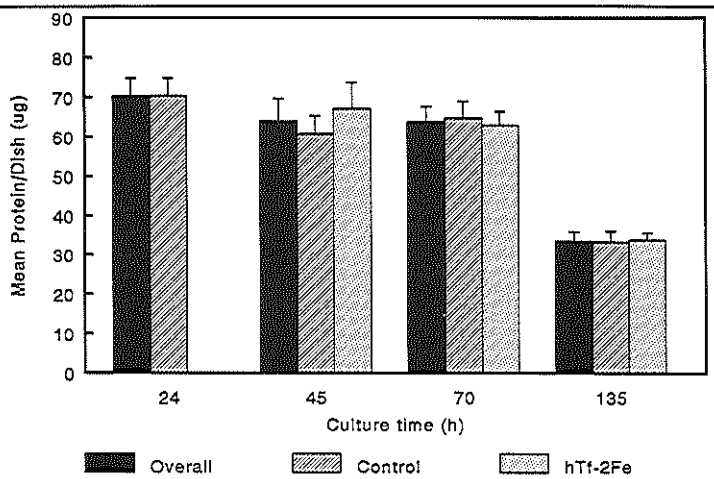


**Figure III-2. Protein content of culture dishes. Influence of culture time and hTf-(2Fe).** Shown are the results of one experiment. Although different in protein quantity, all experiments were highly similar for the trend of protein loss. Depicted are the mean protein contents (± SD) calculated from five dishes per series except for the overall means which were calculated from ten dishes.

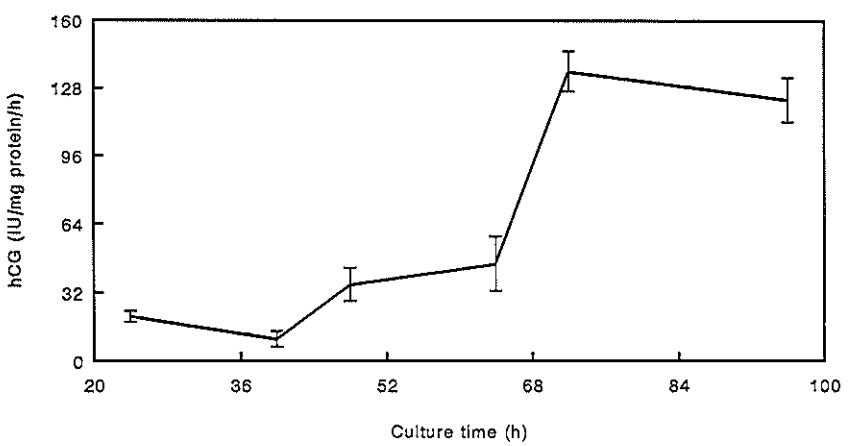


**Figure III-3. hCG production by cytotrophoblasts in culture.**
Cells were cultured in M199 based medium. At indicated times, the medium was removed, centrifuged and hCG concentrations were determined. Fresh identical medium was added to the culture dishes. Calculated were the hCG productions per hour, related to dish protein contents, during the period previous to the removal of the culture medium.

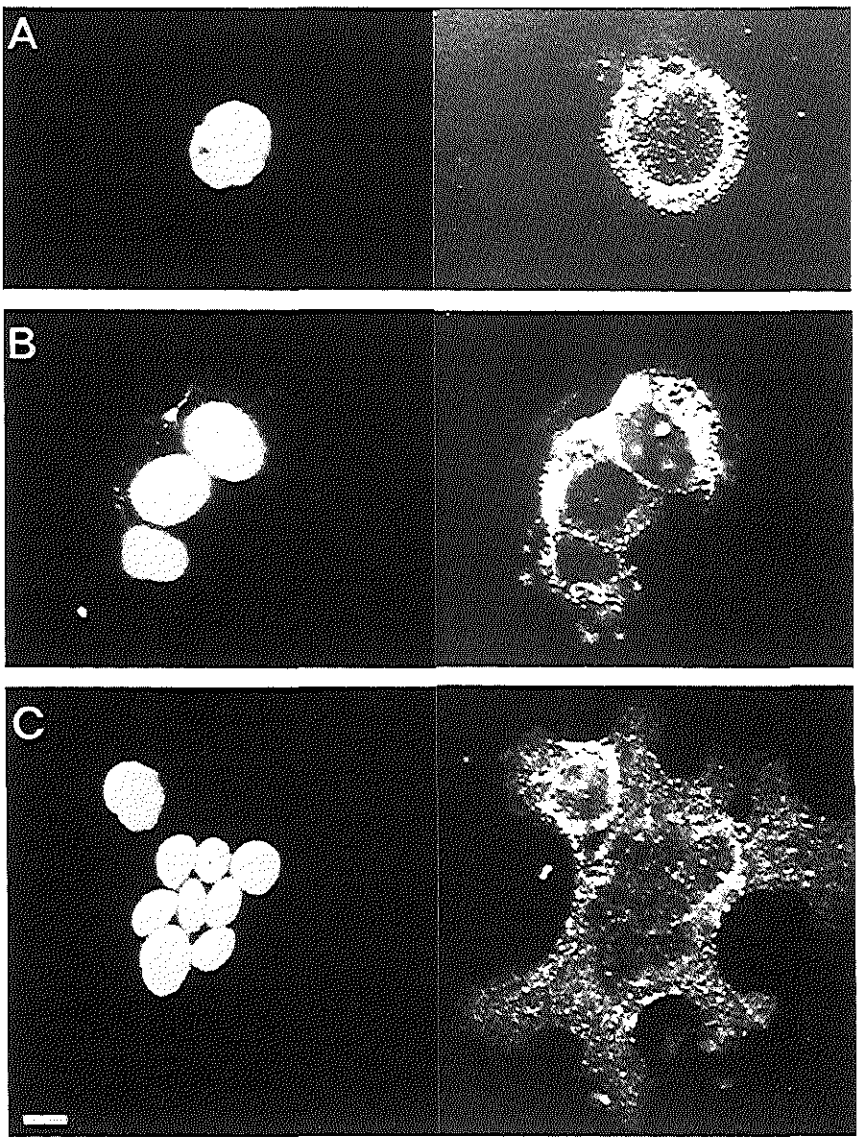


**Figure III-4. Syncytium formation in M199.**
Cells were cultured in M199 based medium as described in materials and methods. Shown are examples of cells at each differentiation stade during syncytium formation. (A: Single cell, 4 h culture; B: Cell aggregate, 20 h culture; C: Syncytium, 50 h culture). The bar represents 10 $\mu$m.

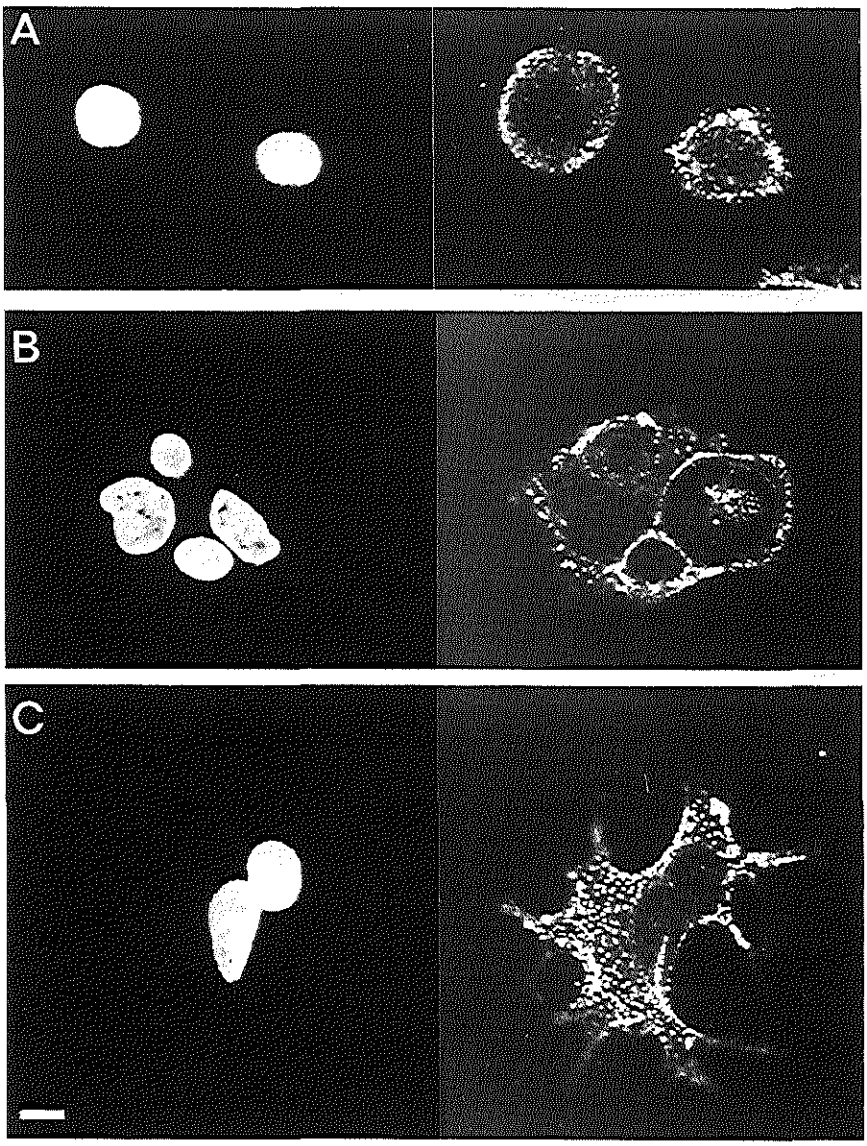


**Figure III-5. Syncytium formation in KGM.**
Cells were cultured in KGM based medium as described in materials and methods. Shown are examples of cells at each differentiation stade during syncytium formation. (A: Single cell, 4 h culture; B: Cell aggregate, 37 h culture; C: Syncytium, 48 h culture). The bar represents 10 $\mu$m.

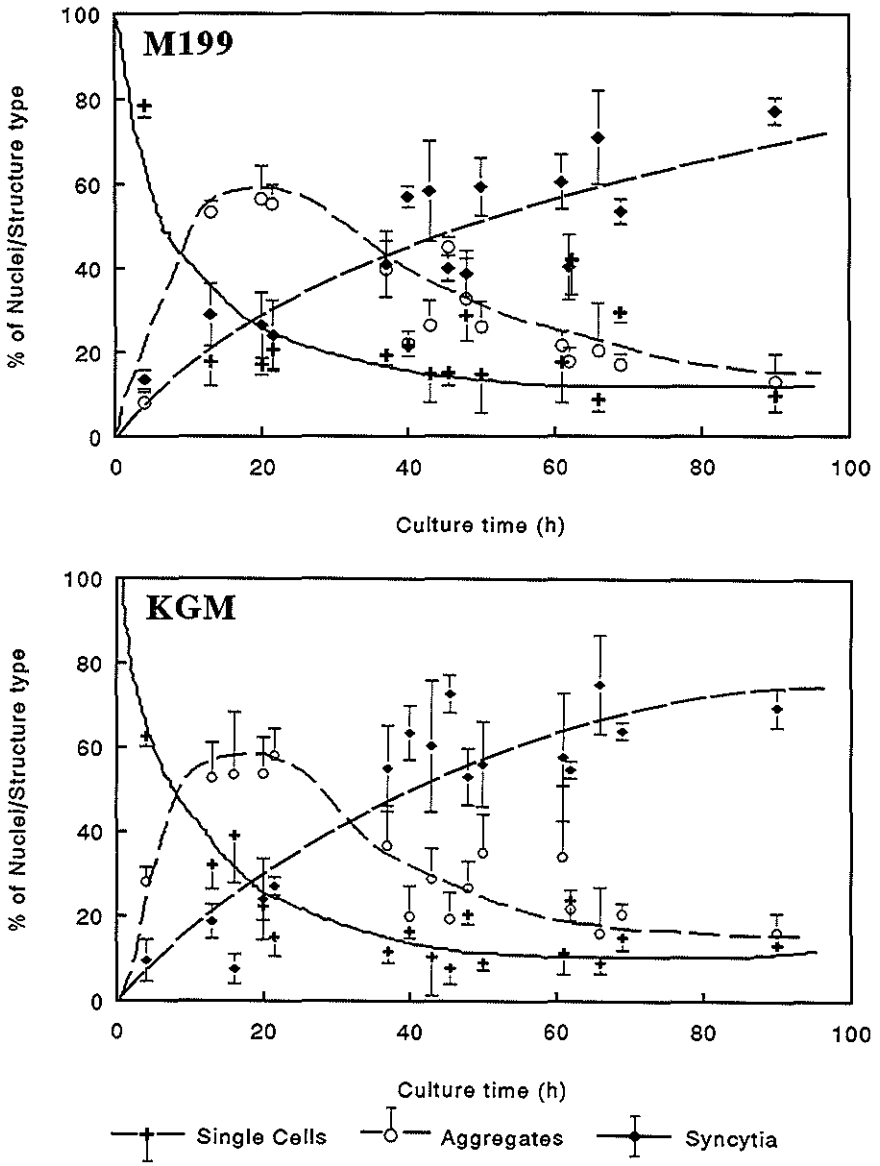


**Figure III-6. Rate of syncytium formation.**
Presented are the combined results of three experiments. Cells were cultured in M199 or KGM based medium as described in materials and methods. The first 100 nuclei were scored for being present in single cells, cell aggregates and syncytia. Shown are the percentages of nuclei in each cell type as a function of culture time and culture medium (M199 versus KGM).

72-90 h. Nevertheless, cultures for up to 160 h are possible without appreciable loss of protein and DNA if the placentae are obtained within 15 min after parturition.

### III-3.2. hCG production

As depicted in Figure III-3, hCG production strongly increased after approximately 40 h. Total hCG concentrations were comparable to previously presented data.[94,211,285] In the fusion experiments, no fluorescence was seen on cell membranes of cells incubated without antibodies, nor on cells incubated with either anti-Desmoplakin I/II or FITC-RAM anti-serum.

### III-3.3. Morphological differentiation

Examples are given of the various stages of cytotrophoblast differentiation in the M-199 based medium (Figure III-4) and the KGM based medium (Figure III-5).

The percentage of single cells, cell aggregates and syncytia in both media is depicted in Figure III-6. Cytotrophoblasts rapidly aggregated, though single cells were seen throughout culture. The majority of the aggregates developed into syncytia, finally leaving approximately 15 % of the nuclei in the aggregated form. Maximally 60-70 percent of the nuclei were found within syncytia. On this point no difference was seen between the two media. However, the final percentage of syncytial nuclei was reached already after 40 h in KGM versus 50 h in M199.

There was a dotted pattern of desmosomes on all cells early in culture, throughout culture on the single cells and on the syncytia formed. Cell-aggregates showed a pavement-like pattern of desmosomes on the intercellular membranes next to the dotted pattern on the upper cellsurface (Figure III-4, Figure III-5). This raised the following question. Light, touching the cell membranes at a tangent, could falsely suggest that these membranes are intercellularly located. Therefore syncytia were checked for the presence of intercellular membranes using a confocal microscope.

With time, the number of nuclei per syncytium slightly increased in cells cultured in M199. In KGM, this number increased less clearly (not significantly) (Table III-2).

The average syncytium size seemed larger in KGM but differed widely and therefore did not differ significantly ($0.50 < p < 0.20$) from the average syncytium size in the

M199 series (162 $\mu m^2$, SD: 70 in KGM; 132 $\mu m^2$, SD: 64 in M199; Students-t test).

| Culture time | M199 | | KGM | |
|---|---|---|---|---|
| | Mn | SD | Mn | SD |
| 48 h | 4.0 | ± 0.1 | 3.2 | ± 0.2 |
| 50 h | 6.0 | ± 0.8 | 4.6 | ± 0.7 |
| 66 h | 6.2 | ± 0.8 | 4.9 | ± 0.8 |

**Table III-2. Number of nuclei per syncytium.**
Cells were isolated and cultured as described in 'Materials and Methods'. Presented are means (Mn) and standard deviations (SD) of the number of nuclei per syncytium calculated from 30 syncytia.

## III-4. DISCUSSION.

Cultured cytotrophoblasts have been used to investigate many placental functions. In general, trophoblasts can be cultured for 72-90 hours without significant loss of protein and DNA (Figure III-2, Table III-1). Mean dish protein contents varied with cell isolation but were comparable to those published by others.[36,288] In some experiments the culture period could be extended to approximately 8 days, though only if the placentae were obtained within 15 minutes after delivery. Because the protein-DNA ratio was stable during at least 72 hours (Table III-1) both can be used as standard for cell number. The combined loss of protein and DNA, after approximately 90 hours, indicated cell loss.

ß-hCG production increased significantly in the first 3 days. The decrease in ß-hCG production in the next few days was highly comparable to the ß-hCG production pattern previously described.[35,211,285]

Specially because cultured cytotrophoblasts differentiate into syncytiotrophoblast like structures the model is suitable for studies on the placental uptake of nutrients (see Chapter IV).[94,395] This differentiation process has both biochemical and morphological characteristics, which however, do not always parallel each other. Even the biochemical aspects of differentiation do not always occur simultaneously.[36,95]

To my knowledge there is only one publication presenting clear evidence of

syncytium formation by cultured cytotrophoblasts.[95] The authors however, do not present detailed information on the number of fused cells nor on the rate of syncytium formation. Others presented data on nuclei distribution in cytotrophoblasts cultured in DMEM-H-G.[211,212] Their data are very similar to the results presented in Figure III-6, but the classification method used, was based on light microscope morphology and not on staining of membranes and nuclei.

In this chapter are shown the effects of M199 and KGM on syncytium formation rates, syncytium size and the average number of nuclei per syncytium in cultured cytotrophoblasts. The major difference between M199 and KGM is the enrichment of the latter medium with Epidermal Growth Factor, Insulin, Hydrocortisone and a poorly defined bovine pituitary extract. Both media have shown to induce biochemical differentiation (see Chapter IV).[35,94,95]

Morphologically, only small differences in effects were seen between KGM and M199. The process of aggregation and syncytium formation occurred more rapidly in KGM (Figure III-6), but the average number of nuclei per syncytium was higher in M199. No significant difference was seen between the average syncytium sizes. Pure subjectively the KGM syncytia seemed larger, but due to the variation in individual surface area sizes (from 62 to 287 $\mu m^2$ in KGM versus 48 to 224 $\mu m^2$ in M199), significance was not reached ($0.50 < p < 0.20$).

In both media, occasionally, large syncytia were seen. The nuclei of these rounded structures (up to 75 per syncytium) were small and unicoloured suggesting a low level of transcription.

Syncytium formation, expressed as percentage of nuclei per structure type, can easily be affected by the selective loss of one type of structure in a specific medium. This would imply loss of protein and DNA. In the first 72 hours of culture, however, neither protein nor DNA is significantly lost (see Figure III-2, Table III-1). No figures are presented on protein or DNA loss in KGM cultured cytotrophoblasts.[95,200] Assuming that no DNA was lost KGM cultured cytotrophoblasts, the results indicate no major difference between the morphological effects of the two media tested.

Although scientifically irrelevant, the minor, statistically not significant, differences in syncytium formation rate, and the average number of syncytial nuclei, in the two

culture media tested, may be explained by the following mechanisms. In culture, cytotrophoblasts migrate, in which transferrin receptors (TfRs) appear to play an important role.[47] KGM is thought to slow down the increase in surface TfR number's.[200] This might affect the migration capacity, independent of the process of cell-differentiation.[47] Subsequently, the reduced mobility could lead to a lower average number of nuclei per syncytium. The increase in cytoplasma volume of cells cultured in KGM could be caused by the supplemented epidermal growth factor.[54,277]

Morphological differentiation of cultured cytotrophoblasts seems independent of the culture media tested in the current study. For studies on the biochemical differentiation and on placental nutrient uptake, as few culture medium additives as possible should be used. Each additive might have its specific (special) effects,[63,346,424] not considering the effects of growth factor combinations or matrices.[115,249,286]

## Chapter IV. TRANSFERRIN RECEPTOR NUMBERS, DISTRIBUTION AND SHEDDING, IN IN VITRO CULTURED HUMAN CYTOTROPHOBLASTS.

### IV-1. INTRODUCTION.

The transport of iron across the placenta requires the binding and uptake of transferrin by the syncytiotrophoblast (see Chapter I, Transferrin receptor).[184] Transferrin uptake is a receptor mediated process, in which the transferrin receptor (TfR) plays an outstanding role (see Chapter I, Cellular iron uptake).

It is generally believed that the number of TfRs at the cell surface membrane reflects the iron need of the cell. Large numbers of TfRs are found in bone marrow on erythrocyte precursors (haem synthesis), on hepatocytes (iron storage), and on syncytiotrophoblasts (maternal-fetal iron transport). In normal individuals, the vast majority of cellular TfRs are found in the bone marrow (80 %).[236,251,349]

As a consequence of the endocytic cycle, TfRs are found at the cell-surface as well as intracellularly. The distribution of TfRs over the locations varies with cell-type and culture condition.[94,106,125]

Next to these two metabolically active sub-populations the existence of another TfR-pool has been proposed by Hirose-Kumagai and Akamatsu.[163] The TfRs in this pool are intracellularly located but do not participate in the endocytic cycle. The presence of TfRs at the basal membrane is controversial. Faulk and Galbraith could not detect any TfRs in the syncytiotrophoblast basal membrane,[117] while Vanderpuye, Kelley and Smith, using isolated basal membrane could.[403] Also in fused BeWo choriocarcinoma cells, TfRs are detected on the basal side of the cells.[62]

A soluble form of the transferrin receptor is present in the circulation.[27,353] These receptors originate from normoblasts and reticulocytes. Enhanced erythropoiesis and circulatory reticulocyte numbers are followed by increased serum TfR concentrations.[27] Reticulocytes shed TfRs in vesicles.[186,188,300,301] This process requires sorting of membrane proteins.[188]

In rats, TfR containing vesicles can be isolated from the plasma.[187] TfRs are released from the vesicles through proteolytic cleavage by membrane-based protease.[65, 66] Virtually all (99 %) of the soluable receptors are in the form of a truncated extracellular domain.[65,347,348]

Serum TfR concentration is a measure of erythropoiesis.[28,172,215,355] Serum TfR is also a useful indicator of functional iron deficiency,[120,172,353] but not of iron absorption.[74] In combination with hematocrit, retic index and erythropoietin, serum TfR concentration can be used to detect multiple mechanisms of anaemia in the same patient.[29]

In healthy pregnant females, serum TfR levels are not elevated as compared with nonpregnant levels, and appear to parallel erythropoietic activity and iron status.[28,60,354] Some reports, however, claim that serum TfR concentration increases during the third trimester of pregnancy.[217] Unfortunately, no other iron status parameters were measured in this study, so iron deficiency may have been the major cause. Although during pregnancy the erythrocyte precursors seem to be the major source of serum TfRs,[28] the rapidly growing placenta could also contribute significantly to this pool of soluble receptors.[351]

If the placenta releases TfRs into the circulation, the syncytium formed by syncytiotrophoblasts would most likely be the source.

Regulation of cellular TfR numbers is also possible by the control of receptor synthesis and degradation. In several cell types, TfR synthesis is controlled by the intracellular iron concentration.[280] Regulation takes place at the translational level by means of iron responsive elements (IREs) on the mRNA of the protein (see Chapter II, Cellular iron homeostasis, and Chapter VII). In trophoblasts regulation of TfR synthesis via a mechanism of iron controlled IREs, has not been identified yet. Down regulation of surface TfR numbers in reaction to transferrin supplementation of the culture medium,[36] which has also been described in HeLa cells,[416] does not contradict the presence of an IRE-involved regulation mechanism.

That some kind of precisely balanced regulation mechanism for transplacental transport is present is very likely, because of the enormous increase in iron transport during pregnancy on one hand and, on the other hand, the potential danger of high levels of free intracellular iron.

Another argument in favour of a regulation mechanism for transplacental iron transport is the observation that fetuses do not suffer from iron induced anaemia, even if the maternal iron stores are depleted.[87,322,413,433] Based on in vitro studies, showing TfR expression to be dependent on cell differentiation as well as iron

supplementation of the culture medium Bierings et al. suggested that the iron transport may be regulated by the alteration of surface TfR numbers similar to the variation of surface TfRs by HeLa cells, K562 cells and human monocytes.[8,36,384,416] Surface TfR numbers could be regulated by changes in the synthesis/degradation ratio, by redistribution of TfRs among (functionally different) sub-pools, and by the number of transferrin receptors shed into the maternal circulation. These mechanisms may, at least in part, function simultaneously.

Redistribution of TfRs has been confirmed in several cell-types and might be influenced by the number of TfRs located in intracellular pools.[80,81,106,279,417,430]

Evidence for receptor shedding by the placenta, as a mechanism of regulation, has been obtained for the tumor necrosis factor receptor.[406]

In the experiments presented in this chapter, we investigated the effects of culture time (differentiation grade) and iron enrichment of the culture medium on TfR shedding, distribution and synthesis/degradation ratio.

## IV-2. MATERIALS AND METHODS.

### IV-2.1. Cell isolation and culture conditions.

The cells were isolated according to procedure A, as described in Chapter II. Culture medium was enriched with supplements as indicated (hTf-(2Fe), FAC, desferrioxamine), and renewed every 24 h. In the experiments on TfR shedding the culture medium was renewed twice daily.

### IV-2.2. Measurement of transferrin receptor shedding.

*Sample preparation*

Two culture conditions were compared (control medium versus hTf-(2Fe) supplemented medium, 1.25 $\mu$M). In each series two dishes were marked. The medium, removed from these two dishes, was separately stored at -20°C until all samples were available for determination of TfR concentration.

To measure total TfR numbers, every time the medium in the remaining dishes was replaced, cells of one dish were lysed in distilled water and harvested with a rubber 'Policeman'. Lysis was checked using an inverted microscope (CK2, Olympus,

Japan). Cell-suspensions were collected in pre-weighed tubes. At the end of the culture period the cells of the marked dishes were collected. All cell-lysates were stored at -20°C until further procedures were carried out. These media and cell-lysates were used for TfR determination as described below.

*Determination of medium TfR concentration*

The cell suspensions were centrifuged for 10 minutes at 10 000 g and the supernatants collected. TfR concentration was measured as described previously,[29] after 1:2 dilution with 0.15 M phosphate-buffered saline (PBS, pH 7.4) containing 0.5 % bovine serum albumin and 0.05 % tween 20. Sonication of the sample in a buffer containing 2 % teric (polyoxyethylène 9-laurylester, Polidocanol, Sigma P9641) did not change the TfR concentration. The tubes with the samples were reweighted to ascertain the sample volume. An aliquot was mixed with an equal amount of 0.2 % Triton X-100 and homogenized by sonication for 10 s in melting ice. Another 250 $\mu$l aliquot was mixed with 750 $\mu$l of a buffer solution resulting in a final composition of 10 mM phosphate buffer (pH 7.4), 0.15 M NaCl, 2 % teric, 1mM iodoacetic acid, 0.5 mM phenylmethansulfonylfluorid and 20 U/ml aprotinin. The mixture was sonicated on melting ice for 30 s at high setting in a Polytron homogenizer (Kinematca, Littau, Switzerland) and assayed for soluble TfR the same day after 1:2 dilution with Tween-PBS containing 2 % teric.

### IV-2.3. $^{125}$I-transferrin binding essays.

To minimize the interference of residual receptor bound transferrin, cells were pre-incubated for 15 min (at 37 °C in 5 % $CO_2$/95 % air) in M199 without additives. Finally, they were rechilled to 4 °C and washed twice with PBS.

*Scatchard analysis*

The cells, cultured in either control medium or hTf-(2Fe) enriched medium, were incubated for 1.5 h at 4°C with increasing concentrations of $^{125}$I-labelled diferric transferrin ($^{125}$I-hTf-(2Fe)). Nonspecific binding was measured in similar samples by addition of a 100 times excess of unlabelled hTf-(2Fe). After incubation the cells were washed three times with PBS. Finally, cells were lysed bij adding 1 ml destilled water and collected with a rubber 'Policeman'. Surface bound radioactivity was measured with a Packard 500C autogamma spectrometer. The concentration

dependent binding was analysed as decribed by Scatchard.[332]

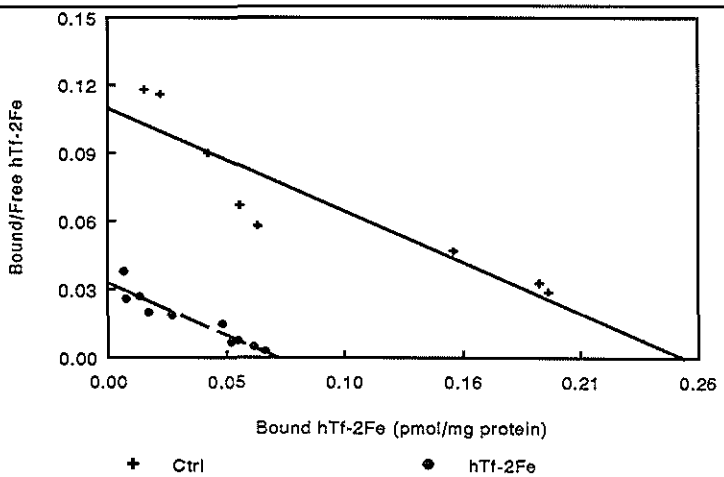


**Figure IV-1. Scatchard analysis of concentration dependent hTf-(2Fe) binding to cultured cytotrophoblasts.**
Cytotrophoblasts were cultured in control medium (Ctrl, ————) or hTf-(2Fe) (— — — —) containing medium. The number of membrane bound transferrin-receptors is clearly decreased. There is no change in affinity of hTf-(2Fe) for its receptor in reaction to hTf-(2Fe) enrichment of the culture medium.

*Surface TfR numbers*

To measure the number of cell-surface TfRs, cells were incubated for 1½ h at 4 °C (long enough to reach equilibrium) with a concentration of 125 nM $^{125}$I-hTf-(2Fe). A transferrin concentration of 10 times $K_D$ would give a 90 percent saturation.[56] The concentration used in the current experiments, was 60 times $K_D$ (Figure IV-1). Under these conditions full saturation was reached.[37] Nonspecific binding was measured by addition of a 100 times excess of unlabelled diferric transferrin and never exceeded 25 % of the total binding of $^{125}$I-hTf-(2Fe). Nonspecific binding amounting up to 25 % is rather high for ligand concentrations used in Scatchard analysis.[332] However, at the ligand concentrations used it is not extreme. The cells were then washed three times with ice-cold PBS to remove unbound $^{125}$I-hTf-(2Fe). Finally, cells were

lysed by addition of distilled water and collected with a rubber 'Policeman'. Surface bound radioactivity was assessed using a Packard 500C autogamma spectrometer.

TfRs were calculated from the amount of specifically bound $^{125}$I-hTf-(2Fe).

*Total TfR numbers*

Cultured cells were washed three times with ice-cold PBS and lysed in 0.5 ml 0.1 % Triton X-100. Lysis was checked using an inversion microscope (CK2, Olympus, Japan). The detergent 'Triton X-100' has been widely used in receptor binding studies and no effects have been reported on receptor-ligand interaction.[163] Cell-suspensions were collected in pre-weighed tubes and homogenised by sonication for 10 s in melting ice. Tubes were reweighed to ascertain the sample volume. Of the cell lysates portions of 0.1 ml were taken for protein determination. Cell lysates were incubated with $^{125}$I-hTf-(2Fe) (final and saturating concentration 125 nM) for 1 h at room temperature. Ammonium-sulfate was added in a 1:1 volume ratio, resulting in a 30 % $(NH_4)_2SO_4$ solution to precipitate the $^{125}$I-hTf-(2Fe)-TfR complex (see Figure IV-2).[315]

Samples were filtered through 1.2 $\mu$m glass microfiber filters (GF-C, Whatman).[315] Filters were rinsed four times with 30 % $(NH_4)_2SO_4$. Radioactivity on the filters was

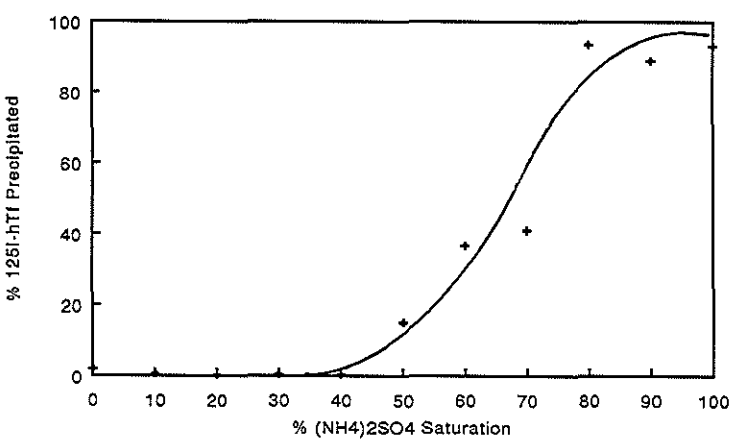


**Figure IV-2. $^{125}$I-hTf precipitation.**
In creasing amounts of fully saturated $(NH_4)_2SO_4$ were added to a solution of BSA (50 $\mu$g/ml) and $^{125}$I-hTf (125 nM) resulting in a final saturation of 0 to 100 %. After incubation for 2 h at 4°C the samples were filtered through Whatman GF/C filters. The filters were rinsed four times and counted for radioactivity. Presented are the percentages of precipitated $^{125}$I-hTf as a function of $(NH_4)_2SO_4$ saturation of the sample.

measured as described above. TfR number was calculated from the amount of specifically bound $^{125}$I-hTf-(2Fe). This method was carefully checked for precipitation of unbound $^{125}$I-hTf-(2Fe) (< 0.5 %) (Figure IV-2).

*Transferrin receptors participating in the endocytic cycle.*

Cells were incubated for 1 h at 37°C in medium M199 supplemented with 250 nM $^{125}$I-hTf-(2Fe) and successively washed three times with

PBS (pH 7.4) at 4°C. To remove surface bound hTf-(2Fe) acid/neutral washes were carried out at 4°C: cells were firstly incubated for 10 min with sodium-acetate buffer (pH 4.5) and secondly for 10 min with PBS (pH 7.4). This procedure was repeated twice. Finally, the cells were lysed in distilled water and collected with a rubber 'Policeman'. Radioactivity in the cell lysates and the combined acid/neutral washes was determined as described above. Nonspecific binding was determined by binding of $^{125}$I-hTf-(2Fe) in the presence of a 100-times excess of unlabelled hTf-(2Fe). The number of surface bound TfRs was derived from the acid/neutral wash labile $^{125}$I-hTf-(2Fe). The number of intracellular TfRs was calculated from the amount of radioactivity resistent to the acid/neutral washes.

### IV-2.4. Protein and DNA determination.

Determinations of sample protein and DNA concentrations were performed as described in Chapter II.

## IV-3. RESULTS.

### IV-3.1. On transferrin receptor shedding.

Figure IV-3 shows the trends of the decrease in protein content of the culture dishes, obtained in 6 experiments on TfR shedding. Using the trends of protein content decrease, the virtual dish protein contents at 0 h and 100 h culture were calculated, and from these results the average protein loss per hour was calculated. There was no difference between the protein loss from cells cultured in iron poor medium or in hTf-(2Fe) containing medium. The average protein loss was 0.22 %/h (from 165 to 129 $\mu$g/dish in 100 h).

The calculated TfR loss was comparable to the average protein loss. The number of

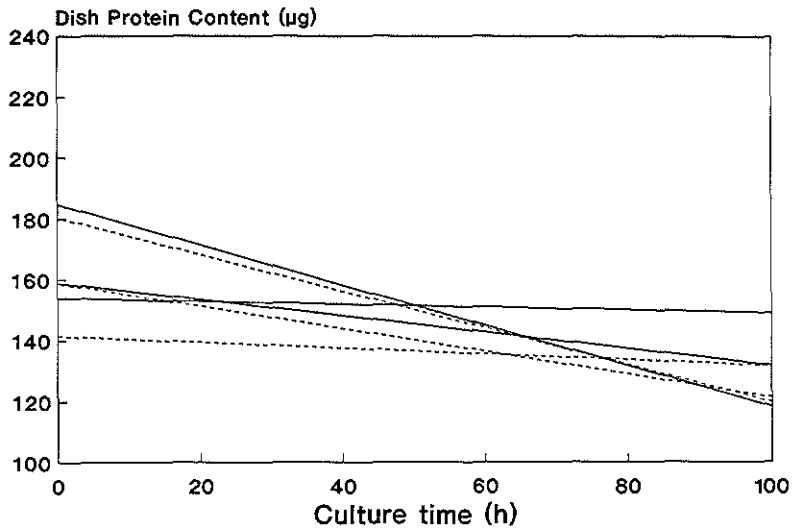


**Figure IV-3. Trends of protein loss.**
Shown are the trends of protein loss in six experiments.
There was no significant difference between the results obtained in the control series (Ctrl, ————) and the diferric transferrin series (hTf-(2Fe), — — — —). The average protein loss was 0.22 %/h.

TfRs in the culture medium was related to the total number of TfRs in the cell-lysate at the beginning of the corresponding culture period. The hourly TfR losses were calculated per period.

The number of TfRs released in the culture medium as percentage of the total number of cellular TfRs are shown in Figure IV-4. Sonication of the samples did not change TfR concentration indicating that no TfRs are externalized in vesicles. The cells in the control series contained 1.60 pmol TfRs per mg protein, in the hTf-(2Fe) series cells contained 1.35 pmol/mg protein. These receptor numbers are close to the results obtained in the $^{125}$I-hTf-(2Fe) binding studies. The average percentage of TfRs lost was 0.26 % per h, showing no curve or tendency. No significant difference (Students-t test: $0.20 > p > 0.10$) in TfR release was seen between cells cultured in iron poor medium or in hTf-(2Fe) enriched medium (Ctrl: 0.18 %/h, SD $\pm$ 0.065 and hTf-(2Fe): 0.35 %/h, SD $\pm$ 0.174 respectivily).

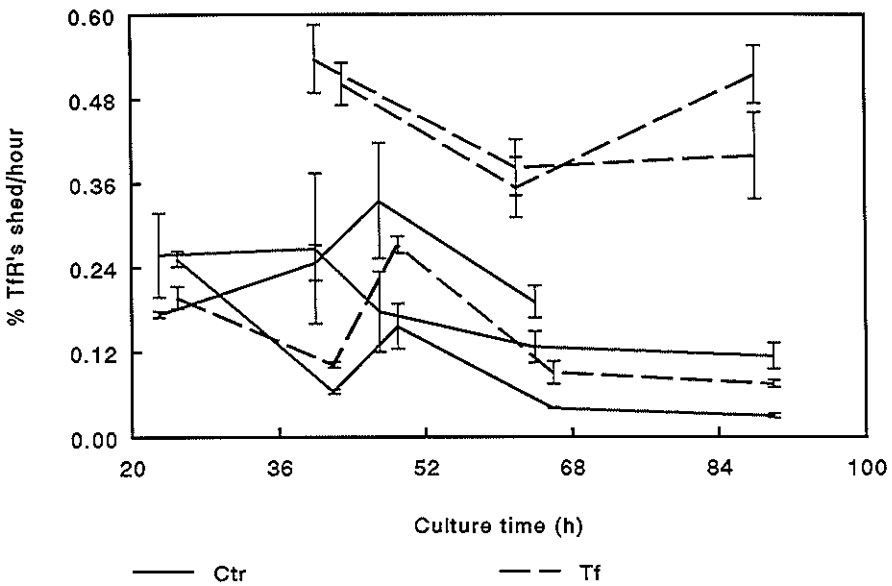


**Figure IV-4. Transferrin receptor losses.**
Shown are the losses of transferrin receptor (TfRs) in six experiments; three control series (————) and three diferric transferrin series (— — — —). TfR losses per hour are expressed as percentage of the total number of cellular TfRs $\pm$ 1 SD. The average percentage was 0.26 %/h. The loss of TfRs in the control series (Ctrl, 0.18 %/h, SD 0.065) did not differ significantly from the loss of TfRs in the diferric transferrin series (hTf-(2Fe), 0.35 %/h, SD 0.174). Students-t test: $0.20 < p < 0.10$.

From the results shown in Figure IV-3 and IV-4, it was concluded that syncytiotrophoblasts do not shed TfRs. The increased protein loss in one experiment (Figure IV-3), could have masked the number of TfRs excreted by shedding. This, however, was not the case because also in the other experiments, with very little loss of protein, the number of TfRs lost did not exceed the dish protein decrease rate. Based on the equal loss of protein and TfRs it is concluded that syncytiotrophoblasts do not shed TfRs.

### IV-3.2. Transferrin receptor numbers.

According to the results presented in Figure IV-1, the number of membrane bound transferrin-receptors is clearly decreased. There is no change in receptor affinity for

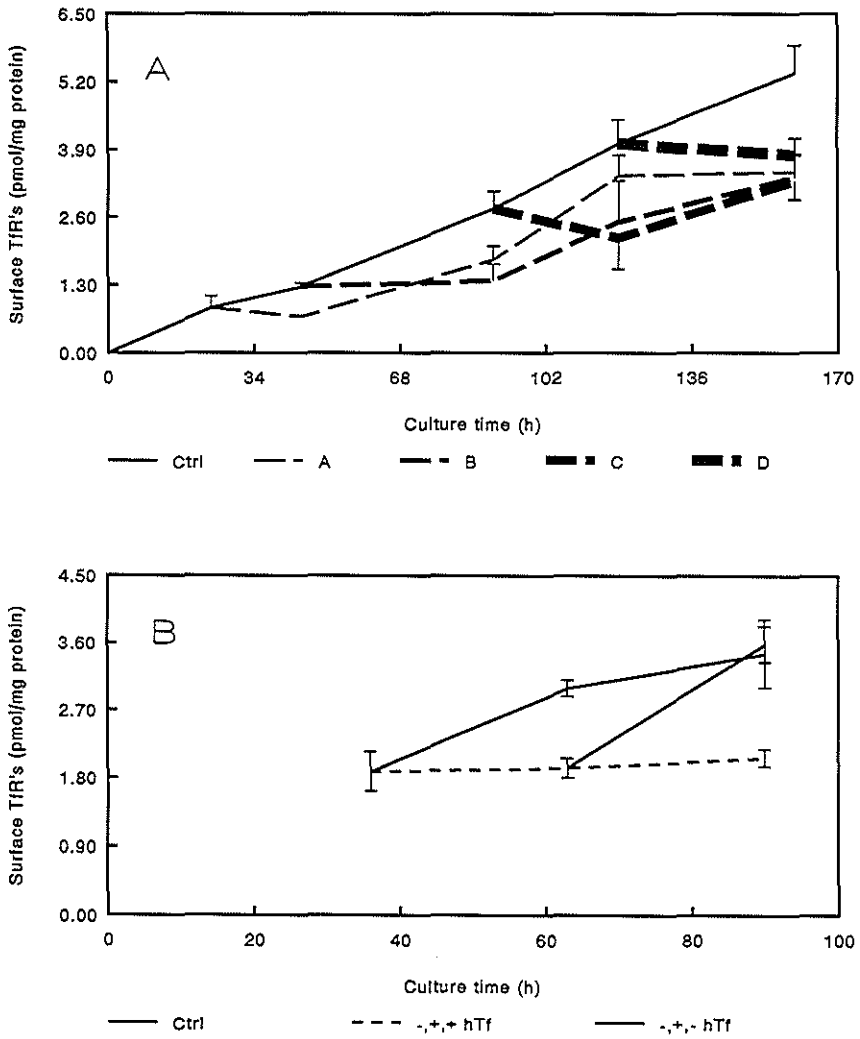


**Figure IV-5. Surface transferrin receptors. Influence of culture time and hTf-(2Fe).**

Figure IV-5A. After indicated culture periods, the control culture medium (————) was replaced by medium containing hTf-(2Fe) (— —; 0.1 mg/ml; Series A to D). Bars represent one standard deviation.

Figure IV-5B. Culture conditions were similar to those described under Figure IV-5A (Control series: -,-,-; hTf-(2Fe) series: -,+,+). In 5 culture dishes the hTf-(2Fe) fortified medium was replaced by iron poor medium at 65 hours (-,+,- series). Bars represent two standard deviations.

hTf-(2Fe), in reaction to hTf-(2Fe) enrichment of the culture medium.

The number of surface TfRs increased with time and was affected by iron to the medium, a process which was reversible (Figure IV-5A, Figure IV-5B). Cells cultured in iron poor medium increased the number of surface TfRs to a significantly higher level (Figure IV-5A; $\alpha_T < 0.05$), compared with cells cultured in hTf-2Fe containing medium. Replacement of iron fortified medium by iron poor medium led to a number of surface TfRs highly comparable with that of cells permanently cultured in iron poor medium (Figure IV-5B). These TfR numbers were significantly higher ($\alpha_T < 0.01$) than those in cells permanently cultured in iron poor medium. In the cells cultured for 2½ h, surface TfRs were not detectable with the procedure used; nonspecific binding in these cells was always comparable to the total binding of $^{125}$I-hTf-(2Fe).

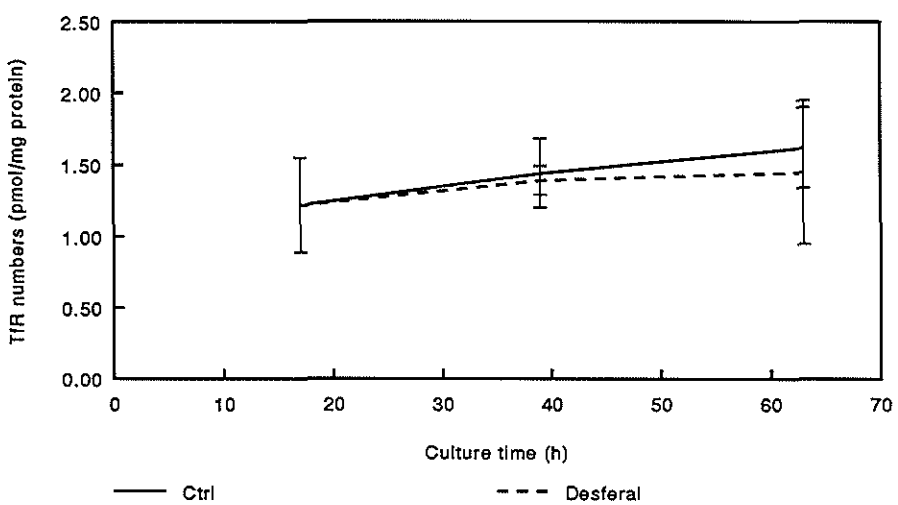


**Figure IV-6. Surface transferrin receptors. Influence of culture time and desferrioxamine.**

After indicated culture periods, the control culture medium (————) was replaced by medium containing desferrioxamine (— —; 50 $\mu$M). Bars represent one standard deviation.

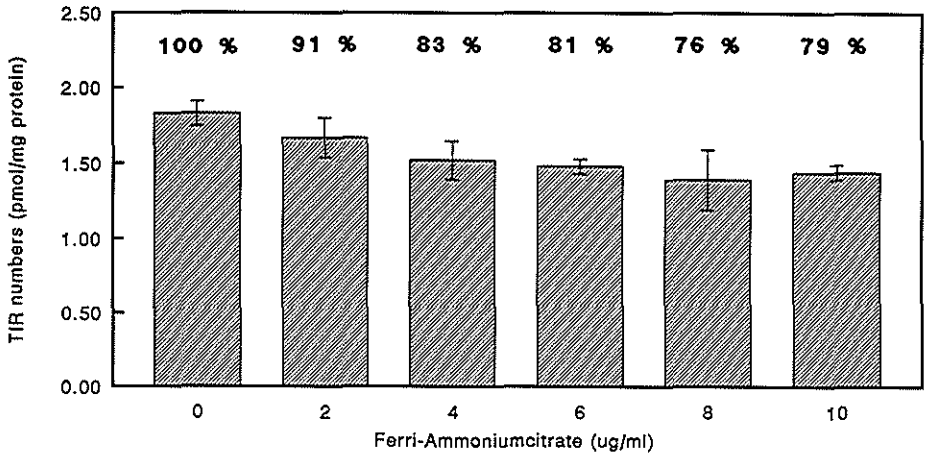


**Figure IV-7. Surface transferrin receptors. Influence of ferricammoniumcitrate concentration.**
After 24 h culture in control medium, culture was continued in ferricammoniumcitrate (FAC) containing medium (concentrations as indicated). Surface TfR numbers were determined as described in materials and methods at 40 h culture.

As compared with iron poor medium, desferrioxamine enriched medium did not further increase surface TfR numbers (Figure IV-6). Therefore, it is likely that cellular iron levels are not decreased by desferrioxamine more than by iron poor M199.
Compared with hTf-(2Fe), the effects of ferricammoniumcitrate (FAC) on surface TfR numbers are small (Figure IV-7). A dose-dependent effect is seen, but surface TfR numbers are only about 20 % lower than in the control series (0 $\mu$g/ml FAC).
Total TfR numbers increased with time (Figure IV-8). This increase was more pronounced when cells were cultured in iron poor medium ($\alpha_T < 0.02$). Here again, no further effect of desferrioxamine was seen (Figure IV-9). In the cells cultured for 2½ h, very small numbers of TfRs were detectable (0.28 pmol, $\pm$ 0.03) TfRs/mg protein).

### IV-3.3. Transferrin receptor distribution.

With time there was a redistribution of TfRs among the functionally different compartments in the trophoblast cell. Figure IV-10 presents the results obtained in

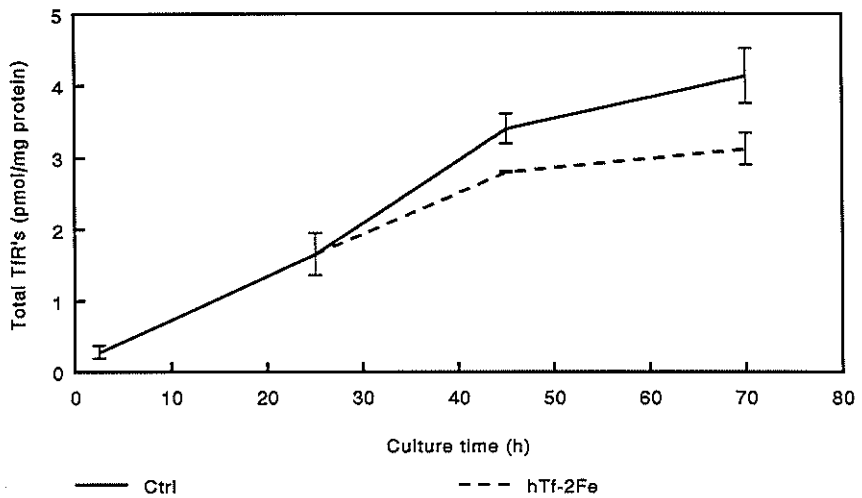


**Figure IV-8. Total transferrin receptors. Influence of culture time and hTf-(2Fe).**
The cells were cultured in iron poor medium (———) for 24 h. From 24 hours on culture was continued in either iron poor medium (———) or hTf-(2Fe) fortified medium (— —) (0.1 mg/ml). At indicated culture times total TfRs were determined as described in materials and methods ($^{125}$I-transferrin binding essays).

one of three studies. The data show that about 45 % of all TfRs actively participated in the endocytic cycle. The other part is stored and functionally inactive. This distribution was stable with time unless cells were cultured in iron poor medium. In this case the percentage of active TfRs increased to about 56, significantly higher than the 46 percent of the hTf-(2Fe)-cultured series ($p < 0.02$). After 24 h about 30 % of the TfRs were located on the cell-surface. With time a significantly increasing part (up to 46 percent) of the active TfRs were found on the cell-membrane ($p < 0.05$), which depended on hTf-(2Fe) enrichment of the culture medium. Finally, the percentage of surface bound TfRs (of the total of TfRs) was significantly higher ($p < 0.05$) when cells were cultured in iron poor medium. The results indicate that this was mainly caused by an increase in the number of TfRs actively participating in the endocytic cycle.

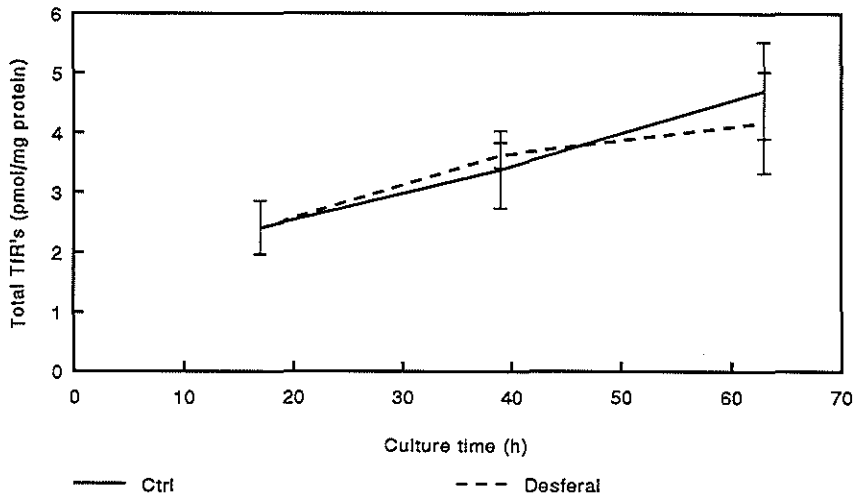


**Figure IV-9. Total transferrin receptors. Influence of culture time and desferrioxamine.**
The cells were cultured in iron poor medium (———) for 24 h. From 24 hours on culture was continued in either iron poor medium (———) or desferrioxamine enriched medium (— —, 50 $\mu$M). At indicated culture times total TfRs were determined as described in materials and methods ($^{125}$I-transferrin binding essays).

## IV-4. DISCUSSION.

Placental transport of iron has to be balanced very accurately. The first step in this process is the uptake of iron by syncytiotrophoblasts, a process which is TfR mediated. TfR expression can be studied in vitro in differentiating cytotrophoblasts. It was shown previously that different iron compounds affect cell-surface TfR numbers.[36,38] Therefore, some kind of regulation mechanism for cell-surface TfR numbers is likely to function in syncytiotrophoblasts.

Theoretically, TfR densities may be controlled by the release of TfRs into the maternal circulation (shedding), by variations in the synthesis/degradation ratio, causing changes in total TfR amounts, and by TfR redistribution among functionally different pools.

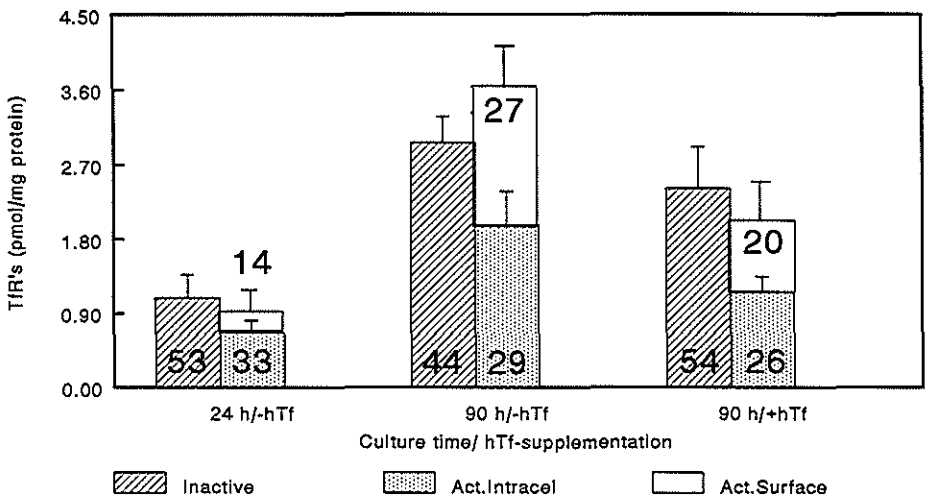


**Figure IV-10. Transferrin receptor distribution. Influence of culture time and hTf-(2Fe).**
Shown are the results of one experiment. Cells were cultured in iron poor control medium. After 24 h, culture was continued up to 90 h, in either iron poor medium or human diferric transferrin (hTf-(2Fe)) supplemented medium (0.1 mg/ml). The figures represent the percentage of TfRs in each sub-group.

### IV-4.1. On transferrin receptor shedding

Serum TfRs may originate from every TfR expressing cell. Common sources of the majority of serum transferrin receptors are erythrocyte precursers and, during pregnancy, the placenta. Serum TfR levels decrease in the first trimester of gestation, but increase again in the third trimeste.[28,217] Most likely this occurs because of the enhanced erythropoiesis, but it may be caused in part by the rapid growth of the maternal fetal interphase.

The exact source of plasma TfRs could easily be revealed if TfRs showed tissue specificity, or if fetal and maternal TfRs differed immunologically. Unfortunately, this is not the case.

A useful model to study TfR shedding is the in vitro culture of cells.[66,216]

In human tumor cell-lines, TfRs are externalized at a rate of 56 to 194 fmol/h/$10^6$ cells, most likely via vesicles.[216] This process strongly depends on temperature and transferrin availability.[25,216] K562-erythroleukemic cells have the highest TfR shedding

rate.[216] Related to cultured cytotrophoblasts these cells have high TfR numbers (0.149 to 0.176 pmol (see above) and 1.8 pmol respectively per $10^6$ cells,[25] of which they shed approximately 10 % every hour (0.194 pmol/h/$10^6$ cells).[216] Cultured cytotrophoblasts do not shed TfRs and therefore this is not a mechanism used to reduce cellular TfR numbers in these cells.

Nevertheless, two remarks have to be made. Firstly, the celltype chosen could have been incorrect. The cells used in this study were isolated from term human placentae. It is possible that trophoblasts change (decrease) TfR shedding towards the end of gestation. As a matter of speculation, this might be one of the factors starting the delivery, for TfRs are thought to play a role in the immunological interactions of the syncytiotrophoblasts with the maternal immune system.[116] To overcome this celltype-problem, cytotrophoblasts should be isolated from pre-term placentae obtained from caesarean sections on maternal indication.

Secondly, although not convincingly proved, the loss of trophoblast cells into the maternal bloodstream could also contribute to the number of circulating TfRs.[311] Simpson, Mayhem and Barnes suggested the possibility of a constant turn-over of syncytiotrophoblasts in a way comparable to the situation present in the intestinal cryptes.[351] In vitro this type of cell loss is difficult to differentiate from cells detached because of decreased viability.

### IV-4.2. Transferrin receptor numbers and distribution

The results with regard to the concentration dependent binding of $^{125}$I-hTf-(2Fe) showed that differentiating cytotrophoblasts reduce surface TfR numbers in reaction to hTf-(2Fe) supplementation of the culture medium (Figure IV-1). No change was seen in receptor affinity. Therefore, it is possible to calculate TfR numbers from experiments using only one (saturating) $^{125}$I-hTf-(2Fe) concentration.

In our studies surface TfR numbers increased until day 4-5 comparable to the production of hCG (Figure III-1 and Figure IV-5a). After this period hCG production fell, suggesting a metabolic/biochemical change,[276] whereas TfR-expression continued to increase or at least stabilized.

The rate of increase in surface TfRs was not influenced by the availability of iron (Figure IV-5A). Replacement of the standard culture medium by hTf-(2Fe) enriched

medium, transiently reduced surface TfR numbers. Soon, however, the normal rate of increase was resumed, a revesible process (Figure IV-5B). As compared with iron poor medium desferrioxamine containing medium did not change surface TfR numbers. This suggests that desferrioxamine does not affect cellular iron homeostasis differently from iron poor M199. FAC iron on the other hand does not affect surface TfR numbers to the same level as hTf-(2Fe), even though the actual amount of iron added to the culture medium in the FAC series was up to 40 times as high (Figure IV-7). Apparently this iron compound is handled differently from hTf bound iron.

The rate of increase as well as the numbers of TfRs were highly comparable with those found by Kennedy, Douglas and King.[200] Their results suggest that syncytium formation is related to a (small) supression of surface TfR densities.[95] This suppression could be explained by the decrease in cell-surface area in fused cells, for TfR numbers are independent of cell-fusion,[35] and were related to cellular protein. The suggestion of Kennedy, Douglas and King that cytotrophoblasts cultured in Ham's/Waymouth's medium remain undifferentiated, and at the same time express TfRs, is highly unlikely because cytotrophoblasts in vivo do not express TfRs.[53,168,200] The results of Kennedy, Douglas and King are more logically explained by culture medium induced differences in biochemical differentiation, independent of the morphological differentiation.[200]

Differences in morphological and biochemical differentiation have been shown in other in vitro models as well. If, for instance, in BeWo choriocarcinoma cells syncytium formation is induced by cAMP-analogons or cAMP-phosphorylase inhibitors, the actual number of surface TfRs is reduced.[106] In cultured cytotrophoblasts, cAMP-analogons as such neither stimulate syncytium formation nor modulate surface TfR numbers, although hCG production is increased 5 to 10 fold,[2,36] which is comparable to the reaction of BeWo cells.

Similar to the surface TfRs, total TfR numbers increased with time as depicted in Figure IV-8 and Figure IV-9, particularly if cells were cultured in iron poor or desferrioxamine containing medium.

Based on the results with regard to total TfR numbers it was concluded that iron affects the TfR synthesis/degradation ratio. However, these results do not exclude

the possibility of TfR redistribution, a process which, as a result of cell differentiation, has been demonstrated before in choriocarcinoma and K562 erythro-leucemic cells.[106,174,279] No changes, however, were found in TfR numbers or in TfR distribution during differentiation of HL-60 human leukemic cells,[109] and in different grades of macrophage activation, respectively.[145]

Time and hTf-(2Fe) had different effects on the TfR synthesis/degradation ratio and receptor redistribution (Figure IV-10). Surface TfR numbers, expressed as percentage of the active TfR sub-population, increased significantly with time ($p<0.05$) and were not influenced by the availability of iron. This might be an effect of cell differentiation. The final distribution is highly comparable to that in BeWo cells.[105,106] The size of the active TfR population, expressed as percentage of total TfRs, was constant when, similar to the in vivo situation, iron was available. Iron shortage caused TfRs to redistribute between the inactive and active receptor pool. Within the pool of active TfRs there was a shift of TfRs to the surface independent of iron availability and therefore most likely caused by a process of differentiation similar to that in BeWo choriocarcinoma cells.[106] Surface TfRs might be increased to a pre-fixed percentage of the active receptor pool which has previously been suggested,[36]

The variation in surface TfRs number, due to iron availability, was independent of cell-differentiation and can be explained by the shift of TfRs between the inactive and active pool. If, within the active TfR subpopulation, the distribution of TfRs over cell-surface and interior remains stable, this shift will automatically lead to an increase in surface TfRs.

Another parameter of importance in the regulation of iron uptake and transfer is the affinity constant '$K_a$' for the binding of hTf-(2Fe) to its receptor. No modification of the $K_a$ value was found in response to iron supplementation of the culture medium (Figure IV-1), nor in response to growth hormones or cAMP-analognes.[36] This is in contrast to the data presented by Kennedy, Douglas and King,[200] which suggest a twofold higher $K_a$ after induction of syncytium formation by keratinocyte growth medium (from 6 to 12 x $10^7$/M). In view of the average serum hTf concentration of 80 $\mu$mol/l, this change can hardly be of any physiological relevance.[89]

Trophoblasts could regulate iron uptake (and by this way transplacental iron-

transport) by variation of surface TfR numbers via changes in total TfR amounts and via redistribution of TfRs among the receptor pools. This is in accordance with the in vivo situation of iron deficient mothers carrying fetuses with normal iron stores. Nevertheless, the results described above, do not prove that there is indeed a causal relationship between the number of surface TfRs and iron uptake (see Chapter V).

When transplacental iron transport processes are studied in cultured cytotrophoblasts, it should be considered that the iron transport system matures simultaneously with a regulation mechanism for cellular iron homeostasis.

## Chapter V. IRON UPTAKE AND TRANSFERRIN RECEPTOR KINETICS.

### V-1. INTRODUCTION.

In man, the major iron source of the placenta is maternal transferrin (hTf).[121] As described in chapter one (Cellular iron uptake), the iron uptake process in the (human) haemochorial placenta is 'receptor mediated endocytosis', for which transferrin receptors are required.

Briefly, receptor mediated endocytosis can be described as follows. HTf is bound by its receptor and internalized in endosomes. Due to acidification of the endosome, iron is released from transferrin and transferred to the cytosol by a process unknown so far. The hTf-TfR complex is recycled back to the cell surface where, due to the environmental pH (7.4), the ligand dissociates from the receptor. Both are then ready to be reused.

Prior to uptake, TfRs are clustered in coated pits. Some receptors, like the asialoglycoprotein receptor, are internalized and recycled, independently of the TfR.[68] Others are internalized in the same endosomes as TfRs and, therefore, intracellular sorting of the receptors has to take place. Even TfRs do not take one pathway.[366] The process of transferrin receptor trafficking has recently been reviewed by Stoorvogel et al.[369]

Because of the characteristics of 'receptor mediated endocytosis', it is obvious that cellular iron uptake rates depend on hTf concentration, hTf iron saturation, cell surface TfR numbers, and on the TfR cycle time.

TfR cycle times, more specific the internalization and externalization rates of the receptor, are used for regulation of cell surface TfR numbers,[58,106,279,383] but reduction of cell surface TfR numbers can take place without changes in these rate contants.[209]

As discussed in chapter IV, cytotrophoblast TfR vary in number and location. Changes in surface TfR densities could be of value in the uptake of iron by these cells. It has even been suggested that the whole process of transplacental transport might be regulated by syncytiotrophoblast cell surface TfR densities.[36] This hypothesis is attractive, because of its similarity with the IRE-involved regulation of intracellular iron levels, by variation of TfR mRNA translation; a mechanism which

has been found in other cell types.

It has been shown that the number of TfRs is not the only factor controlling the rate of iron uptake. In K562 erythroleukemic cells, a 50 % reduction of cell surface TfR numbers in reaction to 4ß-phorbol 12-myristate 13-acetate, did not change the uptake of iron from transferrin.[209] In Friend erythroleukemic cells, iron uptake and TfR numbers are not proportionally increased in reaction to dimethylsulfoxide treatment.[167] In chick embryo breast muscle also TfR recycling kinetics, and possibly TfR distribution, can also affect the uptake of iron.[361]

Therefore, the effects of cell-differentiation and hTf-(2Fe) enrichment of the culture medium on both transferrin mediated total iron uptake and iron uptake rates were investigated.

## V-2. MATERIALS AND METHODS

Cell-isolation and culture conditions were as described in chapter II. Cell-isolation procedure A was used. After the standard recovery period, cells were cultured in 35 mm dishes, either in standard control medium or in hTf-(2Fe) (1.25 $\mu$M) containing medium.

### V-2.1. Iron uptake from diferric transferrin.

Total transferrin mediated iron uptake was determined as follows. Cells, cultured for indicated times, were washed twice with icecold PBS and subsequently incubated with 1.25 $\mu$M hTf-2$^{59}$Fe in M199 at 37°C in 5 % $CO_2$/95 % air. At indicated times the incubation medium was removed and the cells were washed twice with icecold PBS. The cells were lysed by addition of distilled water (checked under an inverted microscope), and collected with a rubber 'Policeman'. Intracellular $^{59}$Fe was determined using a Packard 500c autogamma spectrometer.

### V-2.2. Transferrin receptor kinetics.

To investigate the effects of cell-differentiation on TfR endocytosis and exocytosis rates, the experiments described below were performed 18 h and 65 h after initiation of the cell-culture. The effects of iron availability were studied using hTf-

(2Fe) (1.25 $\mu$M), which was added to the culture medium after the first medium change at 18 h.

*Transferrin receptor endocytosis rates*

To measure TfR endocytosis rates, single cycle experiments were carried out as described by Ciechanover et al.[69] At indicated times, culture medium was removed and the cells were washed twice with icecold PBS (pH 7.4). Cells were incubated for 1½ h at 4°C in DMEM-H with 0.250 $\mu$M hTf-($2^{59}$Fe). After removal of the radiolabeled ligand and three subsequent washes with PBS, the cells were incubated in prewarmed M199 supplemented with 6.25 $\mu$M hTf-(2Fe) at 37°C, for up to 1 h. At indicated times, TfR internalization was stopped by quick removal of the medium and immediate chilling of the cells by PBS washes (pH 7.4) at 0°C. Next acid/neutral washes were performed: the cells were exposed to a sodium acetate buffer (25 mM sodium acetate, 150 nM NaCl, 2mM $CaCl_2$, pH 4.5) for 10 min at 4°C, followed by incubation with PBS (pH 7.4) for 10 min at 4°C. These incubations were repeated twice, and the incubation media combined. Finally the cells were lysed in distilled water (checked under an inverted microscope) and collected with a rubber 'Policeman'. Radioactivity in the combined acid/neutral washes and in the cell-lysates was determined separately, using a Packard 500c autogamma spectrometer. Acid/neutral wash labile radioactivity was considered to originate from the cell-surface. Acid/neutral wash resistant radioactivity was assumed to be intracellularly located.

*Transferrin receptor exocytosis rates*

To measure TfR externalization rates, experiments were performed as originally described by Ciechanover et al.[69] At indicated times, culture medium was removed and the cells were washed twice with PBS (pH 7.4, 4°C). Next the cells were incubated for 1½ h at 37°C, in prewarmed M199 supplemented with 250 nM $^{125}$I-hTf-(2Fe). After removal of the radiolabeled ligand and three subsequent washes with PBS (pH 7.4, 4°C), the cells were incubated with acid (sodium acetate buffer, pH 4.5) and neutral (PBS, pH 7.4) washes as described above. Next the cells were incubated with prewarmed M199, supplemented with 6.25 $\mu$M hTf-(2Fe) at 37°C, for up to 1 h. At indicated times, TfR externalization was stopped by quick removal of the medium and immediate chilling of the cells by PBS washes at 0°C. Again, three

acid/neutral washes were performed. The incubation media were combined. Finally, the cells were lysed in distilled water (checked under an inverted microscope) and collected with a rubber 'Policeman'. Radioactivity in all samples (first acid/neutral washes, externalization media, second acid/neutral washes, and cell-lysates) was determined separately using a Packard 500c autogamma spectrometer. Radioactivity in the different samples was interpreted as described above ('TfR endocytosis rates'). Radioactivity in the second acid/neutral washes was considered to be externalized.

*Calculation of the internalization and externalization rate constants.*

Rate constants were calculated from the rate of internalization and externalization of $^{59}Fe$ and $^{125}I$, respectively, as described below. In the studies on TfR internalization $^{59}Fe$ is preferentially used because it remains in the cell after uptake. TfR externalization is most efficiently measured by using hTf bound $^{125}I$.

In the internalization experiments a substantial amount (approximately 50 %) of the radioactivity bound at 4°C, was released within seconds into the incubation medium, upon warming to 37°C. Comparable amounts were released in TfR internalization experiments in proliferative (50 %) and fused (65 %) BeWo cells.[106] Comparable data were (55 %) were found by v Dijk et al.[89] Excluding this amount of lost $^{59}Fe$, the percentages of radioactivity in the incubation medium, at the cell surface, and in the cell, were calculated. The radioactivity remaining at the cell surface and lost into the culture medium during the hour of incubation were both considered not to be internalized. Therefore, the final percentage of intracellular radioactivity was fixed to 100 % and the previously internalized amounts were adapted. Because the TfR internalization rate is equivalent to the rate of acquisition of acid/neutral wash resistance of the $^{59}Fe$ upon warming of the cells to 37°C, these curves were used to obtain TfR internalization rates.

### V-2.3. Protein determination.

Culture dish protein content was determined using the method of Pierce assay (see Chapter II, Protein determination).

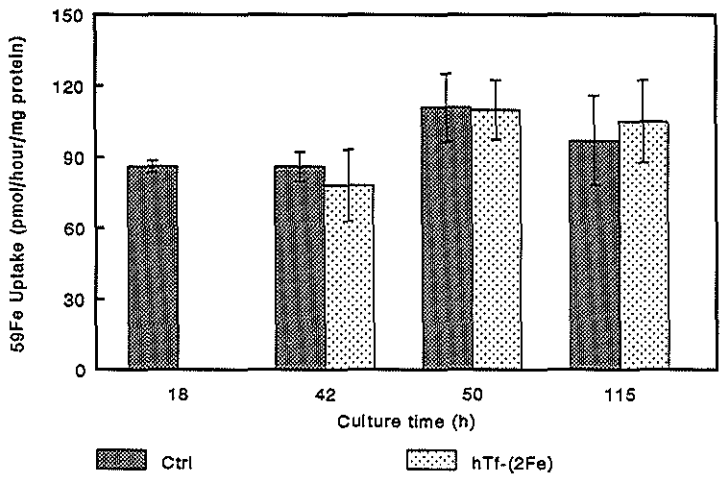


**Figure V-1. Initial iron uptake.**
Iron uptake from hTf-($2^{59}$Fe), in the first hour of incubation, was measured at indicated times, in cells cultured in either iron poor control medium, or in hTf-(2Fe) supplemented medium (1.25 $\mu$M). Neither cell differentiation nor culture conditions significantly affected initial iron uptake.

## V-3. RESULTS.

To measure initial iron uptake, cytotrophoblast cells were incubated for 1 h with 1.25 $\mu$M hTf-($2^{59}$Fe) at indicated culture times (Figure V-1). In the incubation period iron uptake was independent of cell-differentiation and culture conditions (hTf-2(Fe) availability). This implicates that initial iron uptake rates are not affected by these parameters.

Prolonged incubation with hTf-($2^{59}$Fe) (Figure V-2) caused differences in accumulated iron amounts between cells cultured for 18 h and 65 h. In a period of 6 h, relatively undifferentiated cells (18 h culture) accumulated 70 % more iron than differentiated cells did. The composition of the culture medium (hTf-(2Fe) availability) had no influence on iron accumulation during the 6 h incubation period in cells cultured for 65 h. Differences in amounts of accumulated iron in reaction to cell-differentiation, without changes in initial iron uptake, indicate that some kind of iron release mechanism matures during cytotrophoblast differentiation in culture.

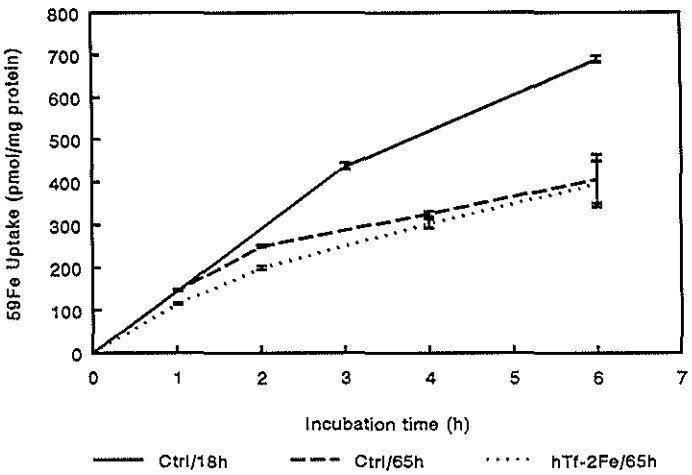


**Figure V-2. Iron accumulation.**
Cells were cultured for 18 h in control medium. Culture was continued in either control medium or hTf-(2Fe) supplemented medium (1.25 $\mu$M). Iron accumulation was measured at indicated times as descibed in materials and methods. Initial iron uptake is not affected by cell-differentiation nor by culture conditions. More iron was accumulated in less differentiated cells, suggesting that some kind of iron release mechanism matures during culture.

TfR internalization rates, determined at 18 h and 65 h culture time, differed with cell-differentiation and iron (hTf-(2Fe)) enrichment of the culture medium. In each of the Figures V-3, V-4 and V-5 results are depicted of four combined experiments. The curves showed the characteristics of a first order process and could easily be fitted in a one exponential curve plot according to the equation $\ln(1-B_t/B_{max}) = kt$ (see Figure V-3). The internalization rate constants ($k_{int}$) derived from these fitted curves were: 0.18/min in cells cultured for 18 h, 0.08/min in cells cultured for up to 65 h in iron poor control medium, and 0.12/min in cells cultured in hTf-(2Fe) enriched medium.

From Figure V-3, V-4 and V-5 it can be concluded that, apart from the amount of $^{59}$Fe rapidly lost upon warming of the culture medium, a small part of the remaining radioactivity is subsequently lost in the following hour. With vd Ende et al. we believe that the differences in the percentages of $^{59}$Fe finally lost during incubation are caused by the variation of the internalization rates.[106] Reduced TfR

internalization rates allow more hTf-($2^{59}$Fe) to dissociate from the receptor.

The rate of TfR externalization can be measured using $^{125}$I-labelled hTf-(2Fe). By saturation of the TfRs participating in the endocytic cycle with $^{125}$I-hTf-(2Fe) at 37°C, and subsequent removal of the surface bound $^{125}$I-hTf-(2Fe) at 4°C using sequential acid/neutral washes, TfR externalization rates are equal to the rate of decrease of intracellular $^{125}$I upon warming to 37°C.

The TfR externalization rates were calculated as follows. The amounts of radioactivity present in the media and the second acid neutral wash were added and corrected for variation in cell protein. These corrected data were transformed to a 100 % scale, taking the amount of radioactivity associated with the cells at time t=0 as 100 %. Since no difference was found between the 20h and 65h control data, the corresponding data points were pooled. Figure V-6 gives the transformed and time averaged data points of three independent experiments.

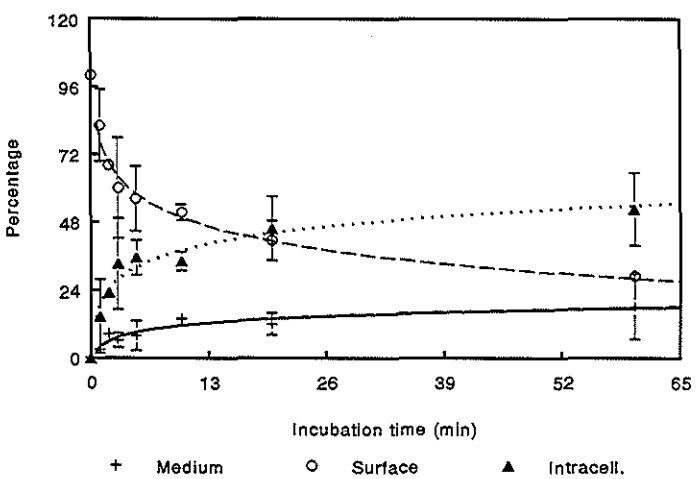


**Figure V-3. Transferrin receptor internalization rate at 18 h culture in control medium.**

Shown are the combined results of three independent experiments. Cells were isolated and cultured for 18 h as described in materials and methods. After incubation with hTf-$2^{59}$Fe at 4°C, cells were incubated in DMEM-H at 37°C for up to 1 h. At indicated times, radioactivity present in the culture medium, at the cell-surface, and intracellularly, was determined. Of each sample are plotted the radioactivity present in each sublocation as a percentage of total radioactivity. The internalization curve fits a one exponential model (k: 0.180/min, $t_{½}$: 3.9 min).

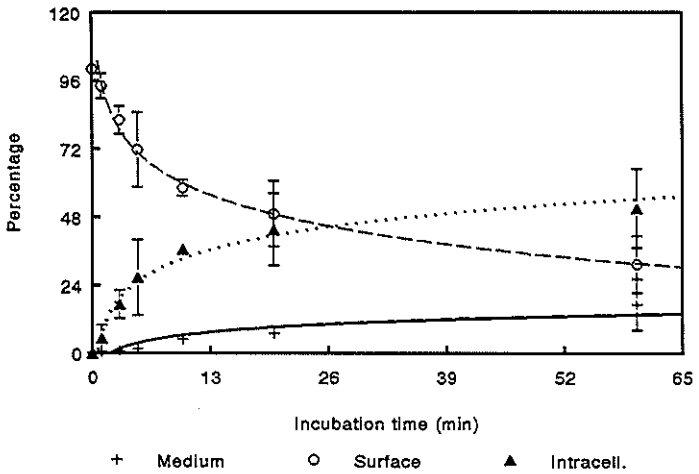


**Figure V-4. Transferrin receptor internalization rate at 65 h culture in control medium.**
Shown are the combined results of three independent experiments. Cells were isolated and cultured for up to 65 h, in iron poor control medium as described in materials and methods, but otherwise treated as described for Figure V-3. The internalization curve fits a one exponential model (k: 0.085/min, $t_{½}$: 8.7 min).

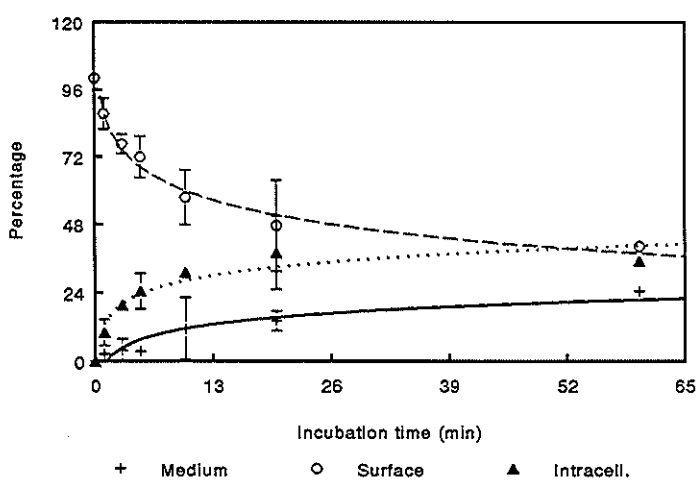


**Figure V-5. Transferrinreceptor internalization rate at 65 h culture in hTf-(2Fe) enriched medium.**
Shown are the combined results of three independent experiments. Cells were allowed to recover for 18 h in control medium. Culture was continued for up to 65 h in hTf-(2Fe) enriched medium (1.25 $\mu$M), as described in materials and methods, but otherwise treated as described for Figure V-3. The internalization curve fits a one exponential model (k: 0.121/min, $t_{½}$: 5.8 min).

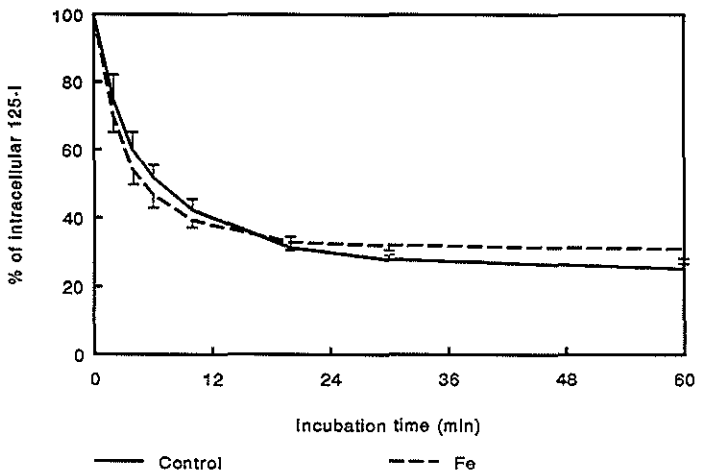


**Figure V-6. Transferrin receptor externalization rates.**
Shown are the combined results of three independent experiments. Cells were isolated and cultured as described in materials and methods. Shown are the $^{125}$I-hTf externalizations in cells cultured in control medium (rate constants did not change during culture) and hTf-(2Fe) enriched medium.

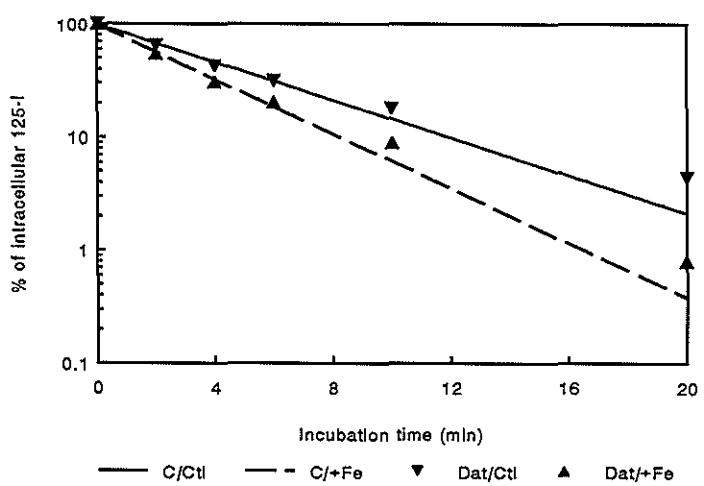


**Figure V-7. Transferrin receptor externalization rate constants.**
Semi-logarithmical plot of the curves shown in Figure V-6 after modification as described in 'Results'. Depicted are the modified curves and the original data after subtraction of the percentage of radioactivity which remained in the cell after incubation for 1 h at 37°C. Externalization rates were k: 0.19/min ($t_{½}$: 3.6 min) and k: 0.278/min ($t_{½}$: 2.5 min) for the control (——) and hTf-2Fe (– – –) series respectively.

The two curves were analysed by non-lineair regression, using the Marquardt algorithm (Statgraphics$^{R}$). Since 25 to 30 % of the radioactivity remains associated with the trophoblasts even after 60 min excubation, and since this fraction tends to diminish only very slowly, we fitted the data to the function: $Y(t)=C1*\exp(-k*t)+C2$. C2 gives the constant part and $C1*\exp(-k*t)$ represents the first order exocytotic part. k is the first order rate constant. Using the estimates of the coefficients C1 and k, the exocytotic part of the function y(t) can be calculated. In Figure V-7 the results of the calculated curves are semi-logarithmically plotted.

As depicted in Figure V-6 and V-7, the TfRs in the Fe supplemented series were more rapidly externalized. In the 18 h cultured cells $t_{½,ext}$ was 3.6 min. Prolonged culture in iron poor medium did not change externalization rate constants. At 65 h culture the $t_{½,ext}$ in the hTf-(2Fe) series was: 2.5 min. The corresponding externalization rates constants were $k_{ext.}$: 0.19/min and $k_{ext.}$: 0.24/min in the control and in the hTf-(2Fe) series, respectively.

| Culture time (h) | 18 | 65 | 65 |
|---|---|---|---|
| hTf-(2Fe) suppl. culture medium | - | - | + |
| $t_{½,\,int.}$ (min) | 3.9 | 8.7 | 5.8 |
| $k_{int.}$ ($min^{-1}$) | 0.18 | 0.08 | 0.12 |
| $t1_{½,\,ext.}$ (min) | 3.6 | 3.6 | 2.9 |
| $k1_{ext.}$ ($min^{-1}$) | 0.19 | 0.19 | 0.24 |
| $T_{cycle}$ (min) | 10.9 | 15.7 | 11.7 |

**Table V-1. Transferrin cycle times.**
Internalization and externalization rates were measured, as described in materials and methods, in cells cultured in control medium for 18 h and 65 h, and in cells cultured for 65 h in hTf-(2Fe) supplemented medium. Total cycle times were calculated using the binding and dissociation rates obtained by Ciechanover et al.[69] rates ($3.0 \times 10^{6}$ $M^{-}$·$min^{-}$ and 2.6 min, respectively).[69]

The consequences of these changes in internalization and externalization rate constants are summarized in Table V-1. TfR total recycle times ($T_{cycle}$) were calculated using the figures of Ciechanover et al. on hTf binding and dissociation rates ($3.0 \times 10^6$ $M^{-1}min^{-1}$ and 2.6 min, respectively).[69] In cytotrophoblasts cultured for 18 h and 65 h in iron poor control medium $T_{cycle}$ times were 10.9 min and 15.7 min, respectively. In the iron enriched series $T_{cycle}$ at 65 h culture time was 13.2 min.

## V-4. DISCUSSION

Syncytiotrophoblasts take up iron from hTf by receptor mediated endocytosis.[72,94,121] Because this is the major mechanism of iron uptake, the rate of iron uptake depends on hTf concentration, hTf iron saturation, surface TfR densities, and TfR cycle times.

In the experiments described in this chapter, hTf concentration and hTf iron saturation were constant during incubation of the cells two factors with potential influence on iron uptake thus remain: surface TfR densities and TfR cycle times.

The effects of cell-differentiation and hTf-(2Fe) availability during culture on TfR numbers and distribution are discussed in Chapter IV. During cell-differentiation surface TfR densities increase and iron poor culture medium leads to higher TfR numbers. These changes in TfR numbers could affect iron uptake rates and transplacental iron transport, as previously suggested by Bierings et al.[36]

The results on TfR accumulation (Figure V-1) do not support the hypothesis that iron uptake is solely regulated by surface TfR densities. Independent of surface TfR numbers, the initial iron uptake is 80-100 pmol/mg protein/h. These uptake rates are similar to those obtained in K562 erythroleukemic cells (39 pmol/h/$10^6$ cells) and BeWo choriocarcinoma cells (50-110 pmol/h/mg protein).[106,209] In BeWo cells iron uptake rates depend on culture conditions, with the highest uptake rates in cells forced to fuse by theophyline supplementation of the culture medium.

Previously, comparable but slightly lower Fe uptake rates (25 pmol/mg protein) were obtained in cultured cytotrophoblasts.[94] This uptake rate could be explained by the lower hTf iron saturation ($\geq$ 75 %) used by these authors.

Iron accumulation during 6 h in relatively undifferentiated cells (Figure V-2) amounted to up to approximately 700 pmol/mg protein. This pattern of iron uptake

was almost linear and very similar to that found in cytotrophoblasts cultured for 48 h in a 1:1 mixture of Ham's and Waymouth's culture medium.[95] In this medium cytotrophoblasts less rapidly differentiate, according to morphological criteria.

Cells cultured for 65 h showed a decrease in Fe accumulation rates during 6 h incubation, irrespective of hTf-(2Fe) supplementation of the culture medium (Figure V-2). This observation suggests that some kind of Fe release mechanism matures during cytotrophoblast culture, which makes Fe release by a process of diffusion, as proposed by Douglas and King, unlikely.[94] Nevertheless, the effects of medium replacement, observed by these authors, indicate that the mechanism of iron release is sensitive to environmental Fe concentrations.

The other factor of influence on iron uptake by means of receptor mediated endocytosis is the TfR cycle time. In several cell types, changes in internalization and externalization rates allow the cell to change surface TfR densities.[58,279,383] These rates are also affected by growth factors like the epidermal growth factor and the platelet derived growth factor.[80] In contrast to the changes in TfR internalization and externalization rates, which parallel theophylline forced fusion of BeWo choriocarcinoma cells,[106] the growth factor induced changes in rate constants occurred rapidly.

The process of receptor-mediated endocytosis comprises a series of reactions, each with its specific rate constant. In combination, these reactions result in the total TfR cycle time, and can be summarized in the equation:

$$T_{cycle} = (k_{bind.} \cdot L)^{-1} + (k_{int.})^{-1} + (k_{ext.})^{-1} + (k_{diss.})^{-1}$$

where $T_{cycle}$ is the total TfR cycle time, $k_{bind.}$ is the rate constant for hTf binding to the transferrin receptor, L the ligand (hTf) concentration (1.25 $\mu$M), $k_{int.}$ the rate constant for TfR internalization, $k_{ext.}$ the rate constant for TfR externalization, and $k_{diss.}$ the rate constant for apo-hTf dissociation from the receptor at the cell surface.[69]

The rate constants for hTf binding and dissociation were not determined in the in vitro model used in our experiments, but were previously obtained by Ciechanover et al. in a human hepatome cell line (HepG2) ($k_{bind.}$ = 3.0 x $10^6$ $M^{-}$·$min^{-}$; $k_{diss.}$ = 2.6/min).[69] Because Ciechanover et al.[69] studied the binding of human transferrin to

human cells (and thus human TfRs), and because no structural differences are described for the TfR, originating from different human cell types, we assume that these rate constants can be used in the cytotrophoblast model.

The experiments on TfR internalization showed that the surface TfRs were internalized with the same rate constant, that was changed in reaction to cell-differentiation and hTf-(2Fe) availability during culture.

In contrast to TfR internalization, TfRs externalization only varied in reaction to iron supplementation of the culture medium. About 30 % of the radioactivity was not released within 1 h. Using the same procedure, vd Ende et al. showed a similar pool of radioactivity (27 %) not released from BeWo cells.[106] This percentage of radioactivety might represent the non-active receptor pool shown in chapter IV.

If intracellularly TfRs are distributed among functionally different pools, sorting must take place. The present knowledge on this process has been reviewed by Stoorvogel et al.[369] In many cell types TfRs have to be sorted from the degradative pathway, and since cultured cytotrophoblasts do not shed their TfRs (Chapter IV), some kind of degradation pathway has to exist in these cells too. One of the other routes might pass through the Golgi complex to enable the cell to resialylate the receptor.[360] If this route through the Golgi complex is taken by the TfRs, these receptors should not be included in the pool of TfRs participating in the endocytic cyle.

It has been described that in HepG2 cells 30 % of the asialoglycoprotein receptor are not involved in the endocytic cycle, and are possibly localized in the trans Golgi reticulum. Remarkably however, in this cell type, no TfRs were detected in these structures.[368] In the human placenta brush border membrane and in the guinea pig, no asialoglycoprotein receptors have been found.[90,238]

Using the internalization and externalization rate constants and the rate constants for hTf binding and dissociation obtained by Ciechanover et al.,[69] TfR cycle times can be calculated (Table V-1). Under the in vitro conditions used, total TfR cycle time was the longest in cells cultured in control medium for up to 65 h (15.7 min), the cells with the highest TfR densities (see Chapter IV). In cells cultured in hTf-(2Fe) supplemented medium for 65 h - conditions which result in less higher surface TfR numbers (Chapter IV)- total TfR cycle time was 11.7 min. The relatively shortest

total cycle time was obtained in 18 h cultured cells (10.9 min), which also had the lowest surface TfRs densities (Chapter IV). These cycle times are very similar to the TfR cycle times in non-fused (21.2 min) and fused (14.6 min) BeWo cells,[106] and indicate that the changes in surface TfR densities are neutralized by variation of the total cycle times. This is in agreement with the results on initial iron uptake by cytotrophoblast in vitro.

It could be that intracellularly TfRs are sorted into a resialylation pathway and/or a degradative pathway,[360,369] from which the $^{125}I$ is released in a free form or bound to hTf-fragments. Resistance of the externalized $^{125}I$ to precipitation with 10 % TCA has not been tested, so no further data are available on protein (fragment) association of the externalized $^{125}I$.

In conclusion: despite the differences in surface TfR densities, induced by cell-differentiation and hTf-(2Fe) availability during culture (Chapter IV), iron uptake rates are not affected by these variables. This can be explained by the variation in TfR cycle times induced by cell-differentiation and culture conditions. Iron uptake by cultured differentiating cytotrophoblasts, and most likely alsothe iron uptake by the syncytiotrophoblasts in vivo, is stable and balanced by changes in surface TfR numbers in combination with variations in TfR cycle times. The changes in iron accumulation rates in reaction to cell-differentiation strongly suggest the presence of some kind of iron release mechanism in cytotrophoblasts.

## Chapter VI. FERRITIN IN HUMAN CYTOTROPHOBLASTS.

### VI-1. INTRODUCTION.

Iron plays important roles in, for instance, DNA synthesis in cells, and in the degradation pathways in lysosomes,[390] but iron is also potentially dangerous. A surplus of free iron could catalyze hydroxyl formation.[144,431] Therefore, the majority of intracellular iron is stored in ferritin where it can do no harm.[282]

As described in Chapter I (Ferritin), ferritin is a protein consisting of twenty-four polypeptide subunits. Each molecule can store maximally 4500 iron-atoms but is normally saturated for 20-25 %.[388] There are two types of subunits: heavy (H) and light (L).[11,98] Corresponding with a predominance of H- and L-subunits, there are H- and L-isoferritins with functional differences. Compared to H-rich ferritins, L-rich ferritins are basic and more stable. They process iron less rapidly, but have higher iron contents.[12,98,151]

Placental isoferritin composition changes during pregnancy to an approximately equal amount of acidic and basic isoferritins at term.[50,218,235]

In cell culture, the form iron is offered in is important for the mechanism of iron-uptake.[91,92,181,428] The implications of the iron compound for the intracellular handling and storage of iron, are unknown. Different effects are expected because nitrilotriacetate iron did not enhance cell proliferation in mouse lymphocytes.[48]

In vivo, the majority of iron is offered to the trophoblast bound to hTf.[121]

According to the IRE hypothesis, the storage of intracellular iron is efficiently regulated by the effects of iron on ferritin mRNA translation (see Chapter VII), as has been shown in cells only in control of their own iron stores.

A different situation occurs in syncytiotrophoblasts, because these cells form the first layer of the maternal-fetal barrier. They have to keep intracellular free iron levels as low as possible but, on the other hand, the transplacental transport of iron should increase to very high levels, especially during the third trimester.[87,322,413]

In this Chapter the effects are shown of diferric transferrin and ferric-ammoniumcitrate on trophoblast ferritin concentration, ferritin iron saturation, and ferritin subunit ratio.

Ferritin synthesis and TfR synthesis are discussed in light of the IRE-hypothesis in Chapter VII.

## VI-2. MATERIALS AND METHODS.

### VI-2.1. Materials.

The required chemicals and products are described in chapter II. Immun-Lite assay kits were obtained from Bio-Rad, Richmond, USA.

### VI-2.2. Placental ferritin isolation.

Placental ferritins were isolated according to Konijn et al.[218] Briefly: placenta tissue was washed twice in phosphate buffered saline and homogenized in distilled water containing 0.1 mM PMSF and 0.02 % $NaN_3$. Insoluble material was removed and ferritin was isolated from the supernatant by addition of ammoniumsulphate (pH 5,2) until 50 % saturation was achieved. The precipitate was resuspended in 29 mM sodiumphosphate buffer (pH 7.0). Insoluble material was removed. After ultracentrifugation (120,000 g, 3.5 h) the pellet was chromatographed on a Sepharose 4 B column. Ferritin subfractions were obtained by ion-exchange chromatography on a DEAE-A25 column (0.154-0.64 M NaCl) and analysed by agarose iso-electric focussing (pH: 4-6) using the Pharmacia Phast system. Gel: 1 % Agarose EF. Running conditions: prefocussing phase: 1000V, 2.0mA, 3.5W, 15°C, 60Vh; application phase: 200V, 2.0mA, 3.5W, 15°C, 15Vh; separation phase: 1000V, 5.0mA, 3.5W, 15°C, 60Vh.

### VI-2.3. Cytotrophoblast cell isolation.

Cytotrophoblast cells were isolated using both procedure A and B (see Chapter II, trophoblast cell isolation).

The cells obtained by procedure B were counted (using a Bürker counting-chamber), and lysed by sonication in PBS containing 1mM Phenyl-Methyl-Sulfon-Fluoride (PMSF) and aprotinin (0.5 U/ml). The cell-lysates were used for trophoblast ferritin subunit-ratio determination (see further). Cells obtained by procedure A were used for cell-culture.

### VI-2.4. Cell-culture conditions.

Isolated cells were counted (using a Bürker counting-chamber), diluted to 6 x $10^5$ cells/ml in culture medium, and plated out in 35 mm and 60 mm Falcon culture dishes (1.5 and 4.5 x $10^6$ cells/ dish respectively). Standard control culture medium was used (see Chapter II, Trophoblast cell isolation).

Cells were allowed to recover from the isolation procedure for 24 h and, prior to the start of the experiments, washed twice with M199 to remove non-adherent cells. Culture was continued as descibed in Chapter II (Cell culture conditions) in control medium, or in medium supplemented with either hTf-(2Fe) (1.25 $\mu$M) or ferric-ammoniumcitrate (FAC, 10 $\mu$g/ml). Culture media were refreshed every 24 h.

### VI-2.5. Trophoblast ferritin isolation.

*Preparation of trophoblast cell lysates*

At indicated culture times, cells in 60 mm dishes were washed twice with PBS and lysed by addition of 1 ml distilled water (checked using an inverted microscope ($CK_2$, Olympus, Japan). The cells were harvested with a rubber 'Policeman' and the dishes were washed twice with 0.5 ml distilled water. Samples (in total 2 ml) were stored at -20 °C until continuation of the procedure. Homogenous samples were obtained by sonication for 10 seconds in melting ice. Of each sample aliquots (50 $\mu$l) were set apart for protein determination.

Ferritin was isolated from the trophoblasts lysates either by ultracentrifugation or by immunoprecipitation as described below.

*Ultracentrifugation procedure.*

The cell-lysates were incubated with DNase (0.3 $\mu$g/ml) for 15 min at 37°C. Subsequently Proteinase-K (1 $\mu$g/80 $\mu$g protein) was added and again the samples were incubated for 15 min at 37°C.[13] Next the samples were incubated for 5 min at 75°C,[135] rapidly cooled to room temperature and centrifuged for 10 min at 10,000 g (Rotor JA-21 Ti Beckman). The supernatants were transferred to clean, iron free tubes suitable for ultra-centrifugation (Rotor 70.1 Ti Beckman). The pellet was resuspended in 1 ml distilled water and again centrifuged for 10 min at 10,000 g. The supernatants were centrifuged for 20 min at 50,000 g. Finally the supernatants of the 50,000 g fraction were centrifuged for 4 h at 120,000 g (Rotor 70.1 Ti Beckman).[306] The supernatants were removed and the pellets were resuspended in

20 $\mu$l LiOH-borate buffer (pH 8.6). Using this procedure, samples prepared from iron loaded cells contain, in addition to ferritin, an iron binding compound. This compound does not react with anti-ferritin antibodies but morphologically it resembles haemosiderin.[319] It is most likely a ferritin degradation product.

*Ferritin-immunoprecipitation*

After sonication and heat-treatment (75°C, 5 min) the cell-lysates were centrifuged (10,000 g, 15 min). Insoluble material was removed. The supernatants were concentrated to a volume of 0.5 ml using a Centricon-10 microconcentrator (Amicon). These samples were incubated for 18 h at 4°C with rabbit anti-human-placental-ferritin IgG bound to protein A-Sepharose CL-4B. Subsequently the samples were centrifuged (1100 g, 5 min). The precipitate was washed twice with distilled water and incubated for 2 h at 20°C with 0.05 M glycin-HCl (pH 2.7) to dissociate the ferritin-antiferritin complex.[206] Finally, the samples were centrifuged (1100 g, 5 min) to remove the anti-ferritin IgG protein-A sepharose complex.

**VI-2.6. Ferritin determination.**

Total ferritin concentration was determined using the Spectro Ferritin enzyme immunoassay (Ramco Laboratories). This ferritin elisa only determines 44 % of the placental ferritins, which means that measured ferritin concentrations should be multiplied by 2.27 to obtain the total ferritin concentration.[375] Despite this disadvantage the Spectro Ferritin kit was chosen, for it detects more of the placental ferritins then other immunoassays.[49,375]

**VI-2.7. Rabbit anti-human-placental-ferritin-IgG purification.**

A New Sealand NZW-rabbit was immunized with purified total human placental ferritin. IgG antibodies were isolated using an Econo-Pac serum IgG purification kit (Bio-Rad, Richmond, USA).

**VI-2.8. Iron determination.**

Total cellular iron contents were spectrophotometrically determined based on the method described by Harris,[149] using ferrozine as a chromogen. The method was slightly modified: the ferritin iron cores in 125 $\mu$l samples were dissolved by addition

of 62.5 $\mu$l 12 M HCL and 37.5 $\mu$l 40 % TCA.

Ferritin iron contents were determined in immunoprecipitated ferritin, using a FAAS-analyser PU-9200X and a Cu-Fe-Zn lamp (Pye-Unicam, UK). Dry phase 110-130°C (30 to 40 s); ash phase 800°C (15 s); atomisation 2200°C (5 s).

### VI-2.9. Determination of trophoblast ferritin subunit ratios.

Ferritin was extracted from freshly isolated cytotrophoblasts using the ultracentrifugation procedure, and its concentrations were determined using the Spectro ferritin immunoassay.

Subunits of approximately 2 $\mu$g ferritin were separated by SDS-PAGE according to Schägger and v Jagow (1987) (10 % T and 3 % C).[335] In this procedure tricine is used as trailing ion.

The subunits were blotted on 0.2 micron nitrocellulose membranes (1 h, 100 V, 250 mA, at room temperature; Bio-Rad, Richmond, USA). The membranes were fixed by incubation with 0.2 glutaraldehyd in PBS (45 min),[104] followed by three washes in PBS (pH 7.4).

Subunits were visualized with the Immun-Lite assay, following the manufacterors instructions. Finally, subunit ratios were determined using the Ultroscan XL Enhanced Laser Densitometer (LKB, Bromma).

### VI-2.10. Protein determination.

Sample protein concentration was measured using the Micro BCA Protein Assay Reagent.

### VI-2.11. Statistics.

The significance of the results was tested by using the Student-Newman-Keuls test.

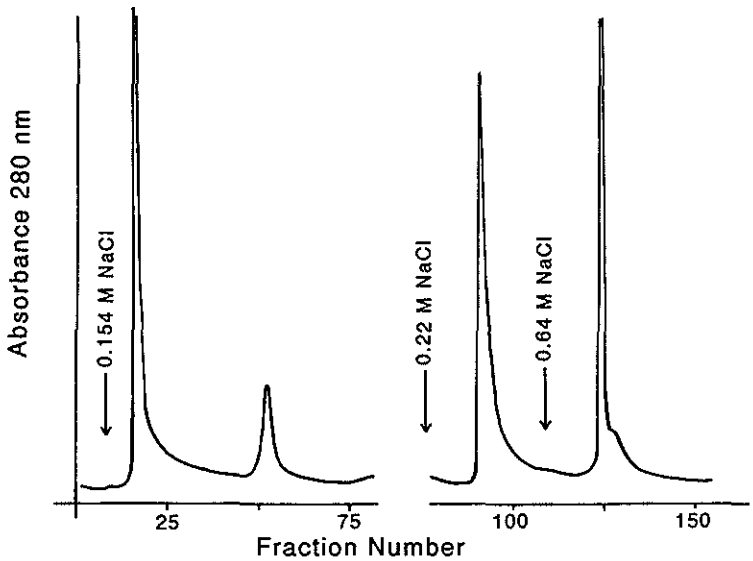


**Figure VI-1. DEAE-A25 chromatography of total placental ferritin.**
Ferritin was isolated and chromatographed as described in materials and methods. A 2 x 40-cm column was used and 5 ml fractions were collected. Arrows indicate beginning of higher NaCl gradient. Four peaks were obtained, designated Basic I, Basic II, Intermediate, and Acid.

## VI-3. RESULTS.

By chromography on a DEAE-A25 Column four ferritin subfractions were obtained, as depicted in Figure VI-1. The subfractions were designated 'Basic I', 'Basic II', 'Intermediate' and 'Acid', similar to Konijn et al.[218] A second 'acid' subfraction could not be obtained, neither by prolonged elution nor by higher NaCl molarities (up to 1.0 M).

Figure VI-2 shows a representative example of an agarose (1 %) iso-electric focussing of the placental ferritin subfractions obtained by DEAE-A25 chromatography and depicted in Figure VI-1. Lane 1: markers, Lane 2: total placental ferritins and Lane 3-6: the four placental ferritin subfractions (3: Basic I, 4: Basic II, 5: Intermediate and 6: Acid). The observed iso-electric points were: total placental ferritin: pH 4.67-5.52, Basic I: pH 5.28-5.52, Basic II: pH 5.07-5.41,

Intermediate: pH 4.77-5.33 and Acid: pH 4.69-5.20.

In Figure VI-3 the results on trophoblast ferritin subunits are depicted, in relation to placental iso-ferritins. Although trophoblast ferritin subunit ratios varied with each placenta, the results showed that trophoblast ferritin was further enriched with H-subunits (L/H ratio: 1.86:1, SD 0.65, n = 3).

Figure VI-4 shows the effect of iron enrichment of the culture medium on total cellular iron contents. Culture medium composition did not affect cell viability, nor cellular protein and DNA contents (results not shown). Addition of hTf-(2Fe) to the medium did not change intracellular iron amounts significantly (from 6.0 to 6.3 nmol/mg protein). The cells cultured in control medium lost a small amount of

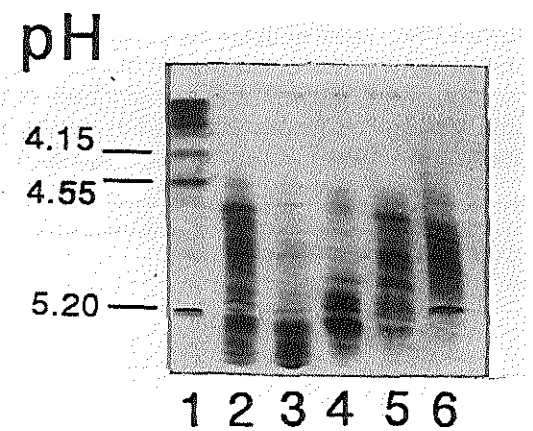


**Figure VI-2. Iso-electric focussing of total placental ferritin and placental ferritin subfractions.**
Placental ferritin subfractions were obtained by DEAE-A25 chromatography as described in materials and methods. (1) Markers, (2) Total placental ferritin, (3) Basic I subfraction, (4) Basic II subfraction, (5) Intermediate subfraction, (6) Acid subfraction. The iso-electric points were: total placental ferritin: pH 4.67-5.52, Basic I: pH 5.28-5.52, Basic II: pH 5.07-5.41, Intermediate: pH 4.77-5.33, and Acid: pH 4.69-5.20.

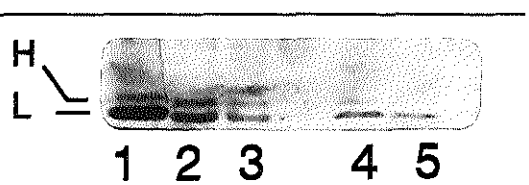


**Figure VI-3. Total placental and trophoblast ferritin subunit composition.**
Trophoblast and placental ferritin were obtained as described in materials and methods. Ferritin subunits were obtained by SDS-PAGE and Western blotting as described in materials and methods. The subunits were visualized using an Immune-Lite assay and by exposure of a photosensitive film to the blot. (1) Basic I subfraction, (2) Intermediate subfraction, (3) Acid subfraction, (4) Trophoblast ferritin, (5) Trophoblast ferritin, prolonged reduction.

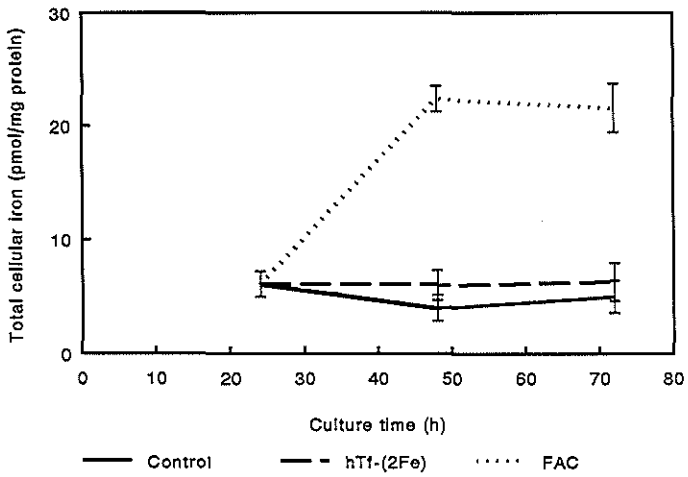


**Figure VI-4. Total cellular Fe-content.**
Shown are the combined results of four independent experiments. Trophoblast iron contents were determined as described in materials and methods. Compared to both the control and the hTf-(2Fe) series, cellular iron contents were increased significantly in the FAC series ($\alpha_T < 0.002$).

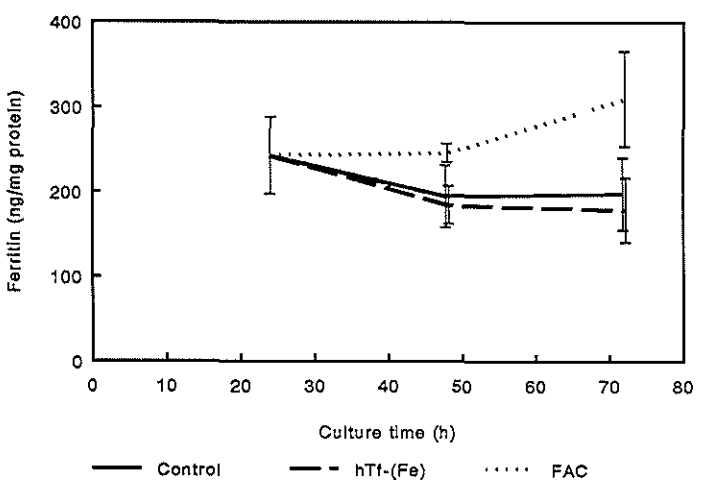


**Figure VI-5. Cellular ferritin concentrations.**
Shown are the combined results of four independent experiments. Cells were isolated and cultured as described in materials and methods. Cellular ferritin concentrations were determined using a Spectro ferritin immunoassay. After 72 h culture, cellular ferritin concentration in the FAC series becomes significantly different from both the control and the hTf-(2Fe) series ($\alpha_T < 0.05$).

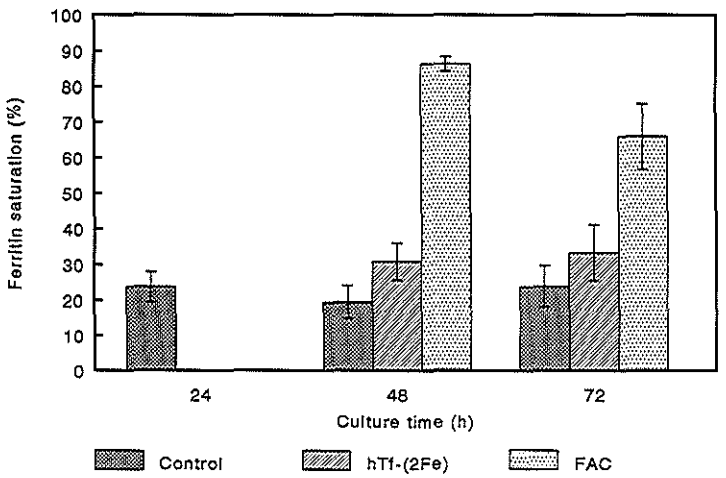


**Figure VI-6. Ferritin iron saturation.**
Shown are the combined results of three independent experiments. Ferritin was immunoprecipitated from cell homogenates, as described in materials and methods. Iron saturation was measured using a FAAS-analyser PU-9200X (Pye-Unicam, UK). Compared to both the control and the hTf-(2Fe) series, ferritin iron saturation was increased significantly in the FAC series ($\alpha_T < 0.01$).

iron (from 6.1 to 5.0 nmol/mg protein). However, cellular iron contents did not differ significantly from those in the hTf-(2Fe) series. In cells cultured in FAC enriched medium total iron amounts were increased (from 6.1 to 21.6-22.4 nmol/mg protein). These values are significantly different from those in both the control as well as in the hTf-(2Fe) series ($\alpha_T < 0.002$).

Cellular ferritin concentrations (Figure VI-5) were similarly effected. Ferritin concentration slightly decreased both in the control and in the hTf-(2Fe) series (from 240 to 190 ng/mg protein). Cells cultured in FAC enriched medium increase ferritin concentrations to over 300 ng/mg protein, which becomes significant after 72 h of culture ($\alpha_T < 0.05$).

During culture, the amount of ferritin bound iron as percentage of total cellular iron slightly decreased from 23.6 percent to 20.9 and 18.9 percent in the hTf-(2Fe) and the FAC series, respectively (results not shown); these differences do not reach significance.

Figure VI-6 shows trophoblast ferritin iron saturation. Full saturation was set at 4500 iron-atoms per ferritin molecule. In the control and the hTf-(2Fe) series, ferritin iron saturation was 23 and 30 percent, respectively. In both series saturation was stable with time. Ferritin iron saturation in the FAC series significantly increased with 75 % ($\alpha_T < 0.01$).

## VI-4. DISCUSSION.

The major function of intracellular ferritin is the storage of iron, which, in its free form, could become dangerous.

Ferritin subunit composition causes heterogeneity. Mid-gestational human placental ferritin mainly contains acidic subfractions.[235] Contradictory results on placental ferritin subunit composition at term have been published. According to Brown et al. term placental ferritin is L-subunit-rich.[50] Konijn et al. published data showing a one-to-one ratio of basic and acidic iso-ferritins.[218]

Although we could not produce a fifth fraction designated "Acid II",[218] our preparation of total placental ferritin closely resembled the placental ferritin characteristics by these scientists. The shoulder in peak 4 might contain this fifth fraction (Figure VI-1). The isoelectric points of the ferritin subfractions, however, are highly comparable, indicating that similar products were obtained (Figure VI-2).

Placenta ferritin L-H subunit-ratios varied slightly with each placenta. Remarkably, it differed from the L-H subunit-ratio of trophoblast ferritin (Figure IV-3). Most likely total placental ferritins are a mixture of ferritins from different cell-types with variations in subunit-ratios. Trophoblast ferritins are H-subunit rich (Figure VI-3). Since H-ferritins handle intracellular iron more rapidly,[12,411] this would fit in with the iron-transport function of the cells.

The results presented in this chapter show that cells cultured in hTf-(2Fe) supplemented medium had a stable iron content, slightly higher than the cellular iron concentration in the control series (Figure VI-4). Because trophoblasts take up iron (Chapter V), this could be explained by iron release from the cells, without iron storage above the direct cellular needs; a mechanism consistent with the function of term syncytiotrophoblasts.

Cells cultured in FAC enriched medium accumulated iron. Apparently, iron bound to ammoniumcitrate is not processed (uptake/storage/transfer) similar to hTf bound iron. A difference in cellular iron processing, caused by the way iron is chelated, has previously been described.[181] On the other hand, when in the FAC series iron uptake continues, it also leaves the cell, because a steady state situation is presumably reached within 24 h (Figure VI-4).

High intracellular iron contents are cytotoxic, unless stored in ferritin. A surplus of iron above the ferritin storage capacity and the need for ferritin synthesis above the cellular capicities eventually lead to uncomplete degradation of ferritin into haemosiderin. Although less efficiently, compared with iron stored in ferritin iron in this form is not as dangerous as iron in a free form. Also in trophoblasts a reaction to the intracellular iron concentrations was seen (Figure VI-5 and Figure VI-6). Cells confronted with high intracellular iron concentrations and high ferritin iron saturations (FAC series), increased their cellular ferritin. Because cellular ferritin levels are not increased in the hTf-(2Fe) series, and iron is taken up by these cells (Figure V-1 and V-2), the effect of FAC on cellular ferritin contents may be induced by a mechanism of self-protection, triggered by the increased intracellular (free) iron levels.

In the hTf-(2Fe) series, cells were able to balance iron uptake and release. Intracellular iron levels did not change and no adaptation of cellular ferritin levels was necessary. Apparently, no extra ferritin is required for the transcellular transport of iron.

Syncytiotrophoblasts are cells specialized in the rapid transport of many kinds of nutrients. Under physiological circumstances term syncytiotrophoblasts do not store iron (anymore).[311] Therefore, when (physiologically) bound to transferrin, iron is taken up, possibly temporarily stored, and transferred through the cell, without loss of control. Only a small amount of iron is required for intracellular processes, which could easily be 'taken' from the iron-pool transported to the fetus. In such a cell type the control of iron uptake and storage do not necessarily have to be coupled. If the uptake of iron is regulated and the intracellular iron amounts are balanced by the release of iron, ferritin synthesis does not have to be regulated simultaneously. If iron is forced into the cell via an unnatural way (unphysiological iron chelators are

most likely taken up by pinocytosis,[70,92,181] it may be impossible for the cell to process the iron via the normal routes. Iron-release may be hampered and, subsequently, intracellular iron concentrations may become to high. Under these conditions of obstructed iron handling the cell can protect itself by ferritin synthesis.
In conclusion: the reaction of cytotrophoblasts cultured in vitro on iron, with respect to the intracellular ferritin levels, depends on the way the iron is chelated. The unphysiological iron chelator FAC increases ferritin synthesis, while hTf bound iron does not. Apparently, the iron-form is important for the intracellular route iron is processed in.
Although there are discrepancies between the results described above and the regulation of ferritin synthesis as proposed in the IRE-hypothesis (see chapter I, Cellular iron homeostasis) conclusions on the presence of an IRE-controlled mechanism of cellular iron homeostasis in syncytiotrophoblasts, cannot yet be drawn. Experiments on the effects of iron on transferrin receptor and ferritin synthesis are required to reach such conclusions (see Chapter VII).

## Chapter VII. THE SYNTHESIS AND DEGRADATION OF TRANSFERRIN RECEPTORS AND FERRITIN.

### VII-1. INTRODUCTION.

Cellular protein concentrations can be controlled at many levels. Two of these levels are mRNA translation and the rate of protein degradation.[440]

In contrast to the mechanisms of degradation, many aspects of the translational regulation of TfRs and ferritin have been elucidated during the last decade. Growth factors and different iron compounds have been shown to affect mRNA translation,[44,223,260] Major functions in this regulation mechanism are performed by the iron responsive element (IRE) and the IRE-binding protein. The IRE is a stemloop of about 28 nucleotides present in the mRNA of TfR and ferritin.[14,280] Similar structures have been found in, for instance, transforming growth factor mRNA and in 5-aminolevulinate synthase mRNA.[133,203] The stemloop configuration is essential for the function of the IRE; mRNA that could not form a stemloop, bound 1000-fold less well to the IRE-binding protein.[23] The structure of the loop, especially of the top, is similar in TfR and ferritin mRNA. The top consists of CAGUGX and an unpaired C is always present in the stem.[61,155]

A cytosolic protein binds specifically to the IRE.[23] This protein has been named iron regulating factor (IRF), ferritin repressor protein (FRP), and IRE-binding protein (IRE-BP). The latter name will be used in this thesis. The IRE-BP has been isolated and purified, and cDNA encoding for the protein has been cloned.[326,327]

The IRE-BP is identified as an approximately 90-kD protein, and it shows many similarities with aconitase, an enzyme of the citric acid cycle.[71,140,199,210,327] The IRE-BP has aconitase activity and aconitase binds to the IRE.[192,441]

Because of the similarities, it has been proposed that cellular energy production is coupled to cellular iron homeostasis.[71] This connection between iron uptake and energy production might contain a clue for the growth stimulating effects of iron.

Regulation of TfR and ferritin synthesis takes place by binding of the IRE-BP to the IRE.[223,281,325,377] Low intracellular iron levels increase the affinity of the IRE-BP for the IRE, possibly by changing its rate of degradation.[359] The iron induced changes in

affinity may be caused by oxidation and reduction of the IRE-BP.[157] The effects of binding of the IRE-BP depend on the location of the IRE in the mRNA. In ferritin mRNA the position of the IRE is very critical for its function.[133,134]

IRE's are located in the 5' side in ferritin mRNA and in the 3' side in TfR mRNA. Dissociation of the IRE-BP stabilizes ferritin mRNA[260] and alows it to be translated, while TfR mRNA becomes less stable and is degraded more rapidly. The overall effect of high intracellular iron levels is, therefore, reduced binding of the IRE-BP to the IRE's, leading to increased ferritin and decreased TfR synthesis. Iron shortage caused by desferrioxamine gradually increases TfR synthesis.[259] However, the compound in which iron is offered to the cells might affect the impact on mRNA translation. Hemin, for instance, also influences ferritin mRNA transcription.[70]

In the IRE-hypothesis, the regulation of iron uptake and storage are combined. The results described in chapter IV and VI, however, strongly suggest that in cultured cytotrophoblasts the regulation of TfR and ferritin synthesis are not controlled strictly according to the IRE-hypothesis. Data are required on cytotrophoblast TfR and ferritin synthesis to obtain evidence on the role of IRE's in the control of trophoblast iron homeostasis and, possibly, in the regulation of transplacental iron transport. Apart from the comparison of iron enriched medium to iron poor control medium, these experiments should comprise the effects of active withdrawal of cellular iron by chelation.

## VII-2. MATERIALS AND METHODS.

### VII-2.1. Cell isolation and culture.

Cytotrophoblasts were isolated using procedure A as described in Chapter II, and cultured for about 18 h in control medium. Culture was continued up to 72 h in control medium, medium supplemented with hTf-2Fe (1.25 $\mu$M), FAC (10 $\mu$g/ml), or medium enriched with desferrioxamine (50 $\mu$M). At indicated culture times TfR and ferritin synthesis was determined as described below.

### VII-2.2. Ferritin, transferrin receptor, and total protein synthesis.

*Transferrin receptor and ferritin synthesis*

Cells were washed twice with icecold PBS. Methionin-free medium was added and the cells were incubated for 1 hour at 37°C, followed by 2 hours incubation with 60-80 $\mu$Ci translabelled $^{35}$S-Methionin (1089 Ci/mmol) per 1.5 x $10^6$ cells in methionin free medium. The medium was removed and the cells were washed twice with PBS. The cells were lysed in 300 $\mu$l lysate buffer consisting of 1 % Triton X-100, 1mM Phenyl-Methyl-Sulfon-Fluoride, 0.5 U/ml aprotinin in PBS (pH 7.4). To remove all cell material, the dishes were washed twice with 100 $\mu$l lysate buffer. Lysis was checked using an inverted microscope. The cell-lysates were centrifuged for 2 min at 10,000 g.

BSA (400 $\mu$g) and L-Methionin ($10^4$ times the original $^{35}$S-Methionin concentration) were added to the supernatants of all the samples. $^{35}$S-Methionin labelled ferritin was precipitated by addition of rabbit-anti-human-placental-ferritin-IgG, bound to Sepharose-CL4B.[101] $^{35}$S-Methionin labelled TfRs were precipitated by addition of a saturating concentration of diferric transferrin bound to Sepharose 4B. Samples were gently rotated during 18 hours at 4°C and subsequently centrifuged for 5 min at 1200 g. The precipitates were washed four times with a solution containing of PBS (pH 7.4), 0.2 % sodiumdesoxycholate, 0.2 % Triton X-100, 1mM PMSF, and 1 mg/ml bovine serum albumin. Equal volumes of electrophoresis sample buffer were added to the precipitates (buffer composition: 4ml distilled water, 1ml 0.5 M Tris-HCl (pH 6.8), 0.8ml Glycerol, 1.6ml 10 % SDS, 0.4ml 2-ß-mercaptoethanol, 0.2ml 0.05 % bromophenol blue) and the samples were boiled for 10 min. Finally, the samples were centrifuged for 2 min at 10,000 g. The supernatants were used for further analysis.

The proteins in 15 $\mu$l of the supernatant were separated by polyacrylamide gel electrophoresis (200V, 45-60 min) on 12 % (for ferritin) and 15 % (for TfRs) homogenous acrylamide/bis gels. Electrophoresis was continued until the frontindicator approached the end of the gel. The gels were fixed for 30 min in a solution consisting of 10 % acetic acid, 55 % ethanol and 35 % distilled water, and subsequently incubated for 15 min in a fluorographic reagent obtained from Amersham Nederland BV, (den Bosch, NL). The fixed gels were (vacuum) dried at

80°C for 2 hours. Light sensitive films (GBX-2, Kodak) were exposed to the gels for 4 days up to 2 weeks before fixation. Finally, the autoradiographes were scanned in an Ultroscan XL Enhanced Laser Densitometer (LKB, Bromma).

*Total protein synthesis*

Samples for determination of the total protein synthesis were handled as follows. To 0.02 ml of the cell-lysate 0.1 ml distilled water and 0.1 ml icecold 20 % trichloroaceticacid (TCA) was added. The samples were centrifuged for 15 min at 1500 g and subsequently put on ice. The precipitate was washed with 0.5 ml 10 % TCA and vigorously mixed with 0.5 ml Soluene-350. Of the final homogeneous sample 0.02 ml was carefully transferred to bottles suitable for liquid-scintilation, and 10 ml Instagel (Packard) was added. ß-radiation was determined in an Isocap 300 counter (Canberra Packard). In general, efficiency was over 85 %, for which the obtained CPM's were corrected. The amount of radiation (DPM) present in the total protein precipitate was taken as measure for total protein synthesis.

**VII-2.3. Transferrin receptor and ferritin degradation rates.**

To estimate the rate of degradation of TfRs and ferritin, proteins were labelled using the procedure for measurement of the synthesis levels. At 48 h culture time the experiment was started. After incubation for 1½ h at 37°C with $^{35}$S-labelled methionin, the radioactive medium was removed and the cells were washed with M199. Subsequently, medium was added, identical to the medium present prior to the labelling procedure, supplemented with a $10^4$ times excess of unlabelled methionin. At 2, 4, 8, and 24 h after labelling cells were lysed and harvested, and TfRs and ferritin were isolated as described above (Ferritin, TfR and total protein synthesis). The amount of radioactivity in the isolated proteins was quantified by electrophoresis, exposure of the gel to a GBX-2 Kodak film, and densitometry of the film as described above for the measurement of TfR and ferritin synthesis.

**VII-2.4. Cellular protein.**

Total cellular protein was measured using the Pierce Micro BSA Reagent (see Chapter II).

## VII-3. RESULTS.

Also in the present experiments total cellular protein remained stable, irrespective of cell differentiation or culture medium additives, but total protein synthesis decreased during culture in desferrioxamine containing medium (to 47 % of the control series at 65 h culture). No differences were seen between the total protein synthesis in the other series (control, hTf-(2Fe), FAC).

Figure VII-1 shows the results of three combined experiments on TfR synthesis, in relation to total protein synthesis as a function of culture time and culture medium composition. Compared to the control series, the introduction of diferric transferrin (hTf-2Fe) to the culture medium significantly decreased TfR synthesis: $\alpha_T < 0.001$ and $\alpha_T < 0.02$ at 42 h and 66 h respectively. In the FAC series no effect on TfR synthesis was seen. In the desferrioxamine series, TfR synthesis was significantly increased ($\alpha_T < 0.001$, at 42 h).

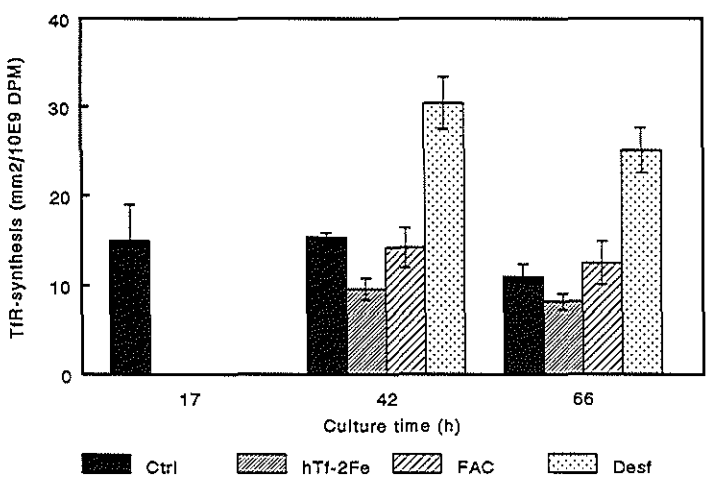


**Figure VII-1. Trophoblast transferrin receptor synthesis.**
Cells were isolated and cultured as described in materials and methods. TfRs were labelled using translabelled $^{35}$S-methionine and precipitated using hTf-(2Fe) bound to Sepharose-4B (see materials and methods). Shown is the synthesis of TfRs related to total protein synthesis. Compared to both the control, and the FAC series, TfR synthesis was significantly lower in the hTf-(2Fe) series ($\alpha_T < 0.02$). TfR synthesis highly significantly increased in the desferrioxamine series ($\alpha_T < 0.001$).

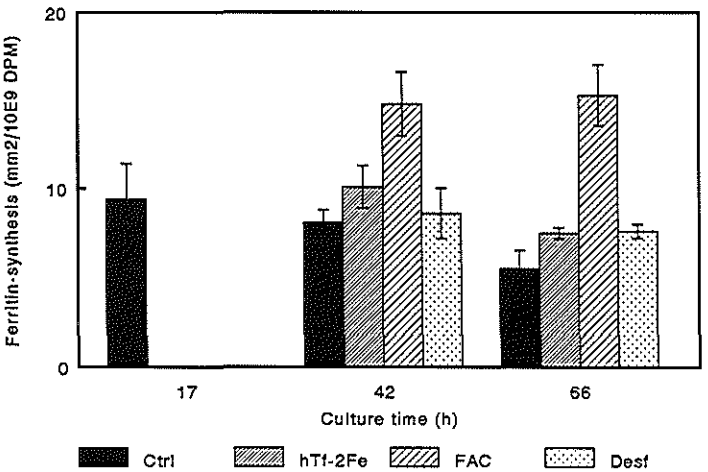


**Figure VII-2. Trophoblast ferritin synthesis.**
Cells were isolated and cultured as described in materials and methods. Ferritin synthesis was measured by $^{35}$S-methionine labelling and immunoprecipitation (see materials and methods). Shown is the synthesis of ferritin related to total protein synthesis. No effects on ferritin synthesis were seen in the control, the hTf-(2Fe), and the desferrioxamine series. Compared to these series, ferritin synthesis was significantly increased in the FAC series ($\alpha_T < 0.001$).

In Figure VII-2 the results are depicted of three experiments on ferritin synthesis. Related to total protein synthesis, ferritin synthesis slightly decreased with time and showed no statistical significant differences between the control, the hTf-(2Fe), and the desferrioxamine series. In the FAC series, however, ferritin synthesis was strongly increased ($\alpha_T < 0.001$).

Similar to the variation in total placental and trophoblast ferritin L-H subunit ratios, the ferritin subunit ratio in newly synthesized ferritin varied with each cell-isolation from 0.30 to 1.12. Within each cell-population subunit ratios were stable during culture, and did not change upon addition of hTf-(2Fe), FAC or desferrioxamine to the culture medium (Table VII-1).

Preliminary results (one experiment) on TfR and ferritin turnover suggested that differences in degradation rates were induced by variation of the iron availability during culture. The effects of cell-differentiation were not investigated. TfR degradation seemed more rapid in cells cultured in iron enriched medium (Control,

desferrioxamine: $t_{½}$ 14 h versus hTf-(2Fe) and FAC: $t_{½}$ 9 h). Compared with TfR turnover, ferritin degradation was less rapid ($t_{½}$: 23 h). It was not affected by the culture medium iron supplementation, though desferrioxamine increased the rate of ferritin degradation markedly (11 h).

| Series | Ctrl | hTf-(2Fe) | FAC | Desf |
|---|---|---|---|---|
| Cuture time h | $mm^2/10^6$DPM (SD) | $mm^2/10^6$DPM (SD) | $mm^2/10^6$DPM (SD) | $mm^2/10^6$DPM (SD) |
| 17 | 0.92 (0.030) | - | - | - |
| 42 | 0.93 (0.106) | 0.89 (0.109) | 1.02 (0.102) | 0.72 (0.026) |
| 66 | 0.89 (0.017) | 1.12 (0.184) | 1.11 (0.131) | 0.92 (0.227) |

**Table VII-1. Trophoblast ferritin subunit synthesis.**
Cells were isolated and cultured as described in materials and methods. Newly synthesized ferritin was labelled during 1½ h, using translabelled $^{35}$S-methionine, and isolated by immunoprecipitation. Subunits were obtained by reduction of the ferritin and separated by electrophoresis. The amount of labelled ferritin subunits was measured by exposure of a Kodak film to the gels for about 2 weeks, followed by densitometry of the films. To determine ferritin subunit amounts in the samples, peak area's were measured and related to total protein synthesis as described in materials and methods.

## VII-4. DISCUSSION.

In various cell-types, cellular iron uptake and storage are regulated by iron itself.[241,245,281] Major roles in this regulation mechanism are played by the iron responsive element (IRE) (a stemloop structure in the mRNA), and the protein binding to this structure, the IRE-binding protein (IRE-BP) (reviewed by Hentze).[158] Iron changes the conformation of the IRE-BP, reducing its affinity for the IRE.[157] Due to the location of the IRE, the translation of ferritin mRNA (IRE in the 5' side),[325] and TfR mRNA (IRE in the 3' side) are differently affected. Dissociation of the IRE-BP (high intracellular iron levels) unblocks ferritin mRNA translation, and it becomes

increasingly synthesized.[325] Under these circumstances TfR mRNA stability is reduced and for that reason it is degraded more rapidly.[281] Withdrawal of iron induces the opposite reaction. Thus, one of the major characteristics of the IRE-involved regulation of cellular iron homeostasis is the coupling of iron uptake and storage in response to iron availability.

Ferritin subunit ratios in newly synthesized proteins varied but were approximately 1 and stable during culture (Table VII-1). This indicates that mainly acidic ferritins are synthesized, which is in accordance with the results regarding ferritin subunit ratios (Figure VI-3).

TfR and ferritin synthesis in cultured cytotrophoblasts shows discrepancies with the IRE-involved reaction pattern upon iron supplementation and withdrawal.

According to the results obtained in the control series, both TfR and ferritin synthesis do not significantly change between 17 h and 66 h of culture (Figure VII-1 and VII-2), which indicates that there is no effect of cell-differentiation on TfR or ferritin synthesis during this period. Since cytotrophoblasts do not express TfRs, TfR synthesis is apparently initiated and stabilized within the first 17 h after isolation. A stable production of TfRs during cell-differentiation was also suggested by the steady increase in surface TfR numbers in cultured cytotrophoblasts (Figure IV-5A).

The effects of iron supplementation depend on the type of iron form offered, but do not match the pattern of TfR and ferritin synthesis as proposed in the IRE-hypothesis. Transferrin bound iron reduces TfR synthesis, but it does not increase the synthesis of ferritin. Iron dissolved as ferricammoniumcitrate, on the other hand, leaves TfR synthesis undisturbed, but significantly increases ferritin synthesis.

Also the cellular reactions on desferrioxamine supplementation of the culture medium are inconsistent with the IRE-hypothesis; TfR synthesis is increased more than two fold, without any effects on ferritin synthesis.

Iron offered as hTf-(2Fe), a physiological form as compared with FAC iron, apparently affects the pool of iron accessible for cellular processes. FAC iron induces a pool of intracellular iron, inaccessible to the normal physiological cellular processes involved in the regulation of iron homeostasis. Similar results have been obtained with nitrilotriacetate in mouse lymphocytes.[48] The cell can only protect itself by increasing its ferritin synthesis. Nevertheless, TfR synthesis is still required, for

accessible iron has to be present as well.

Although the specific effects of hTf-(2Fe), FAC, and desferrioxamine on TfR and ferritin synthesis vary, it can be concluded that in syncytiotrophoblasts the synthesis of these two proteins is not simultaneously regulated. Either TfR synthesis is affected (hTf-(2Fe) and desferrioxamine series) or the synthesis of ferritin (FAC series). This implicates that an IRE-involved regulation mechanism does at least not solely control iron homeostasis by regulation of TfR and ferritin mRNA translation. Also for other levels of the TfR synthesis, alternative regulation mechanisms have been described.[44]

It can be questioned whether it is the mode of iron supplementation that causes the effects described. Previously, it was shown that the cellular handling of iron depends on the iron-compound offered. Iron taken up from nitrilotriacetate, for instance, cannot be used for cell proliferation by mouse lymphocytes.[48,298]

The IRE-involved regulation of TfR and ferritin synthesis has been studied in many cell types, using unphysiological iron-chelators like hemin, FAC, nitrilotriacetate and desferrioxamine. Furthermore, it is remarkable that in all of these studies either TfR synthesis or ferritin synthesis has been investigated. Only in K562 cells both the regulation of TfR synthesis and ferritin synthesis have been subject of investigation, but unfortunately in two separate studies, using hemin and desferrioxamine as stimulators of the ferritin and transferrin receptor, respectively.[259,260] The effect of desferrioxamine in K562 cells is as would be expected. TfR synthesis is increased. But how, in these cells, desferrioxamine affects ferritin mRNA translation is still unknown. Hemin clearly affects ferritin mRNA translation. In K562 cells, a simultaneous effect on TfR-synthesis has to be proven.

In contrast to the effects on TfR and ferritin synthesis, neither cell differentiation nor variation of culture medium iron concentrations changed the catabolism of these proteins. Little is known about the degradation rates of both TfRs and ferritin. In the majority of the reviews on TfRs and ferritin, degradation as part of cellular protein metabolism is ignored.

To my knowledge there is only one review that mentions half life times for the TfR (14 h to 2-3 days).[171] Unfortunately, the original articles this review refers to do not support these figures.

The only article dealing with ferritin kinetics gives 12 h for the half life time of this protein.[324] The ferritin iron release half time (11 h) was highly comparable to the ferritin half life time, which suggests that, at least in K562 cells, ferritin has to be degraded to release the majority of its iron. Although preliminary, the results on TfR and ferritin degradation in cytotrophoblasts suggest the presence of a regulation mechanism. Irrespective of the form iron was offered in, TfR degradation is more rapid in cells cultured in iron enriched medium: 9.5 h versus 14 h in the control and desferrioxamine series. In contrast to ferritin synthesis, its degradation seems to be affected by a lack of iron, caused by desferrioxamine.

In conclusion: the regulation of TfR and ferritin synthesis are uncoupled in cultured cytotrophoblasts, and possibly the degradation of TfR can be affected by the availability of iron. The question remains, whether or not the results described in this chapter are compatible with the transport function of these cells. This question will be dealt with in Chapter VIII, together with the other results described in this thesis.

## Chapter VIII. GENERAL CONSIDERATIONS.

### VIII-1. INTRODUCTION.

Transplacental iron transport is a complicated one-directional process. Iron, originally bound to maternal transferrin, is transported to fetal transferrin. On its way from the maternal to the fetal circulation, it passes through several tissue layers. At term, two of these are cell layers.

The complete transport route is far from understood. Overall transport studies have revealed some of its characteristics The placenta is autonomous in iron uptake,[432] and the major source of iron is maternal transferrin.[121] In man, iron is passed on to the fetus, but the maternal transferrin is not.[100] Maternal Tf binds to TfRs expressed by syncytiotrophoblasts.[409] The actual uptake of iron is thought to take place via receptor mediated endocytosis,[17,69] although data have been published on transferrin iron release at the cell-surface membrane.[404]

### VIII-2. THE PLACENTA AND CULTURED CYTOTROPHOBLASTS.

As discussed in Chapter III, several models can be used for transplacental transport studies. For studies on cellular processes, the in vitro culture of cells is one of the more suitable models.

Two groups of cells suitable for in vitro studies on transplacental iron transport can be distinguished. The choriocarcinoma cell-lines (BeWo, JAR) and the freshly isolated cytotrophoblasts.[211,303] Both cell-types have their (dis)advantages, specially in studies on iron metabolism.

Choriocarcinoma cells proliferate, which implicates that results obtained in different experiments are comparable because identical cells are used. BeWo cells do have the extra advantage that they can be forced to fuse, to form a syncytium, morphologically similar to the placental syncytium in vivo.[105]

Nevertheless, this in vitro model may not be the most suitable for studies on cellular iron metabolism. The results of experiments on this subject are difficult to extrapolate to the physiological situation since proliferating cells do need an

appreciable amount of iron for cell-growth and duplication.

In culture, freshly isolated cytotrophoblasts spontaneously differentiate into syncytiotrophoblast-like structures. This is not only based on morphological but also on biochemical criteria.[95,211] The major disadvantage of this cell-type is their incapability to duplicate and their tendency to die.[211] Therefore the majority of the experiments are carried out in non-identical cells which originate from placentae grown under a wide variety of circumstances. Specially maternal anaemia and diabetes mellitus might affect cellular iron transport processes.[395]

Compared with experiments using BeWo cells, extrapolation to the in vivo situation of the results on iron metabolism, obtained with freshly isolated cytotrophoblasts, is more reliable.

One point of interest remains; most of the experiments were started 17-24 h after isolation. At that stage cells are still differentiating. Therefore, both cell-differentiation and cellular regulating mechanisms for iron metabolism could cause the effects observed and should be considered. The possibility that the mechanisms of transplacental iron transport mature during pregnancy has not been investigated in this thesis. Studies on this subject require first and second trimester placentae without fetal pathology. In particular, second trimester placentae are very difficult to obtain within half an hour after spontaneous abortion or delivery.

### VIII-3. TRANSFERRIN RECEPTORS IN CYTOTROPHOBLASTS.

Every step in iron uptake, storage and release by syncytiotrophoblasts is potentially available for control mechanisms regulating transplacental iron transport. Not all of them (e.g. shedding of TfRs into the maternal circulation, Figure IV-4), are involved in the control of this process.

In cellular iron uptake, TfRs do play an important role.[69] During in vitro differentiation, cytotrophoblasts express TfRs, and surface TfR numbers are affected by supplementation of the culture medium with diferric transferrin (hTf-(2Fe)).[36] In Chapter IV it was shown that surface as well as total TfR numbers steadily increase during culture. Addition of hTf-(2Fe) to the culture medium reduces total TfR numbers, but does not affect the rate of increase (Figure IV-7). The question arises,

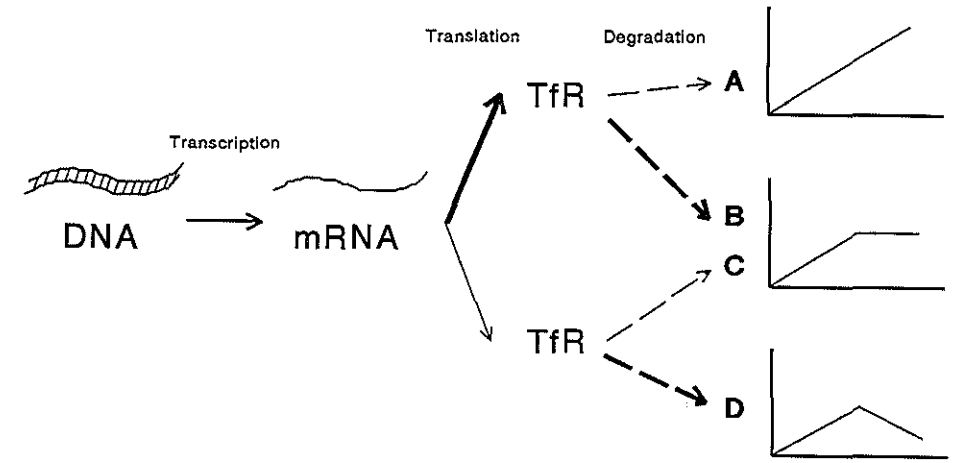


**Figure VIII-1. Regulation of total TfR numbers.**
Schematic drawing of the processes involved in TfR metabolism. Assuming constant, cell-differentiation dependent, TfR DNA transcription, TfR numbers are balanced by TfR mRNA translation and TfR degradation rates (thick and thin arrows represent high and low rates, respectively).
Independent of the rate of TfR synthesis and degradation, from the moment on they are in balance, the number of TfRs will be stable (B,C). The number of TfRs continues to increase, if TfR mRNA translation dominates over TfR degradation (A). The opposite occurs, when TfR degradation is more rapid (D).

if this is caused by (temporarily) decreased TfR synthesis or increased TfR degradation until a new equilibrium has been reached (Figure VIII-1). In Chapter VII it is shown that TfR synthesis is surely, and TfR degradation might be affected (still subject of investigation).

As depicted in Figure VIII-1, TfR synthesis can potentially be affected at the levels of transciption and translation. Regulation of transcription would be possible at the 5' and 3' side of the DNA coding for the receptor. The sequences of the 5' side of the gene are responsible for the proliferation-dependent regulation of TfR synthesis, presumably at the level of transcription (reviewed by Testa, Pelosi and Peschle).[387] An intact 3' side of the gene, coding for the untranslated mRNA Iron-Responsive-Element, is required for the iron controlled regulation of TfR synthesis, at the level of translation. Regulation at this level takes place via IRE-binding proteins in the cytosol.

The following hypothesis for the regulation of TfR expression is based on the findings described above. The rate of TfR gene transcription (the production of mRNA) is a differentiation-phase dependent process. Once produced, the mRNA becomes more stable if cells are cultured in iron poor culture medium. Its turnover rate is low and many TfR proteins can be translated prior to its degradation. HTf-(2Fe) supplementation of the culture medium reduces TfR mRNA stability, the rate of degradation is increased and less TfRs are synthesized. Regulation via this mechanism is reversible.

A necessity for this mechanism to function is the availability of an effective concentration of IRE-BP, even early in differentiation. If the iron-sulphur protein aconitase is the IRE-BP, its permanent presence could easily be explained, because aconitase is required for energy production in the Krebs-cycle.

There is no simple explanation for the difference in effect of hTf-(2Fe) and FAC on TfR synthesis. Possibly, the IRE-BP is incapable of binding ammoniumcitrate chelated iron because of its structure and size. Only a part of the iron offered will destabilize TfR mRNA and succesfully decrease TfR synthesis.

## VIII-4. IRON UPTAKE AND ITS REGULATION BY CYTOTROPHOBLASTS.

It is an old dogma that cellular iron needs are reflected in their surface TfR densities, and that changes in surface TfR numbers can affect iron uptake rates. Therefore, not only the total number of TfRs is important, but also the cellular distribution of the receptors. In Figure IV-5 it is shown that in cells cultured in iron poor medium surface TfR numbers are increased, which is caused in part by a redistribution of TfRs (Figure IV-9).

The distribution of TfRs is a highly dynamic process. TfRs are distributed among functionally different pools localized at the cell surface as well as intracellularly (Chapter IV). In particular this latter sub-group of TfRs is intriguing for it rises the question in which cellular organelles these receptors are present.

The receptors actively participating in the endocytic cycle are present at the cell surface or in endosomes. Part of these receptors, however, are trafficked into other routes (resialylation, degradation). The localization of the inactive TfRs, though

capable of binding transferrin, is more unclear. It seems likely that part of these receptors is present in the Golgi complex or other organelles involved in protein synthesis (reviewed by Stoorvogel et al., 1991).[369] Another part may be trafficked to a basal pool of TfRs participating in an independent receptor cycle.[62,403] These TfR locations may in part explain the 25 to 30 % of receptors not externalized within 1 h. The functional implications of basolateral TfRs are not yet understood. Firstly, there is no transplacental transport of maternal transferrin, which TfRs could mediate. Secondly, if these receptors have identical characteristics as those at the apical side, one would expect iron also to be transported to the mother. This is because, at term, hTf concentrations in the maternal and fetal circulation are similar, but hTf iron saturation is much higher in the fetus (20 % in the mother versus 60 % in the fetus).

The glycosylation of both the TfR,[175] and serum Tf have been shown to influence affinities.[89] There is evidence for considerable interindividual variability in chain complexity.[297] It has been shown in both guinea pig and human placenta that, compared to higher glycosylated transferrins, the affinity for the microvillous plasma membrane TfR of less glycosylated transferrins is higher.[89,238] Since in the last trimester of pregnancy the concentration of high glycosylated transferrin in the fetal serum is higher than that in the maternal, it is likely that this reduces the binding of transferrin to the basal membrane TfRs, and prevents iron from being transported back to the mother.

Remains the question, to what purpose TfRs are present on the basal membrane. For instance, they could be useful, if trophoblasts synthesize transferrin. This could be the case because transferrin mRNA has been isolated from placental homogenates.[341] If so, a basolateral endocytic cycle could participate in the externalization of hTf to the fetal circulation, comparable to the synthesis and externalization of transferrin in the liver.[284]

Despite the differences in surface TfR numbers, the initial rate of iron uptake does not vary in reaction to cell-differentiation or iron enrichment of the culture medium (Figure V-1). TfR cycle times are as important as surface TfR densities (Table V-1), which implicates that the cellular iron needs are not reflected solely by cell surface TfR densities.

The uptake rate of iron is regulated by cellular TfR distribution and TfR kinetics. In case the cellular iron concentration is low, TfR synthesis is stimulated by stabilization of its mRNA. More TfRs are trafficked to the cell surface and iron uptake would increase if TfR cycle times were not adapted (decreased). By combining the regulation of both TfR numbers and cycle times, trophoblasts become capable of controlling iron uptake even more precisely than by regulation of TfR mRNA translation alone. These mechanisms for regulation of iron uptake make syncytiotrophoblasts highly autonomous in iron uptake.
The effect of TfR gene transcription on TfR numbers and iron uptake by syncytiotrophoblasts may be of importance in the long term, but it does not contribute to the regulation of iron uptake in the short culture periods used in the experiments presented.

**VIII-5. IRE'S AND FERRITIN METABOLISM IN CYTOTROPHOBLASTS.**

If IRE's are involved in the regulation of TfR synthesis, also the synthesis of ferritin should be controlled via this system. The effects of hTf-(2Fe), FAC and desferrioxamine on TfR and ferritin synthesis, however, do not support this hypothesis. Enrichment of the culture medium with one of these additives affects either TfR or ferritin synthesis. In particular the decrease in TfR synthesis in reaction to hTf-(2Fe) without a simultaneous increase in ferritin synthesis argues in favour of a regulation mechanism for transplacental iron transport independent of the regulation of cellular iron homeostasis.
Ferritin does not take part in the control of transferrin iron transport in a way comparable to the situation in the gut, where a surplus of iron is stored in ferritin and finally lost in the lumen when mucosal cells are extruded. Nevertheless, ferritin can temporarily store iron on its way to the fetus, protecting the cell from damage by hydroxyl radicals.
The absence of an effect on ferritin synthesis of hTf-(2Fe) supplementation of the culture medium (Figure VII-2) suggests that ferritin is not used as a vehicle for the transfer of iron to the fetal circulation. The main localization of ferritin in syncytiotrophoblasts in vivo (close to the apical cell membrane) further supports this

hypothesis.

The effects of FAC on ferritin synthesis (Figure VII-2) and accumulation (Figure VI-5) might be explained by the incapability of the cell to cope with this iron form. Since its extrusion seems difficult (Figure VI-4), the cell has to protect itself from potentially toxic free iron by an increased ferritin synthesis.

## VIII-6. IRON RELEASE BY CYTOTROPHOBLASTS.

Little is known about cellular iron release mechanisms, not by complete destruction of the cell. Since the uptake of iron is regulated between very narrow limits, the primary locus for the regulation of syncytiotrophoblasts iron contents could be situated in the basal membrane. Regulation of cellular iron extrusion could control transplacental iron transport.

There are experimental results in favour of such a regulation mechanism. Control of the transfer of iron across the basal membrane would most likely be sensitive to the concentration of iron in the fetal interstitial fluids. It has been shown in cultured cytotrophoblasts that replacement of the culture medium increases iron release,[94] and fetectomy causes accumulation of iron in syncytiotrophoblasts.[131,432] Also the results on iron accumulation indicate that some kind of iron release mechanism matures simultaneously with cell-differentiation (Figure V-2).

The effects of FAC enrichment of the culture medium suggest that the release of iron cannot be adapted to the intracellular iron levels in the short term. This is a characteristic of an active transport process, requiring the synthesis of specific vehicles, although incapability of the cell to cope with FAC could also explain these results.

Since it is unclear if trophoblasts in culture polarize, and because the experiments were performed in one chamber settings, it has to be assumed that for cultured cytotrophoblasts the composition of the "maternal" and "fetal" "interstitial fluids" are identical. To pursue this hypothesis, cultured cytotrophoblasts might cope with the media offered as being fetal fluids. In this respect, it could well be that, it is not the maternal blood iron concentration that causes the adaptation of the transport system, but the availability of iron in the fetus.

**SUMMARY.**

Despite the fact that research on the transplacental iron transport has been performed for decades, its cellular mechanisms and regulation are far from understood.

An in vitro model became available in 1986, highly specific for the placental syncytium formed by syncytiotrophoblasts. Cellular iron transport processes could be studied. In this model cytotrophoblasts are isolated. During culture cytotrophoblasts differentiate into syncytiotrophoblast-like cells as among others, concluded from transferrin receptor (TfR) expression. TfRs are required for iron uptake since transferrin (hTf) is the major iron source of the placenta. Iron, bound to hTf, is taken up by the cell via receptor mediated endocytosis. Intracellularly, iron is transferred to the cytosol by a process unknown sofar. In the cytosol it is bound to a 'Low-Molecular-Weight pool', most likely composed of many different molecules. Iron is passed on to ferritin and the basal side of the syncytium. The mechanisms involved in iron transport across the syncytiotrophoblast basal membrane and through the interstitial tissues to the fetal circulation are still unknown. All that has been found is that iron in the fetal circulation, iron is bound to fetal transferrin.

In *Chapter I* an overview is presented of the current ideas on iron metabolism in man and in particular in the placenta. The various proteins involved in chelating iron under physiological conditions are discussed, as are the mechanisms of (cellular) iron uptake, iron transport in the circulation, and iron storage. Also the characteristics of the different placenta types are discussed. Finally, the knowledge of placental iron transport is summarized.

In *Chapter II*, the required materials and the methods generally used are discussed. The procedures of the specific experiments are presented in the matching chapters.

*Chapter III* deals with morphological and biochemical aspects of the in vitro model, used in most of the experiments discussed in this thesis. Cytotrophoblasts cultured in Medium-199 form aggregates and fuse to form multinuclear structures. During

this differentiation process, cellular protein and DNA concentrations are stable until at least 90 h of culture, although prolonged culture up to 160 h is possible. Cell viability is highly dependent on the time loss between delivery of the placenta and the start of the isolation procedure.

During cytotrophoblast differentiation TfRs are expressed. Several characteristics of this process are discussed in *Chapter IV*. Surface and total cellular TfR numbers in relation to cell-differentiation are presented as well as TfR shedding and cellular TfR distribution.
Syncytiotrophoblasts do not shed TfRs. Compared with cells cultured in diferric transferrin (hTf-(2Fe)) containing medium , the cells cultured in iron poor medium increase surface as well as total TfR numbers.
Two functionally different TfR pools are defined, one active and one inactive. The TfRs in the active pool participate in the endocytic cycle, the TfRs of the other pool are inactive although capable of binding hTf. Trophoblasts increase the number of TfRs in the active pool in reaction to low iron supplies by redistribution of the receptors between the active and inactive pool.

In *Chapter V* the effects of the variations in surface TfR numbers on trophoblast iron uptake are discussed. Initial iron uptake rates does not differ in reaction to cell-differentiation nor iron supplementation of the culture medium. This is explained by changes in TfR cycle times, which compensate for the number of surface TfRs. Using both TfR numbers as well as TfR cycle times as tools for the control of iron internalization, the placenta becomes autonomous in iron uptake. It seems likely that a mechanism for iron release matures simultaneously with cell-differentiation.

*Chapter VI* deals with ferritin and its possible role in the transplacental transport of iron. Trophoblast ferritins are highly enriched with H-subunits. Cellular iron and ferritin concentrations are not affected by additon of hTf-(2Fe) to the culture medium. FAC supplementation of the culture medium, however, increases intracellular iron levels and ferritin iron saturation, upon which the cells reacted by increasing cellular ferritin concentrations.

It is concluded that syncytiotrophoblast can handle hTf bound iron without any problem. Iron uptake and release are in balance. Apparently, it is difficult for syncytiotrophoblasts to cope with ammoniumcitrate chelated iron. Iron release is hampered and the cells can only protect themselves by increasing cellular ferritin concentrations in order to store the abundantly present iron.

The results on TfR and ferritin synthesis (*Chapter VII*) further support the findings on TfR numbers and ferritin concentrations, as described in Chapter IV and Chapter VI. Surprisingly, the effects are not completely in accordance with the Iron-Responsive-Element hypothesis, which claims that TfR and ferritin synthesis are simultaneously regulated by IRE's and IRE-BP's. HTf-(2Fe) enrichment of the culture medium, decreases TfR synthesis but does not affect ferritin synthesis. FAC increases ferritin synthesis but does not influence TfR synthesis. Also the changes in synthesis of TfRs (increased) and ferritin (no effect) in reaction to desferrioxamine are not consistent with the IRE-hypothesis. It is concluded that the simultaneous regulation of TfR and ferritin synthesis are uncoupled in syncytiotrophoblasts. It could be that the regulatory mechanisms involved, have different sensitivities for iron.

In *Chapter VIII* the results of the experiments are reviewed in light of the regulation of iron transport across the placenta. The hypothesis that the factor controlling transplacental iron transport could be localized in the basal membrane has been proposed.

## SAMENVATTING

Ondanks het feit dat er reeds tientallen jaren onderzoek plaatsvindt naar het transplacentair ijzertransport, zijn de betrokken cellulaire mechanismen en de regulatie ervan nog verre van begrepen.
In 1986 kwam een in vitro model beschikbaar, specifiek voor het placentaire syncytium dat gevormd wordt door syncytiotrophoblasten. In dit model worden cytotrophoblasten, de voorloper cellen van de syncytiotrophoblasten, geïsoleerd en gekweekt zodat de cellulaire ijzertransport processen bestudeerd konden worden.
In kweek differentiëren cytotrophoblasten in syncytiotrophoblasten wat onder andere geconcludeerd kan worden uit het feit dat ze transferrine receptoren (TfRen) tot expressie brengen. TfRen zijn noodzakelijk voor de opname van ijzer omdat transferrine (hTf) de belangrijkste bron van ijzer voor de placenta is. IJzer gebonden aan hTf, wordt door middel van receptor gemedieerde endocytose opgenomen door de cel. Intracellulair wordt het ijzer vanuit de endosomen via een nog onbekend proces overgebracht naar het cytosol. Daar wordt het gebonden door een pool van laag moleculaire stoffen, de "Low-Moleculair-Weight fraction" en uiteindelijk doorgegeven aan ferritine danwel naar de basale zijde van het syncytium.
De mechanismen betrokken bij het transport van ijzer over de basaal membraan en door het interstitiele weefsel naar de foetale circulatie, zijn nog volledig onbekend. Het enige dat bekend is, is dat ijzer in de foetale circulatie gebonden is aan ijzer.

In hoofdstuk I is een overzicht gegeven van de huidige ideeën ten aanzien van het ijzermetabolisme in de mens en met name de placenta. De diverse eiwitten betrokken bij de chelatie van ijzer onder fysiologische omstandigheden en de mechanismen van (cellulaire) ijzeropname, van ijzertransport in de circulatie en van de opslag van ijzer zijn beschreven. Ook zijn de caracteristieken van de verschillende placenta-types besproken. Tenslotte is een samenvatting gegeven van de kennis over transplacentair ijzertransport.

In hoofdstuk II zijn de algemeen gebruikte materialen en methoden besproken.

Procedures gebruikt voor specifieke experimenten zijn in de corresponderende hoofdstukken gepresenteerd.

Hoofdstuk III behandelt de morphologische en biochemische aspecten van het in de meeste experimenten gebruikte in vitro model. In kweek vormen cytotrophoblasten aggregaten waarna ze fuseren tot multinucleaire structuren. Tijdens dit differentiatie proces zijn tot zo'n 60 uur na aanvang van de kweek de cellulaire eiwit en DNA concentraties nagenoeg stabiel. Langere kweektijden tot 160 uur zijn desondanks mogelijk, hetgeen vooral afhankelijk is van het tijdsverlies tussen geboorte van de placenta en aanvang van de cel-isolatie procedure.

Tijdens differentiatie komen TfRen tot expressie. Enige characteristieken van dit proces komen in hoofdstuk IV ter sprake. Gepresenteerd zijn het aantal TfRen op het cel-oppervlak en het totaal aantal cellulaire TfRen in relatie tot cel-differentiatie. Tevens zijn de TfR uitscheiding en cellulaire verdeling van TfRen besproken.
Syncytiotrophoblasten scheiden geen TfRen uit door middel van "shedding".
Vergeleken met cellen gekweekt in diferric transferrin (hTf-(2Fe)) houdend medium verhogen cellen gekweekt in ijzer-arm medium hun oppervlakte zowel als hun totaal aantal receptoren.
Twee functioneel verschillende TfR subgroepen worden gedefinieerd; één aktief en één inaktief. De TfRen in de aktieve subgroep nemen deel aan de endocytose cyclus. Hoewel de TfRen in de andere subgroep inaktief zijn kunnen zij wel hTf binden. In reaktie op een laag ijzer aanbod verhogen syncytiotrophoblasten het aantal TfRen in de aktieve subgroep door middel van herverdeling van de receptoren over beide subgroepen.

In hoofdstuk V is de invloed van de variatie in het aantal TfRen op het celoppervlak op de ijzeropname door trophoblasten beschreven. Cel-differentiatie noch ijzer aanbod via het kweek medium had enige invloed op de initiele ijzeropname. Dit wordt verklaart door het feit dat TfR cyclustijden zodanig aangepast worden dat voor het aantal oppervlakte TfRen gecompenseerd wordt. Door zowel TfR aantallen als TfR cyclustijden te reguleren wordt de placenta autonoom voor ijzeropname.

Waarschijnlijk rijpt er parallel met de cel differentiatie ook een mechanisme uit dat de afgifte van ijzer reguleerd.

Hoofdstuk VI behandelt ferritine en haar mogelijke rol in het transplacentair ijzertransport. Trophoblast ferritine is sterk verrijkt met H-ketens. Toevoeging van hTf-(2Fe) aan het kweek medium beïnvloed de hoeveelheid cellulair ijzer noch de cellulaire ferritine concentratie. Ferricamoniumcitrate (FAC) daarentegen verhoogd de cellulaire ijzerconcentratie en de ferritine ijzerverzadiging, waarop de cel reageert door de ferritine concentratie ook te verhogen. Geconcludeerd is dat syncytiotrophoblasten hTf gebonden ijzer zonder problemen kunnen hanteren en dat de ijzeropname in balans is met de afgifte. IJzergebonden aan ammoniumcitrate kan minder makkelijk door de cellen worden verwerkt. Doordat de afgifte van ijzer trager verloopt dan de opname zijn de cellen gedwongen om de ferritine concentratie te verhogen om zich te beschermen tegen vrije radicalen.

De resultaten ten aanzien van TfR en ferritine synthese (Hoofdstuk VII) ondersteunen de metingen uit hoofdstuk IV en VI. Opvallend is dat de gemeten effecten niet volledig in samenspraak zijn met de "Iron Responsive Element hypothese, welke inhoud dat TfR en ferritine synthese simultaan gereguleerd worden door ijzer. Verrijking van het kweekmedium met hTf-(2Fe) namelijk verlaagt TfR synthese maar beïnvloedt de TfR synthese niet. FAC verhoogt de synthese van ferritine maar beïnvloedt de TfR synthese weer niet. Ook de effekten van desferrioxamine op de synthese van TfRen (verhoogd) en ferritine (geen effekt) zijn niet conform de IRE-hypothese. Geconcludeerd is dat de parallelle regulatie van TfR en ferritine synthese ontkoppelt is in syncytiotrophoblasten. Het is mogelijk dat de gevoeligheid van de betrokken regulatiemechanismen voor ijzer verschilt met de vorm waarin het ijzer aangeboden wordt.

In hoofdstuk VIII tenslotte is een overzicht gegeven van de gevonden resultaten in verband met het transport van ijzer door de placenta. De hypothese dat het controle mechanisme van het transplacentaire ijzertransport gelegen is in de basaal membraan van de syncytiotrophoblast is verder uitgewerkt.

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## CURRICULUM VITAE.

Sander Starreveld werd op 18 april 1963 te Amsterdam geboren. Op 3 jarige leeftijd verhuisde hij naar Zeist waar hij in juni 1982, aan de Katholieke Scholengemeenschap 'de Breul', het VWO diploma behaalde. Nog datzelfde jaar werd hij toegelaten tot de studie geneeskunde aan de Rijksuniversiteit Utrecht. In augustus 1986 behaalde hij het doctoraal diploma geneeskunde. In de periode voor het behalen van zijn artsenbul in november 1989, liep hij stage in een ruraal ziekenhuis te Mantsoñyane, Lesotho, (Zuidelijk Afrika), werkte als student-assistent op het Netherlands Institute for Drugs and Doping Research, en verrichtte onder leiding van Dr A.J.P.M. Smout, onderzoek op de afdeling Gastro-Enterologie van het Academisch Ziekenhuis Utrecht. Aansluitend aan het behalen van zijn artsexamen voerde hij, voor het Integraal Kankercentrum Midden Nederland, een pilot studie uit naar de haalbaarheid van lange termijn follow-up van mammatumoren. Per 1 maart 1990 werd hij aangesteld als Assistent In Opleiding, op het instituut voor Chemische Pathologie aan de Erasmus Universiteit Rotterdam. Onder leiding van Prof Dr H.G. van Eijk en supervisie van Dr J.P. van Dijk werd op dit instituut het onderzoek uitgevoerd, dat geresulteerd heeft in het voor u liggend proefschrift.

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