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INVESTIGATION OF INTERLEUKIN-10 RECEPTOR EXPRESSION IN HUMAN PLACENTA, ISOLATED CYTOTROPHOBLAST CELLS AND HUMAN KERATINOCYTES

Ph.D. Thesis

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PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

Full-length papers

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Quotable abstracts of scientific presentations

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CONTENTS

ABBREVIATIONS

AEC	3-amino-9ethyl-carbazol
AP	alkaline phosphatase
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BSA	bovine serum albumin
CTB(s)	cytotrophoblast(s)
DMEM	Dulbecco`s Modified Eagle Medium
DMSO	dimethylsulfoxide
DNase I	deoxyribonuclease I
EDTA	ethylenediamine tetraacetic acid
FACS	fluorescnce activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluoresceine isothiocyanate
HBSS	Hanks` balanced salt solution
hCG	human chorionic gonadothropin
HEPES	[2-hydroxyethyl]piperazine-N`-[2-ethanesulfonic acid]
HLA	human leukocyte antigen
hPL	human placental lactogen
HRP	horseradish peroxidase
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IL-10R(s)	the receptor(s) for interleukin-10
IL-8R(s)	the receptor(s) for interleukin-8
KGM	Keratinocyte Growth Medium
MCP-1	monocyte chemotactic peptide-1
MHC	major histocompatibility complex
MMP-9	matrix metalloproteinase-9
mRNA	messenger ribonucleic acid
PBS	phosphate-buffered saline solution
PGE2	Prostaglandin E2
RANTES	Regulated on Activation and Normally T-cells Expressed and presumably Secreted
SDS	sodium dodecyl sulfate
SDS/PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis
STB(s)	syncytiotrophoblast(s)
TBS	Tris-buffered saline solution
TNF	tumor necrosis factor
Tris	Tris-(hydroxymethyl)-aminomethane
WHO	World Health Organization

1 INTRODUCTION

During embryo implantation and haemochorial placentation trophoblast cells interact both with the endometrial stroma and the uterine spiral arteries [5]. Both processes represent a biological paradox which cannot be explained easily with our present knowledge of cell biology [46]. The biochemical and physical duet of the mother and the fetus in the formation of the placenta is one of the most carefully orchestrated phenomena in fetal development. It represents the cooperation of two distinct individuals to form a single structure that protects one and enables the genes of the other to live on. This example of evolution at its finest involves a highly regulated balance between trophoblast proliferation, motility, change in adhesive properties and differentiation [3].

Placental development, which ultimately brings maternal and fetal circulation in close proximity to each other, starts with the process of implantation [132]. The uterus of almost all mammals is ready for implantation only during a limited period of time (implantation window) [124]. Before or after this period the uterus is either indifferent or hostile to the embryo. The period during which an embryo is capable of implanting is usually much longer but varies considerably from species to species. The absolute necessity of a sinchronization between embryonic and uterine development is the most important limitation of the otherwise evident evolutionary advantage of viviparity.

In humans, implantation is interstitial and antimesometrial: the embryo implants deeply in the body of the uterus, most frequently in the upper part of the posterior wall near the mid sagittal plane [25]. In all mammals, including humans, the implantation process can be divided in two phases: an attachment phase and a penetration phase.

During the attachment phase the blastocyst first "finds" its implantation site and then anchors itself to the apical surface of the uterine epithelium. In humans, the attachment of the blastocyst to the uterine lining via its embryonic pole usually occurs on the 6^{th} or the 7^{th} day after fertilization [110]. From a biological point of view this process consists of two distinct steps: apposition and adhesion. During apposition, no visible connections are established between the blastocyst and the endometrium, and the blastocyst can be dislodged easily by simple washing of the uterine cavity. In contrast, adhesion is the step during which functional connections are established although the nature of these connections is still very speculative.

Thought to play a role are carbohydrate lectin interactions, stabilized by binding of integrins to their extracellular matrix ligands. Trophoblasts also express proteoglycans and other carbohydrate structures thought to be critical in binding [40].

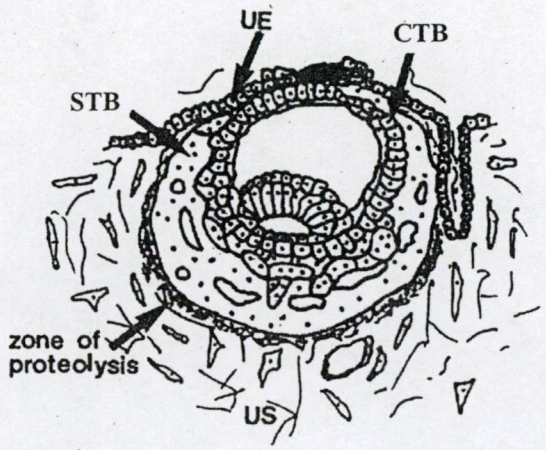


Figure 1. Schematic representation of a human implantation site at approximately nine days after conception (adapted from [3]). The outer layer of the embryo consists of syncytiotrophoblast. Adjacent to the outer syncytiotrophoblast surface, at its interface with the maternal uterine stroma, is a zone of tissue degradation. Beneath the syncytium is the CTB layer.

Legend: CTB - cytotrophoblast
STB - syncytiotrophoblast
UE - uterine epithelium
US - uterine stroma

After initial attachment of the embryo to the uterine lining, the outer cell layer of the blastocyst, consisting of mononuclear trophectodermal cells that surround the inner cell mass, give rise to a second population of cells that divide in the absence of cytokines. They form a multinucleated STB [58] which surrounds the entire embryo, and forms projections which progress between the adjacent endometrial epithelial cells to reach the underlying basement membrane. At this point trophoblast pauses at the residual basal lamina of the uterine luminal epithelium before progressing into the endometrial stroma [132]. After crossing the basement membrane trophoblast cells invade the uterine stroma and finally breach the wall of maternal blood vessels (Fig. 1.). Concomitantly, the remaining unfused trophectodermal cells (*e.g.* mononuclear CTBs) spread along the basement membrane to form the trophoblastic plate [31].

At eight days after ovulation the blastocyst is completely embedded in the uterine stroma and the site of entry is covered by fibrin over which the uterine epithelial cells grow. During the second week of gestation, proliferating CTBs form primary villi, which are trabeculae of mononuclear cells covered by a layer of syncytium, interposed between the fluid-filled, and later maternal blood-filled lacunae formed in the multinucleated mass of the invading STB. The extraembryonic mesoderm begins to project into these structures

transforming them into secondary villi. When blood vessels begin to enter the mesodermal core, the villus is known as tertiary villus (Fig. 2.) By the third week of pregnancy, the newly formed tertiary villi undergo branching, increasing the surface area of the chorion that is exposed to maternal blood. The villous tree undergoes stromal alterations and remodeling leading to the formation of different types of villi throughout pregnancy [14, 32]. The mature villi (Fig. 3.) consist of an outer syncytial layer and an inner layer of CTB cells surrounding the fetal stroma which contains blood vessels [25].

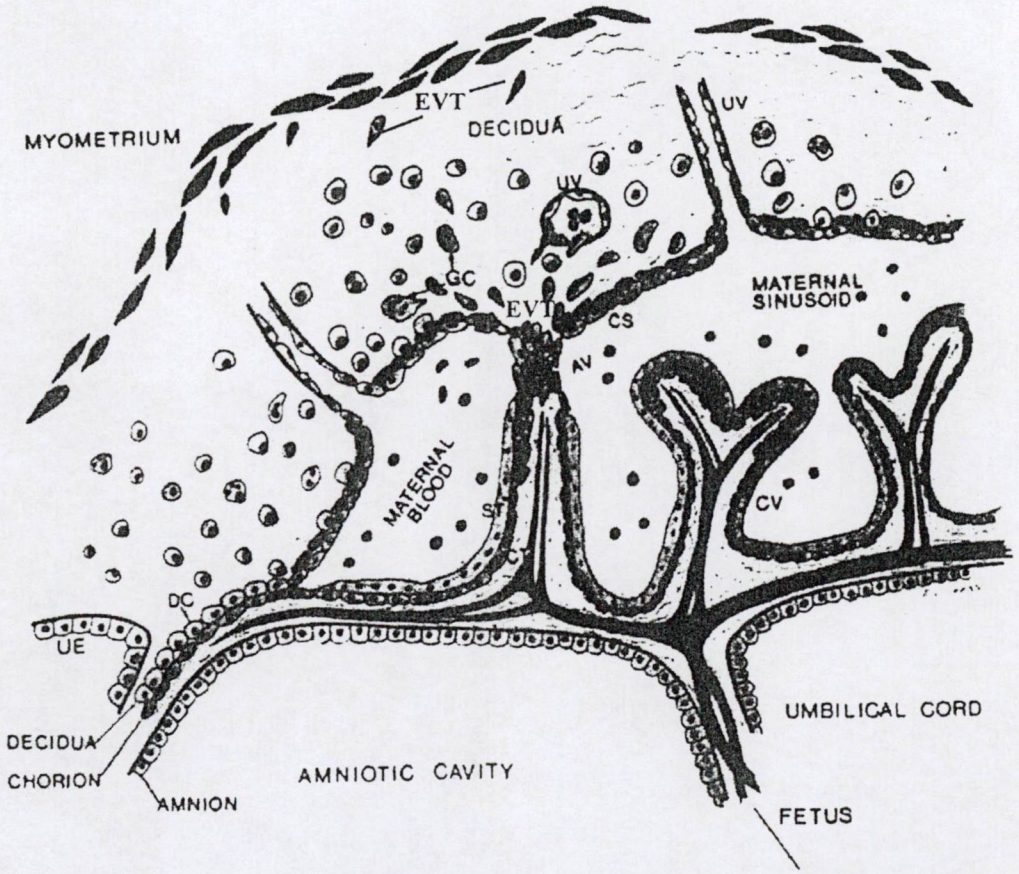


Figure 2. Schematic diagram of the placenta at the end of first trimester showing various trophoblast subpopulations (adapted from [86]). Anchoring villi serve to maintain the attachment of the placenta to the uterine wall. Extravillous trophoblast cells migrate out of the tips of anchoring villi initially as cell columns, and then form the cytotrophoblastic shell, an organized cell layer or become dispersed within the decidua as isolated interstitial trophoblast cells which are highly invasive. Other extravillous trophoblast cells invade uterine vessels, replacing the endothelium. Some extravillous trophoblast cells can invade as far as the myometrium, while others fuse in the decidua to form placental bed giant cells which are presumably noninvasive.

Legend: AV – anchoring villus; CV – chorionic villus; CS – cytotrophoblastic shell; DC – decidual cells; EVT – extravillous trophoblast; GC – giant cells; UE – uterine epithelium; UV – uterine blood vessels

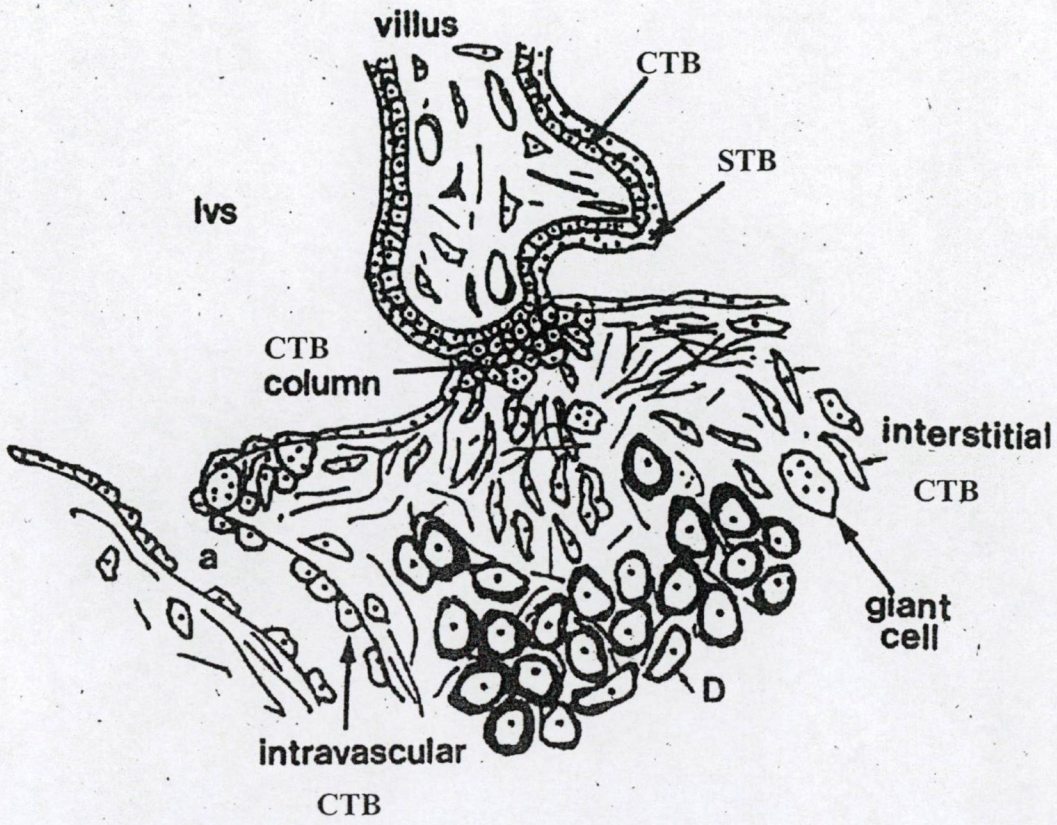


Figure 3. Schematic representation of the materno-fetal interface approximately four weeks after fertilisation, showing the tip of a placental anchoring villus and the adjacent placental bed (adapted from [3])

Legend: CTB - cytotrophoblast; D - decidua; ivs - intervillous space; STB – syncytiotrophoblast

Three major trophoblast populations can be identified during placentation: CTB stem cells and two differentiated derivative cell types – the STB and the extravillous CTB [153]. The undifferentiated trophoblastic stem cell of the placenta, the CTB cell, is the first fetal cell type arising during embryogenesis. It derives from the external trophectodermal cell layer of the blastocyst, thus it is extraembryonic in origin. It represents the earliest epithelium and forms a variety of different structures *e.g.*, placental villi and fetal membranes [14]. Just as the undifferentiated basal layer of the skin gives rise to differentiated keratinocytes, the CTB stem cell of the placenta undergoes a multistep differentiation and finally gives rise to villous (non-invasive) and extravillous (invasive) trophoblast cell populations [160, 162] (Fig. 4.).

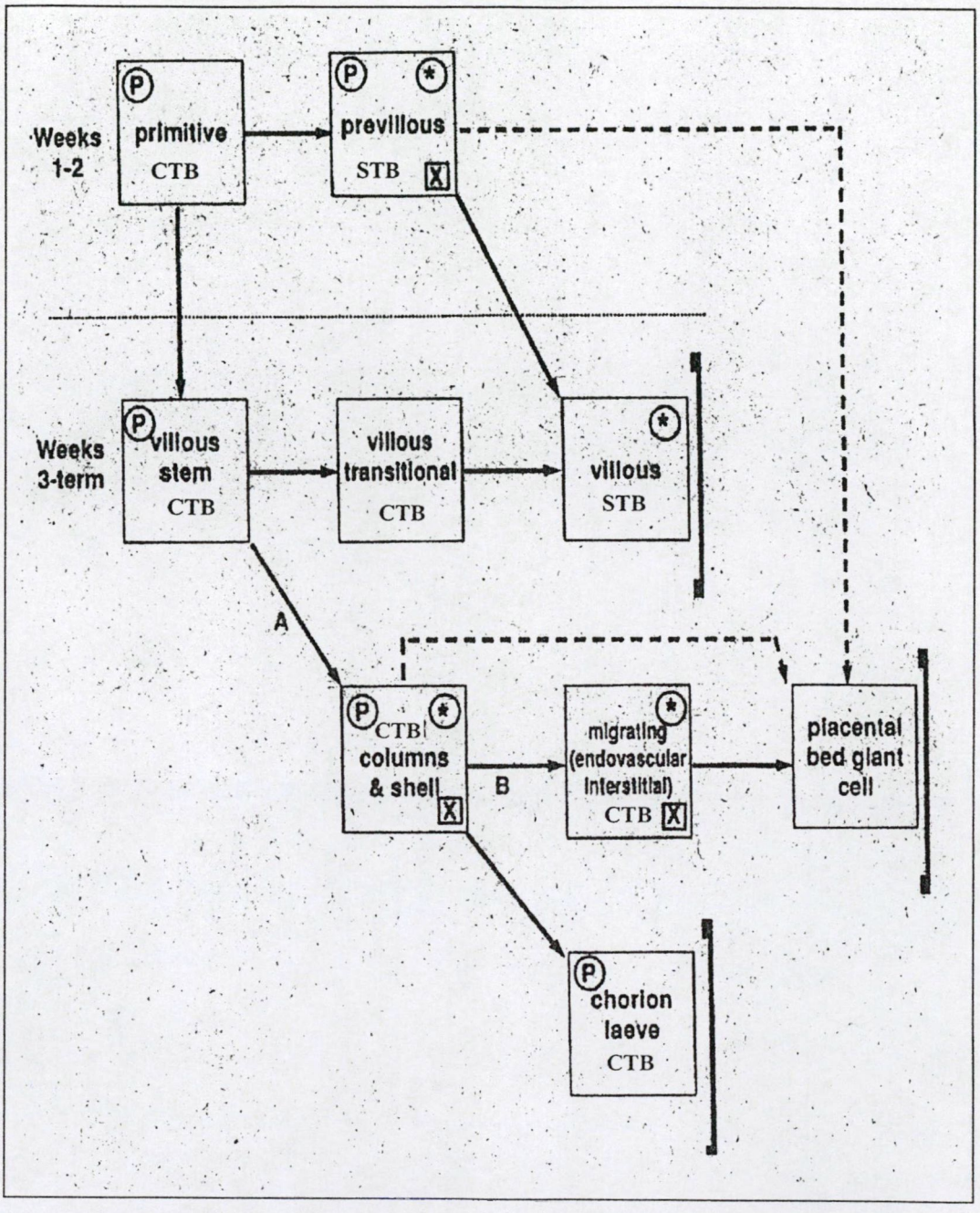


Figure 4. Scheme of human trophoblast differentiation (adapted from [3])

Legend: filled arrows – established pathways; broken arrows – possible minor pathways; unlabeled arrows – intrinsic differentiation pathways; labeled arrows: A – short-range (decidual) stimulus; B – maternal vascular stimulus; P – proliferating cells; * - cells active in remodeling the maternal tissue environment; X – cells exhibiting prominent motile activity; thick brackets – terminus of a pathway; CTB - cytotrophoblast; STB – syncytiotrophoblast

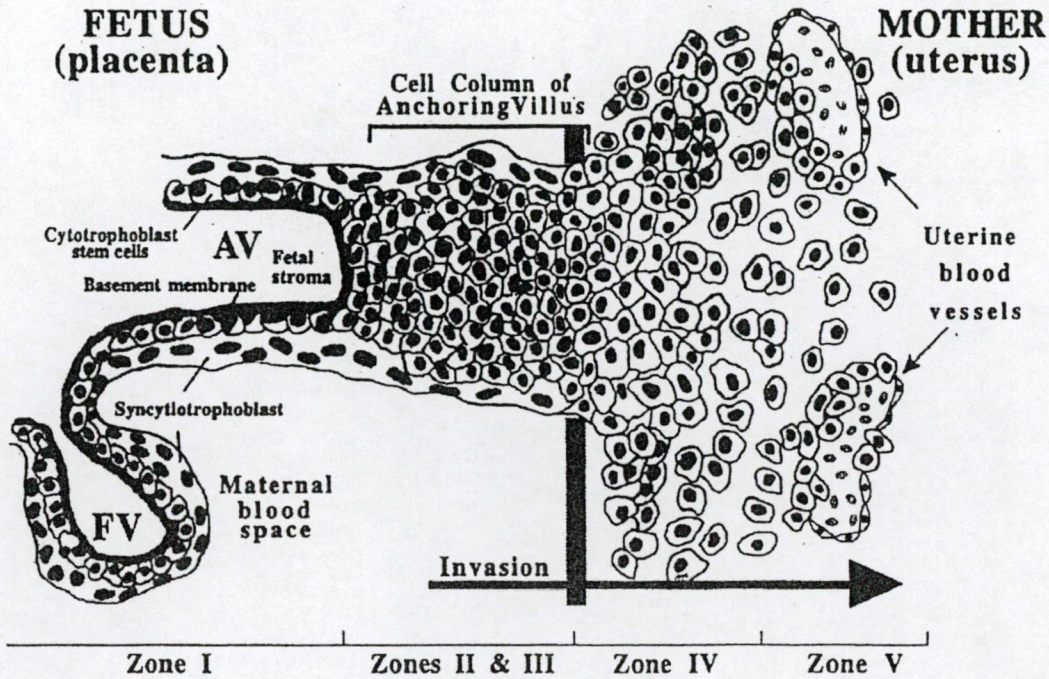


Figure 5. Diagram of a longitudinal section of an anchoring chorionic villus at the fetal-maternal interface at approximately 10 weeks gestational age (adapted from [164]). The anchoring villus functions as a bridge between the fetal and maternal compartments, whereas floating villi are suspended in the intervillous space and are bathed by maternal blood. CTB in anchoring villi (Zone I) form cell columns (Zones II and III). CTB then invade the uterine interstitium - decidua and first third of the myometrium (Zone IV) and maternal vasculature (Zone V). Zones designations mark areas in which CTB have distinct patterns of adhesion receptor expression as described in the Discussion section.

Legend: AV – anchoring villus; FV – floating villus

In the human placenta proliferating CTB stem cells are attached to an extensive basement membrane that surrounds the stromal core of two types of chorionic villi (Fig. 5.). In floating villi, CTBs differentiate by fusion to form an overlying layer of multinucleated STB. These cells are in immediate contact with maternal arterial blood that bathes the floating villi and their primary function is to perform nutrient, waste and gas exchange between the maternal and fetal circulations. In anchoring villi, beginning with the third post-ovulatory week, a subset of CTBs at the distal tips of the villi proliferate and differentiate by leaving their basement membrane to form multilayered cell columns

consisting of highly migratory, non-polarized, invasive CTBs, covered by a thin layer of syncytium. The cell columns expand distally, and populate the decidualized endometrium and the first third of the myometrium, thereby anchoring the villous tip to the uterine wall [26, 41]. The penetrating cells spread over the maternal decidual cells, forming the cytotrophoblastic shell. Some of these deeply invasive cells penetrate the inner walls of the maternal spiral arteries up to their myometrial segments, and by replacing the endothelial lining and the smooth muscle cells, transform them in maximally dilated inert tubes. This process is termed endovascular invasion [3, 14, 24, 55, 72, 123]. This unusual invasive behaviour has two important roles: (1) CTB invasion physically anchors the placenta to the uterus, and (2) by replacing the endothelial lining of the spiral arteries invasive extravillous CTBs create the large-diameter, low-resistance vessels that carry blood to the floating villi at the maternal-fetal interface.

Extravillous trophoblast includes multiple cell populations:

1. the cytotrophoblastic shell
2. the residual trophoblastic elements within the chorion laeve
3. the endovascular trophoblast
4. the interstitial mononuclear and multinucleated trophoblast cells, which have invaded the decidual stroma (decidual trophoblast) and the inner third of the myometrium (interstitial trophoblast) [3, 96]
5. large, multinucleated trophoblast cells in the extravillous locations (placental bed) termed placental bed giant cells [60, 96]; they are presumably non-invasive and remain essentially non-productive with respect to hormones (hCG, hPL) [96].

The controlled invasion of the extravillous trophoblast into the endometrium is essential both for embryo implantation and haemochorial placentation and resembles the invasion of malignant tumor cells [17, 59, 80, 85, 92]. However, unlike tumor invasion, trophoblast invasion is precisely developmentally regulated [59, 85], is confined spatially to specific areas in the uterus (endometrium and inner third of the myometrium), and temporally to early pregnancy (first trimester and early second trimester of gestation) [3, 54]. However, the mechanisms regulating CTB proliferation and commitment to one or another differentiated trophoblast lineage, thus having a profound impact on the invasive-degradative behaviour of trophoblasts, is still a mystery. The elucidation of these mechanisms is subject

of numerous studies because disregulation of these processes can seriously affect the pregnancy outcome. Disorders such as preeclampsia [163] and approximately half the cases of intrauterine growth retardation [57] are associated with abnormally shallow CTB invasion. In contrast, an overly extensive invasion can result in both benign and malignant trophoblast tumors [13].

The hypothesis that cytokines may play a pivotal role in regulating human placental development and implantation has been much discussed in recent years [36, 70, 127]. Indeed the human placenta is an important site of both production and action of various cytokines and growth factors. Although numerous work has been published on the biological role of cytokines in the regulation of critical reproductive events like trophoblast proliferation, differentiation and function, the complex autocrine, paracrine and juxtacrine regulatory mechanisms involving these molecules are still far from being completely understood. Whilst the concept of cytokines influencing implantation and placentation is attractive, it has proved difficult to elucidate the mechanisms involved because it is not clear which cell type will respond to which cytokine. Therefore, a necessary initial step in the investigation of cytokine function is to define those cell populations which express relevant cytokine receptors as this would identify the potential responsive targets.

Among the various cytokines and growth factors the recently described pleiotropic cytokine IL-10 has been proposed to be a factor that might protect the semiallogeneic fetus from maternal allorecognition and rejection by driving the maternal (both local and systemic) immune reaction toward a Th2-type immune response [30, 94].

IL-10 was described initially as a "Cytokine Synthesis Inhibitory Factor" that shifts the body's immune reaction away from an inflammatory response (reviewed in 52; 66, 111, 113). Although its serum levels were found to be stable throughout the menstrual cycle, once the pregnancy is established the circulating levels of IL-10 rise significantly [102]. IL-10 inhibits proinflammatory cytokine production including IL-1β, IL-6, IL-8, TNF-α and IFN-γ [146, 155] therefore prevents the development of Th1-type immune reactions deleterious for both the establishment and maintenance of pregnancy [125, 156]. Several studies have demonstrated that IL-10 can prevent naturally occurring fetal wastage in a murine model of immunologically mediated spontaneous early pregnancy loss [34]. Earlier studies [142, 143,

144] stressed the role of IL-10 as a possible mediator of the immunological pregnancy-protective effect of progesterone.

In human pregnancies, the protective role of IL-10 has been demonstrated by several findings concerning the disregulation of IL-10 production in some reproductive pathologies, including recurrent spontaneous abortion [64, 101], intrauterine growth restriction [63] and intrauterine infection-associated preterm labor [61]. Roth *et al.* were the first to describe that highly purified human CTBs secrete physiological amounts of IL-10 *in vitro*, independently of gestational age [130]. Furthermore, trophoblast-derived IL-10 was able to suppress IFN-γ production of alloreactive lymphocytes in a mixed lymphocyte reaction, indicating that IL-10 may contribute to the placental immune protection of the semiallogeneic fetus in a paracrine manner. Recently, these authors [129] have shown that CTB cells express IL-10R mRNA *in vitro* suggesting that, as well as its role as a regulator of materno-fetal relationship, this cytokine might also possess autocrin regulatory properties.

Another epithelial cell which just as placental CTB stem cells undergoes a continuous process of proliferation and differentiation is the epidermal keratinocyte. Epidermis is subject to a life-long self renewing process during which quiescent stem cells laying on an extensive basement membrane are triggered to produce transiently amplifying cells, which then give rise to early stages of differentiation followed by terminal differentiation and death. Cytokines are likely candidates for regulating these processes and their role is even more evident in pathological conditions associated with a disregulation of keratinocyte proliferation and/or differentiation.

Psoriasis is a common cutaneous disorder characterized by inflammation and abnormal epidermal proliferation with a prevalence of 2-3% in the general population [37]. Several observations indicate that T cells and cytokines [22, 84, 117, 131, 151, 152, 158] are of major importance in the pathogenesis of this chronic skin disease. These observations are supported by the beneficial effects of systemic administration of immunosuppressive drugs like cyclosporin A [145], FK506 [68], and fumaric acid esters [2, 6, 42], known to act on T cells and to influence the cytokine pattern.

According to the predominant expression of IL-2 and IFN-γ and the lack of IL-4 in skin lesions, psoriasis is believed to be characterized by a type 1 cytokine pattern [128, 131,

151]. Moreover, the involvement of other proinflammatory cytokines like IL-1, IL-6, IL-8, and TNF-α has been demonstrated [50, 89, 119].

In contrast to proinflammatory cytokines, there is only limited knowledge regarding the role of anti-inflammatory cytokines in psoriasis. Remarkably, there is rising evidence that a relative IL-1 receptor antagonist deficiency could be of major importance [83]. IL-10, another anti-inflammatory cytokine, is a potent inductor of IL-1 receptor antagonist [69], suggesting low or absent IL-10 expression in psoriasis. However, conflicting data have been published. Whereas IL-10 mRNA was detected rarely or not at all by some authors [131, 151], overexpression has been reported by others [89, 120]. Recent publications showed that subcutaneous administration of IL-10 has clinical efficiency in psoriasis [7]. The presence and functionality of the IL-10R in human keratinocytes as well as its decreased expression in psoriatic epidermis has already been established [105]. The possibility of raising IL-10R transcript levels by the treatment of epidermal cells with antipsoriatic compounds such as vitamin D3, calcipotriol [105] and steroids [104] has also been described. Therefore, the loss of negative regulatory elements such as the cell cycle control gene p53 [106] and the IL-10R in the pathogenesis of psoriasis might have at least the same importance as the excessive synthesis of proinflammatory and mitogenic signals, such as IL-8/IL-8R.

On the basis of these data, the general aim of the present thesis was to expand our knowledge about the expression, functionality and possible pharmacological modulation of IL-10Rs in two epithelia: trophoblast cells and epidermal keratinocytes.

2 SPECIFIC AIMS

2.1. The first aim of this thesis was the establishment of a reproducible method suitable for the simultaneous isolation of villous and extravillous CTB cells from first trimester human placenta.

2.2. The purpose of further work was to investigate the expression of IL-10R at the protein level by CTB cells *in vitro* and to elucidate the placental localization of IL-10R expression *in vivo*, to define the subset of placental CTBs constituting the predominant target for the autocrine regulatory influence exerted by IL-10 during the first trimester of pregnancy.

2.3. The functional characterization of the IL-10R expressed by human first trimester CTBs with special emphasis on the possible role played by IL-10 in placental growth control through the regulation of CTB cell proliferation.

2.4. The HaCaT cell line is widely used in experiments examining keratinocyte physiology and studying keratinocyte proliferation and differentiation. Since normal human keratinocytes express cell-surface IL-10Rs we sought to investigate the IL-10R expression by HaCaT keratinocytes at the protein level, as the presence of cell-surface IL-10Rs on these cells would open the possibility to use HaCaT cells as a model for studying the effects of IL-10 on keratinocyte physiology.

2.5. As IL-10 is able to suppress the proliferation of normal human keratinocytes and in psoriasis a relative deficiency of IL-10 and a decreased expression of its receptor can be demonstrated, we set out to examine the possible effect of the potent antipsoriatic compound dithranol on the expression of IL-10Rs in human HaCaT keratinocytes.

3 MATERIALS AND METHODS

3.1 Isolation of first trimester human CTB cells

CTB cells were isolated from first trimester placenta as described by Bischof *et al.* [18]. Trophoblastic tissue was obtained from legal terminations of pregnancies performed in accordance with the Hungarian Abortion Law between 6-12 weeks of gestation. Villous tissue was dissected manually, rinsed and minced in HBSS (pH=7.4) containing 200 U/ml penicillin and 200 μg/ml streptomycin (both from Sigma, Budapest, Hungary). The minced tissue was then incubated at 37 °C four times for 20 minutes in HBSS containing 0.25% trypsin (Sigma, Budapest, Hungary), 50 U/ml DNase I (Sigma), 4.2 mM magnesium sulfate (Sigma), 25 mM HEPES (Sigma) and antibiotics (200 U/ml penicillin and 200 μg/ml streptomycin). The supernatant containing the dissociated mixed placental cells was collected, and the trypsin activity was neutralized by addition of 10% FCS (Gibco, Life Technologies, Vienna, Austria). The neutralized supernatant was centrifuged at 800 × *g* for 10 minutes and the resulting cell pellet was resuspended in DMEM containing 25 mM HEPES, 200 U/ml penicillin, and 200 μg/ml streptomycin. The cell suspension obtained was

placed immediately into an incubator and was maintained at 37 °C until the end of the entire dissociation procedure. Concomitantly, the remaining villous tissue was subjected to another 20 min trypsinization step. At the end of the dissociation procedure the remaining villous fragments were discarded. The four fractions of cell suspensions were pooled, filtered over a 100 μm nylon mesh to remove remaining villous fragments, centrifuged at 800 × *g* for 10 minutes, and resuspended in 2-3 ml of the same medium without FCS. This cell suspension was layered over a 5% to 70% preformed discontinuous Percoll gradient, according to the method of Kliman *et al.* [82]. The fraction containing the CTB cells (densities 1.048-1.062 g/ml) was washed and resuspended in DMEM. These Percoll-enriched cells were immunopurified immediately and cultured, were stored in liquid nitrogen until immunopurified or were further used for flow cytometric analyses and/or immunohistochemical or immunofluorescent studies. The viability estimated by Trypan-blue exclusion was consistently >95%.

3.2 Storage of cells in liquid nitrogen

The Percoll-purified cells were suspended in cold FCS containing 10% DMSO (Sigma) to give 5-10 × 10^6 cells/ml. One ml aliquots of this cell suspension in 1.8 ml cryogenic vials (Corning Inc., N.Y., USA) were placed in insulated cardboard containers and kept at –70 °C overnight. Next day the tubes were rapidly transferred to liquid nitrogen for long term storage. When required, a tube was removed from the liquid nitrogen and thawed in a water bath at 37 °C. The thawed cells were diluted 1/10 in DMEM containing 20% FCS and centrifuged at 400 × *g* for 5 minutes. The cell pellet was resuspended in fresh culture medium to the required cell density.

3.3 Immunopurification of CTB cells

Cells (either freshly isolated or thawed after storage in liquid nitrogen) suspended in ice-cold DMEM (15-17 × 10^6 cells in 2 ml) were incubated for 30 minutes at 4 °C with 100 μl of a monoclonal antibody to leukocyte common antigen (LCA, CD45, Dako A/S, Glostrup, Denmark) with occasional gentle shaking. In some experiments designed to obtain pure villous CTBs, the monoclonal anti-CD45 antibody was replaced with 50 μl of a monoclonal anti-HLA-ABC (clone W6/32) and 50 μl of monoclonal anti-HLA-DP, DQ, DR (clone CR3/43; both from Dako A/S). All the primary antibodies were raised in mice and

were added undiluted to the cell suspension. After incubation, the cells were washed with PBS (pH 7.4) containing 0.1% BSA (radioimmunoassay grade, Sigma). Prior to separation, 100 µl of magnetic particles coated with a goat anti-mouse antibody (Dynabeads M-280, Dynal, Oslo, Norway) were washed twice with 5 ml PBS containing 0,1% BSA and stored at 4 °C until used. The cell suspension was then incubated with the prewashed magnetic particles at 4 °C for 30 minutes with occasional gentle shaking. At the end of the incubation the tube was clamped to a magnetic concentrator (Dynal Laboratories, Oslo, Norway) for 5 minutes. The supernatant containing the immunopurified CTB cells was removed from the tube and centrifuged at 800×*g* for 10 minutes at room temperature. The pelleted cells were resuspended in culture medium and plated as required (see bellow).

3.4 Cell culture

Purified CTBs were cultured in 24 well culture plates (Costar, Cambridge, MA., USA) under a 5% carbon dioxide and 95% air atmosphere in an incubator at 37 °C. The plating density was 0.5×10^6 viable cells/ml. When CTB differentiation was to be avoided the cells were cultured under serum-free conditions in DMEM containing 25 mM HEPES, 200 U/ml penicillin, and 200 µg/ml streptomycin. In some experiments set out to study CTB differentiation, highly purified cells were cultured in 20% (v/v) FCS-containing DMEM or in KGM (Gibco) supplemented with 17% (v/v) FCS and antibiotics. Every other day the culture supernatant was gently aspirated and replaced with fresh media. After centrifugation at 800×g for 10 minutes in order to remove occasionally detached cells and any insoluble material, the culture supernatants were aliquoted and stored at –70 °C until assayed for hCG content or analysed for the presence of gelatin degrading proteases by zymography. For immunocytochemical characterization of cultured CTBs, the cells were plated on 13 mm cell culture treated plastic coverslips (Menzel-Glaser).

The spontaneously transformed human epidermal cell line HaCaT (kindly provided by Dr. N. E. Fusenig, Heidelberg, Germany) was cultured in DMEM containing 10% FCS.

3.5 Hormone assays

Total human chorionic gonadotropin (hCG + β hCG) was measured in the culture supernatant with a microparticle enzyme immunoassay kit kindly provided by the WHO (WHO Matched Assay Reagents for the Immunoassay of Hormones). The sensitivity of the

assay was 2 mIU/ml. The average intra- and interassay coefficient of variation was 3,6 % and 7,2 %, respectively.

3.6 Zymography

The presence of gelatinolytic metalloproteinases in the supernatants of CTBs cultured for 24 hours was assessed by zymography with a modification of the technique described by Fisher *et al.* [54]. Briefly, 10% polyacrylamide gel in 0,1% SDS containing 1 mg/ml gelatin (all from Sigma, Budapest, Hungary) were cast at a final dimension of 80 × 85 × 1 mm. After the polymerization of the 10% running gel, a 2% polyacrylamide stacking gel was cast above the previous one. Thirty microliters of molecular weight standards (Pharmacia, Uppsala, Sweden) and samples were incubated 5 minutes at room temperature with 5 µl sample buffer containing 17.4% SDS, 7% sucrose, and phenol red in distilled water. Twenty five microliters of the incubate were applied to the gel. After electrophoresis, gels were subjected to six 5-minute washes in 2.5% Triton X-100 in distilled water to allow proteins to renature, followed by three 5-minute washes in PBS containing 0.9 mmol/l calcium chloride and magnesium chloride and were incubated overnight at 37 °C. Next morning the gels were stained with Coomassie Brilliant Blue G250 (0.1% in 25% methanol and 10% acetic acid in distilled water) and destained in 5% methanol and 7.5% acetic acid in distilled water.

3.7 Analysis of IL-10R expression on first trimester CTBs

The expression of IL-10R was determined for Percoll gradient purified CTBs using an IL-10 Fluorokine Kit (R&D Systems Gmbh, Wiesbaden, Germany) according to the manufacturer's protocol. Briefly, the freshly isolated cells were incubated overnight at 37 °C in serum-free culture medium, under conditions that did not allow them to attach (*i.e.* in solution, on rocking platform or on agarose coated culture dish). Next day the cells were washed (in order to remove any residual growth factors present in the culture medium), and separated into two groups to which the biotin-conjugated IL-10 or the biotin-labeled control protein (soybean trypsin inhibitor) were added. After 1 hour incubation at room temperature avidin-FITC was added, followed by an additional 1 hour incubation, washing and resuspension in a specific buffer designed by the manufacturer to stabilize specific staining and minimize background. Analysis was conducted on a FACStar^{PLUS} (Becton Dickinson, Erembodegem-Aalst, Belgium) flow cytometer with a 488 nm argon-ion laser excitation

device. The cells were first analyzed for forward and side scatter to gate out debris, and to select the CTBs based on their relatively large size and primitive cytoplasmic structure.

3.8 Immunofluorescent staining of purified CTBs

Immunopurified CTB cells (2,5-5×10^4 cells/100 µl PBS) were centrifuged at 700 × *g* onto glass slides with a cytospin centrifuge. After drying, the obtained cytospin smears were fixed in acetone at -20 °C for 15 minutes. For the characterisation of CTBs cultured 24 hours on 13 mm plastic coverslips, cells were fixed 15 minutes in a 1:1 mixture of ethanol and acetone at –20 °C. Until stained, both the cytospin smears and the coverslips with cultured CTBs were stored at –20 °C. Before staining the cells were rehydrated 15 minutes in PBS (pH 7.4) at room temperature. In the mean time primary antibodies were diluted in PBS containing 1% FBS. The coverslips and cytospin smears were incubated with 20 µl of the primary antibody in a humid chamber at 37 °C for 1 hour. The primary antibodies (mouse anti-human cytokeratin, clone MNF116 diluted 1/10 and rabbit anti-human β-hCG undiluted, used for the detection of trophoblast cells and mouse anti-human vimentin, diluted 1/20, which labelles stromal cells) were from Dako A/S. After 60 minutes the cells were washed three times in PBS, for 10 minutes each, followed by a 45 minute incubation with the FITC-conjugated second antibody (goat anti-mouse Ig, 1/100, Sigma; sheep anti-rabbit Ig, 1/16, Miles-Yeda Ltd.) at 37 °C. After two 10 minute washes in PBS, the cells were stained with Evans Blue (diluted 1/10 000 in PBS) (Sigma) in order to reduce the intensity of non-specific fluorescence. After mounting with an aqeous mounting medium, the cell preparations were examined under a Leitz UV microscope and the results were expressed as the percentage of positive cells per 300 total cells counted.

3.9 Immunohistochemistry

The IL-10R monoclonal antibody, specific for the extracellular, ligand binding domain of the cell-surface IL-10R, was obtained from R&D Systems. The mouse monoclonal antibodies to α_5 (CD 49e, clone SAM1) and α_6 (CD 49f, clone 4F10) integrin subunits were obtained from Immunotech (Coulter, Marseille Cedex, France) and Serotec (Kidlington, Oxford, UK), respectively. The anticytokeratin antibody (clone MNF116) used to identify CTB cells within chorionic villi was obtained from Dako A/S. A monoclonal antibody against the proliferation marker Ki-67 (clone Ki-S5) was from Boehringer Mannheim

(Vienna, Austria). Negative control slides were stained with mouse IgG class-matched irrelevant antibodies, obtained from Dako A/S. All antibodies and negative control reagents were diluted to a working concentration of 1 μg/ml in TBS, containing 0.1% Triton X-100 and 0.5% BSA (both from Sigma).

Samples of first trimester placental tissue (6-12 weeks of gestation) were taken from routine vaginal terminations of pregnancies. For the investigation of the role played by IL-10 in the regulation of CTB proliferation, chorionic villi were placed on filter papers within 24 well plates (Costar) and cultured under serum-free conditions in 2 ml of penicillin-streptomycin containing DMEM. The placental explants were cultured in medium alone, in the presence of increasing concentrations of IL-10 (0.1-10 ng/ml) (R&D Systems) and some cultures were treated concomitantly with 10 ng/ml IL-10 and 10 μg/ml of an IL-10R specific function blocking antibody (R&D Systems). After 24 hours the CTB proliferation was assessed by immunohistochemistry using a monoclonal antibody directed against the proliferation marker Ki-67. Both freshly obtained and cultured placental tissue fragments were immediately frozen in Cryomatrix (Shandon, Cheshire, UK). Six μm frozen sections were thawed, air dried, fixed in acetone for 10 minutes at 4 °C, and rehydrated in TBS containing 0.1% Triton X-100. Slides were then incubated for 30 minutes at 4 °C with human IgG (0.5 mg/ml, heat aggregated at 65 °C for 20 minutes) (Jackson ImmunoResearch Laboratories Inc., West Baltimore Pike, USA), in order to block cell-surface Fc receptors. Excess liquid was blotted without washing, and the sections stained for IL-10R and Ki-67 were incubated overnight at 4 °C. Slides stained for cytokeratin, α_6 and α_5 integrin subunits were incubated for 1 hour at room temperature. The antigen-antibody reaction was revealed by using a StreptABComplex Duet (mouse and rabbit) Reagent Set (Dako A/S). Briefly, the slides were incubated for 1 hour at room temperature with the biotin-labeled second antibody (goat anti-mouse/rabbit Ig). After washing, avidin-HRP was added and the incubation was performed in similar conditions as described for the second antibody. The peroxidase was developed with AEC as substrate (Sigma). Finally, the sections were washed in tap water, counterstained with hematoxylin, rewashed and mounted in DAKO Glycergel® aqeous mounting medium.

3.10 Western blot analysis

Total protein extracts from cultured chorionic villi were prepared in a lysis buffer of 1.5% SDS, 62.5 mM Tris-HCL pH 6.8, 5mM EDTA, 5% 2-mercaptoethanol, 1 μg/ml antipain, 1 μg/ml chymostatin, and 1 μg/ml leupeptin (all from Sigma). Lysates were precleared by centrifugation at 12 000 × *g* for 15 minutes and supernatants were aliquoted and stored at -20°C. The constituent proteins of the chorionic villi extracts were separated by SDS/PAGE on a 6% separating gel and then transferred to nitrocellulose membrane (Bio-Rad Laboratories). In order to verify the equivalent loadings of proteins in the wells, the gel and the nitrocellulose was stained by Coomassie Brillant Blue and Ponceau S, respectively (Sigma). Membranes were blocked by incubation in TBS (150 mM sodium chloride, 25 mM Tris-HCl, pH 7.4), containing 0.05% Tween 20 (Sigma) and 2% nonfat dry milk (Fluka Chemie AG, Neu-Buchs, Switzerland), for 2 hours at room temperature and subsequently incubated overnight at 4 °C with the monoclonal antibody specific for the S-phase marker Ki-67, diluted to a working concentration of 1 μg/ml in blocking buffer. AP-conjugated rabbit anti-goat IgG (Sigma) was used as secondary antibody at 1:2500 dilution in the blocking buffer. After incubation with the second antibody for 2 hours at room temperature, blots were developed by using BCIP/NBT as substrate (Sigma).

3.11 Analysis of IL-10R expression on HaCaT keratinocytes

The subconfluent HaCaT keratinocytes were washed with PBS under sterile conditions and were incubated for 30 minutes at 37 °C in a humid 5% carbon dioxide atmosphere incubator with increasing concentrations of dithranol (0.1-0.5 μg/ml) (Hermal Chemie, Reinbek, Germany) in DMEM containing 0.5% FCS. Dithranol was always freshly dissolved in acetone and used immediately. Control cells were treated with the solvent (acetone) only or left untreated.

Dithranol treated cells were then washed with PBS and were incubated in DMEM containing 10% FCS for 2 and 4 hours at 37 °C before immunohistochemical staining for IL-10R. The staining protocol for the detection of IL-10R was identical with that used for the characterization of IL-10R expression in first trimester chorionic villi.

4 RESULTS

4.1 Isolation and characterization of first trimester CTB cells

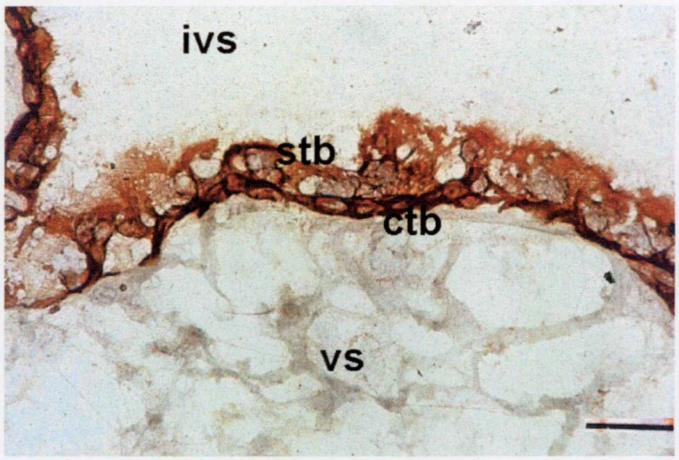


Figure 6. Frozen section of placental villi stained with monoclonal antibody to cytokeratin. Cytokeratin is strongly expressed by both the cytotrophoblast monolayer (ctb) and the overlying syncytiotrophoblast (stb).

(vs, villous stroma; ivs, intervillous space). Bar = 50 μm

In order to isolate first trimester CTBs an enzymatic dissociation method followed by density gradient centrifugation and subsequent immunomagnetic purification was applied. Cell purification was monitored by staining the cells with an anticytokeratin antibody specific for cytokeratins 5, 6, 8, 17 and probably also 19 (Clone MNF116). The antibody has been selected on the basis of our immunohistochemical data which showed that within first trimester placental villi it stains only trophoblasts (Fig. 6.), a finding which was already reported by others [1]. For the characterization of freshly isolated CTB cells the experiments were repeated at least three times with different CTB preparations obtained from pooled placental tissues. The density gradient centrifugation yielded a cell suspension consisting of 78-86% CTBs as judged by cytokeratin positivity of cytospin smears obtained from freshly isolated cells. Approximately one third (32-45%) of the Percoll-enriched cell suspension stained positive with the HLA-ABC specific monoclonal antibody, indicating either an extravillous origin of trophoblastic cells or the presence of lymphomyeloid contaminants. Since placentas are usually obtained from first trimester terminations of pregnancy, the most likely contaminants of CTB cell preparations are lymphomyeloid cells. One of the most reliable markers used to detect lymphomyeloid cells is the leukocyte common antigen CD45. After Percoll gradient centrifugation the proportion of CD45 positive cells in our CTB enriched cell suspensions was 16-32% (data not shown).

Percoll-isolated cells were further purified using an immunomagnetic procedure by which the CD45-positive cells were eliminated. In order to obtain pure villous CTB cells, in some experiments the immunopurification was performed using a 1:1 mixture of a monoclonal mouse antibody to the HLA-ABC epitope of class I MHC antigens and an antibody specific for the HLA-DP, DQ, and DR epitopes of the class II MHC loci. This step took advantage of the fact that villous trophoblast is one of the few tissues to lack MHC antigens on its surface [28, 51, 141]. Highly purified CTBs, irrespective of the antibodies used during immunopurification, were 95-99% cytokeratin-positive, the HLA-ABC positive cells fell bellow 3% of total cells counted and the CD45-positive lymphomyeloid cells became undetectable, as shown both by immunocytochemistry and immunofluorescent studies of cytospin smears. The low level contamination by vimentin-expressing fetal stromal cells was not affected by the immunopurification and accounted for < 6% of total cells counted (data not shown).

After the elimination of CD45-positive contaminating cells the remaining CTB cell population consisted of 18-22% α_5-integrin expressing and 25-38% α_6-integrin expressing CTBs, showing that the isolated cell population consisted of villous and extravillous cells (data not shown).

As shown by Trypan blue exclusion, >95% of the Percoll-isolated cells retained viability, which was not affected neither by immunopurification, nor by storage under liquid nitrogen.

4.2 Culture of first trimester CTB cells

Similar to the characterization of isolated CTBs, cell culture experiments were repeated at least three times with different cell preparations. In the presence of 20% (v/v) FCS, >80% of mononuclear CTBs adhered to the culture plastic within 6 hours of culture. After 12-24 hours, the cells flattened out and formed colonies of 5-6 aggregated cells, which were cytokeratin positive as shown by immunofluorescent staining (Fig. 7A. and 7B.). Over 6 days in culture, the number and the size of the morula-like cellular aggregates increased continuously, and on the 6th day single cells represented < 15% of the total cultured CTBs. Contaminant spindle shaped fibroblasts were < 3% of total cells. Beginning with the 4th day of culture the cell boundaries became hardly distinguishable under phase contrast microscopy (Fig. 8B.) and during subsequent culturing syncytium-like structures appeared.

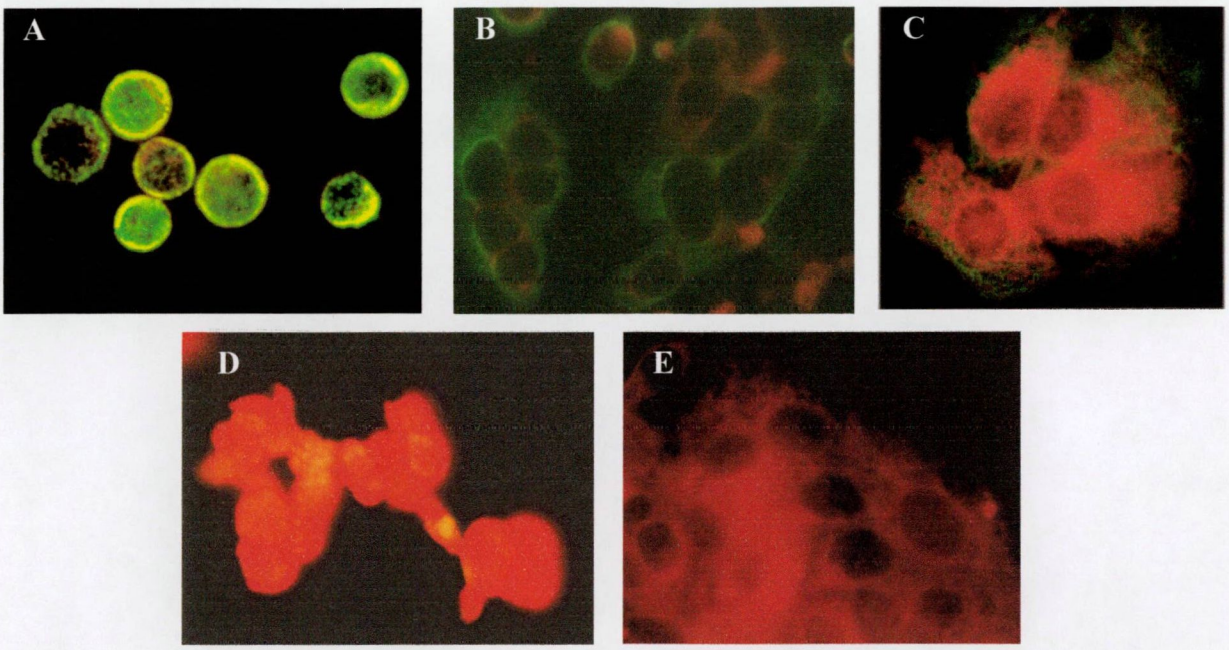


Figure 7. Immunofluorescent staining of highly purified CTB cells. (**A**) Cytospin smear of freshly isolated CTBs stained for cytokeratin. (**B**) Cytokeratin staining of CTBs cultured 24 h in 20% (v/v) FCS containing DMEM on coverslips. (**C**) Cytokeratin staining of CTBs cultured 6 days in KGM containing 17% (v/v) FCS, on coverslips. (**D**) Immunofluorescent staining of βhCG. CTB cells were cultured 6 days in KGM supplemented with 17% (v/v) FCS on coverslips. (**E**) Multinucleated cells resembling syncytiotrophoblasts. After 5 days of culturing in KGM supplemented with 17% (v/v) FCS on coverslips, cells were stained with Evans Blue.

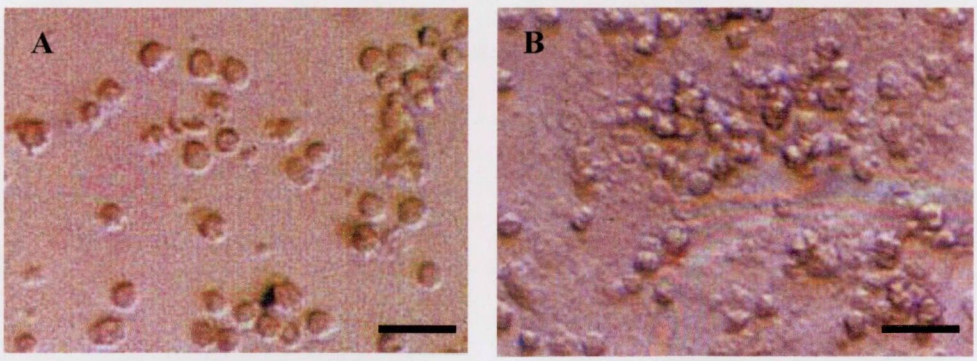


Figure 8. Phase contrast micrographs of first trimester human cytotrophoblast cells cultured for 4 days in serum-free Dulbecco's Modified Eagle Medium (**A**) or in presence of 20% (v/v) fetal calf serum (**B**).
Bar = 50 μm

In the absence of serum < 80% of CTBs adhered after 12 hours of culture and the majority of cells remained round-shaped. After 24 hours some mononuclear CTBs formed small colonies of aggregated cells. Over time the number and the size of the aggregates remained essentially unchanged and the cell boundaries were well distinguishable with phase contrast microscopy (Fig. 8A.). The percentage of single cells remained around 30-40%.

When HLA-ABC and HLA-DP, DQ, DR depleted pure villous CTBs were cultured in KGM containing 17% FCS, extensive cell aggregation was noticed. After 5 days of culturing

multinucleated cells resembling STBs formed (Fig. 7E.). The trophoblastic origin of these cells was confirmed by cytokeratin and β hCG staining (Fig. 7C. and 7D., respectively).

The secretion of the pregnancy hormone hCG by the CTB cell population depleted of CD45-positive lymphomyeloid cells increased abruptly when the cells were cultured in DMEM containing 20% FCS (Fig. 9.). On day 4 the concentration of total hCG in the culture supernatant was twice as much as in the 2nd day supernatant and by the 6th day the concentration of the hormone was six times more than that measured on the 2nd day of culture. In contrast, the amount of hCG secreted by CTB cells cultured under serum-free conditions increased slowly: twice the concentration of the hormone in the 2nd day culture supernatant was recorded only by the 6th day of culture. This pattern of hCG secretion correlated well with the rate of syncytium formation and hCG positivity. In some experiments the 24 hours hormone production of the cultured CTB cells was also measured. Our results indicate that during 24 hours the hCG production of the cells was as small as 143.12 ± 104.56 IU/L (average ± SD, N=4 cell preparations from 9 different placentas) and differed significantly from one cell preparation to another. During the first 24 hours of culture the hCG secretion by CTB cells was not changed by the addition of 20% FCS to the culture medium. Immunopurification did not change the secretion of hCG by CTB cells (data not shown).

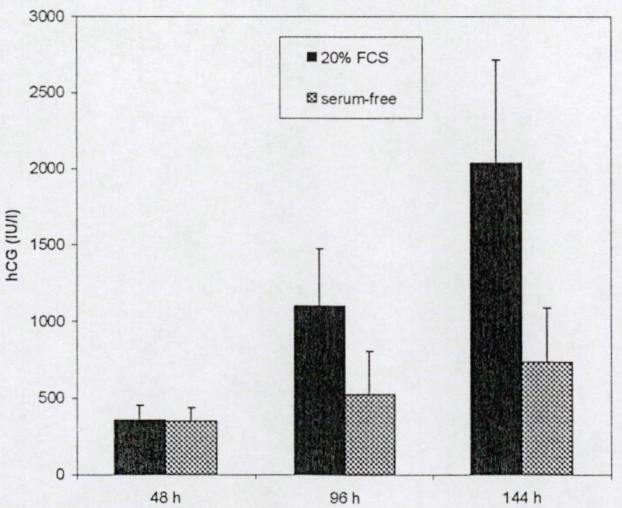


Figure 9. Secretion of hCG by first trimester cytotrophoblast cells cultured in Dulbecco's Modified Eagle Medium under serum-free conditions or in the presence of 20% (v/v) fetal calf serum. Results are means (N=3), and error bars represent standard deviations.

Metalloproteinase secretion is an important characteristic of CTB cells, thus for a more complete characterization of our CTB preparations we set out to examine the array of cell-secreted metalloproteinases using substrate gels copolymerized with gelatin. For this purpose isolated cells depleted of CD45 expressing contaminants, which might also secrete gelatin-degrading proteases, were cultured for 24 hours in serum-free DMEM. Analysis of gelatin-degrading activity in CTB-conditioned media revealed a single digestion band in the 90 kD region (Fig. 10.). These findings are in accordance with the previously reported data by Fisher *et al.* [54] showing that early placental CTBs secrete only the 92 kD matrix metalloproteinase (MMP-9) into the culture medium.

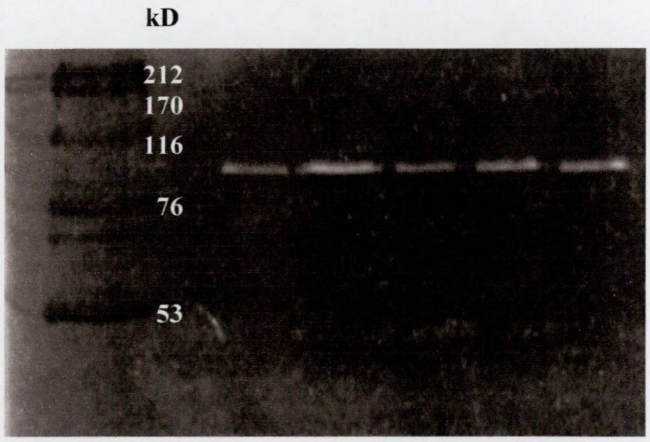


Figure 10. Zymographic analysis of gelatin-degrading activity in CTB-conditioned media from N=5 different CTB preparations. CD45-depleted CTBs were cultured for 24 hours in serum-free DMEM as described in "Materials and Methods".

Taken together, the described morphological data, as well as differentiation studies, hCG and metalloproteinase secretion patterns demonstrate that the isolated cells are CTBs.

4.3 Flow cytometric analysis of IL-10 receptor expression *in vitro* on first trimester CTB cells

Binding experiments performed on Percoll-purified first trimester CTBs using biotinylated IL-10 and avidin-FITC have demonstrated, that above 75-80% of the cells were able to bind the labelled cytokine, as indicated by the FACS hystogram (Fig. 11b.). Cells were also stained with a monoclonal antibody against cytokeratins (clone MNF116). Of the cells, 75-80% showed positive staining (Fig. 11d.) relative to the isotype control (Fig. 11c.), indicating that the cytokeratin positive population (*i.e.* CTBs) and IL-10 receptor expressing cells might be the same. Unspecific binding was ruled out by the negative control experiments, where the

biotinylated IL-10 was substituted by biotin-labelled soybean trypsin inhibitor (Fig. 11a.). This protein is unable to bind specifically to any known cell-surface receptor, therefore the fluorescence of these cells is identical to that of the background. For the specific binding it was essential to maintain the cells in serum-free media overnight under conditions that did not allow them to attach (*i.e.* in solution, on rocking platform or on agarose coated culture dish). Freshly isolated cells, as well as cultured cells, treated with trypsin to remove them from the culture plates, did not bind labelled IL-10 at all (data not shown). This finding indicates that IL-10Rs expressed by early human CTB cells are trypsin sensitive.

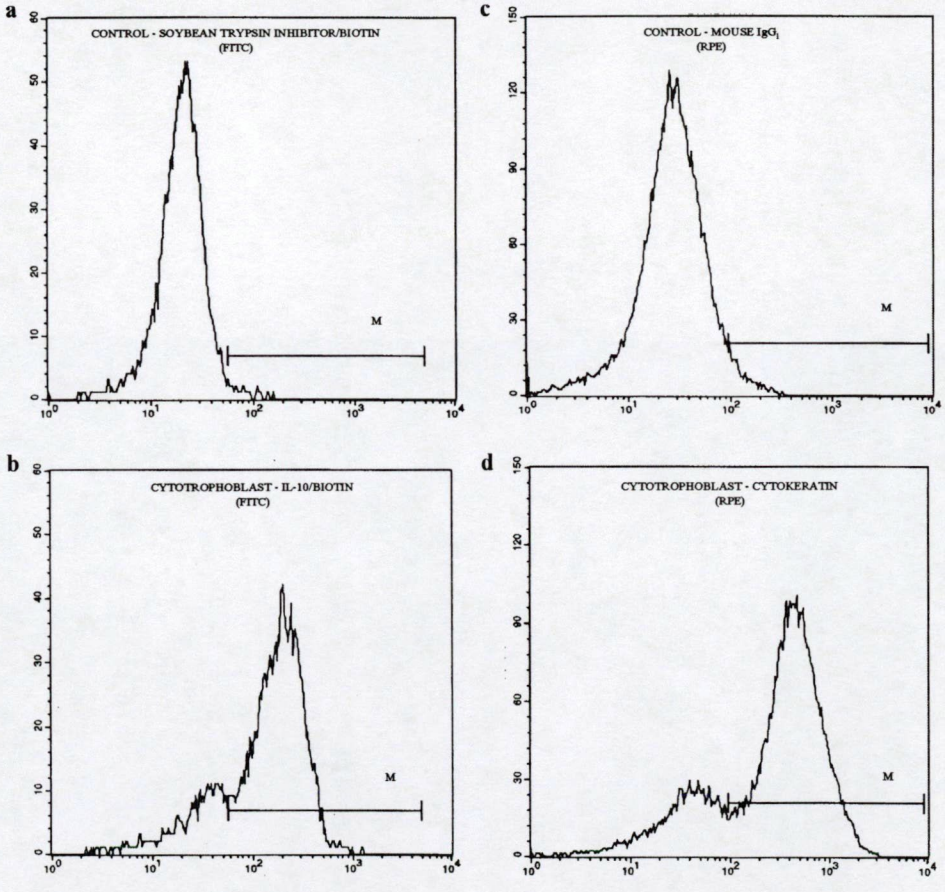


Figure 11. Negative controls of cytotrophoblast cells stained with biotin-labeled soybean trypsin inhibitor (**a**) and non-immune mouse IgG_1 (**c**) were used to determine background staining. Representative experiment of cytotrophoblast cells analysed by flow cytometry for IL-10 receptors (**b**). Representative experiment of cytotrophoblast cells permeabilized overnight in 70% ethanol at -20 °C, and analysed by flow cytometry for cytokeratins (**d**). x axis = fluorescence intensity, y axis = relative number of cells, M = marker.

4.4 Immunohistochemical detection of IL-10Rs in human first trimester placental tissues

Reactivity to IL-10R specific monoclonal antibody was detected in all (N=12) first trimester preparations (Fig. 12. shows the result of a representative experiment). The most intense staining was observed in trophoblast cells forming the internal layer of villi (*i.e.* villous CTB), meanwhile the external STB layer was negative (Fig. 12A.). The CTB cells which expand from the tip of anchoring villi shortly after implantation and form CTB cell columns also reacted with the IL-10R specific monoclonal antibody (Fig. 12B.). The most intense reactivity was observed in the proximal cells of these columns, located nearest the villous stroma. In contrast, cells located more distally stained only weakly, if at all. In all experiments, reactivity to IL-10R specific antibody was localized to the cell membrane with no reactivity in the cytoplasm of the cells. Moreover, CTB cells resting on the villous basement membrane (villous CTBs and the first row of CTB cell columns) stained intensely along the apical and lateral microdomains of their cellular membranes. This staining pattern suggests that CTB cells express cell-surface IL-10Rs in a polarized manner. Cells of the villous stroma were always negative, showing that in the first trimester of pregnancy these cells do not express IL-10Rs.

4.5 Expression of α_5 and α_6 integrin subunits in human first trimester placentas

In order to specify more precisely the subsets of IL-10R bearing CTB cells *in vivo*, in parallel we have studied the pattern of integrin expression in our first trimester placental tissue preparations. Villous CTB cells resting on villous basal lamina expressed α_6 integrin subunit clustered along the basement membrane (Fig. 12E.). Proximal cells of CTB cell columns resting on the basement membrane of the villous tip have preserved this pattern of expression. In contrast, more distal but still closely packed CTBs of cell columns expressed α_6 integrin subunit in an unclustered way. Concomitantly, a gradual reduction of staining intensity towards the distal, deeply invasive cells was observed. Villous STBs and deeply invasive cells of CTB cell columns were always negative.

When serial sections stained for α_5 integrin subunit were examined and compared to those stained for α_6 integrin, a reciprocal staining pattern was observed: α_5 integrin

expression reached its maximum in distal cells and gradually decreased towards the proximal part of cell columns (Fig. 12F.). In villous CTB no staining for α_5 integrin subunit was seen.

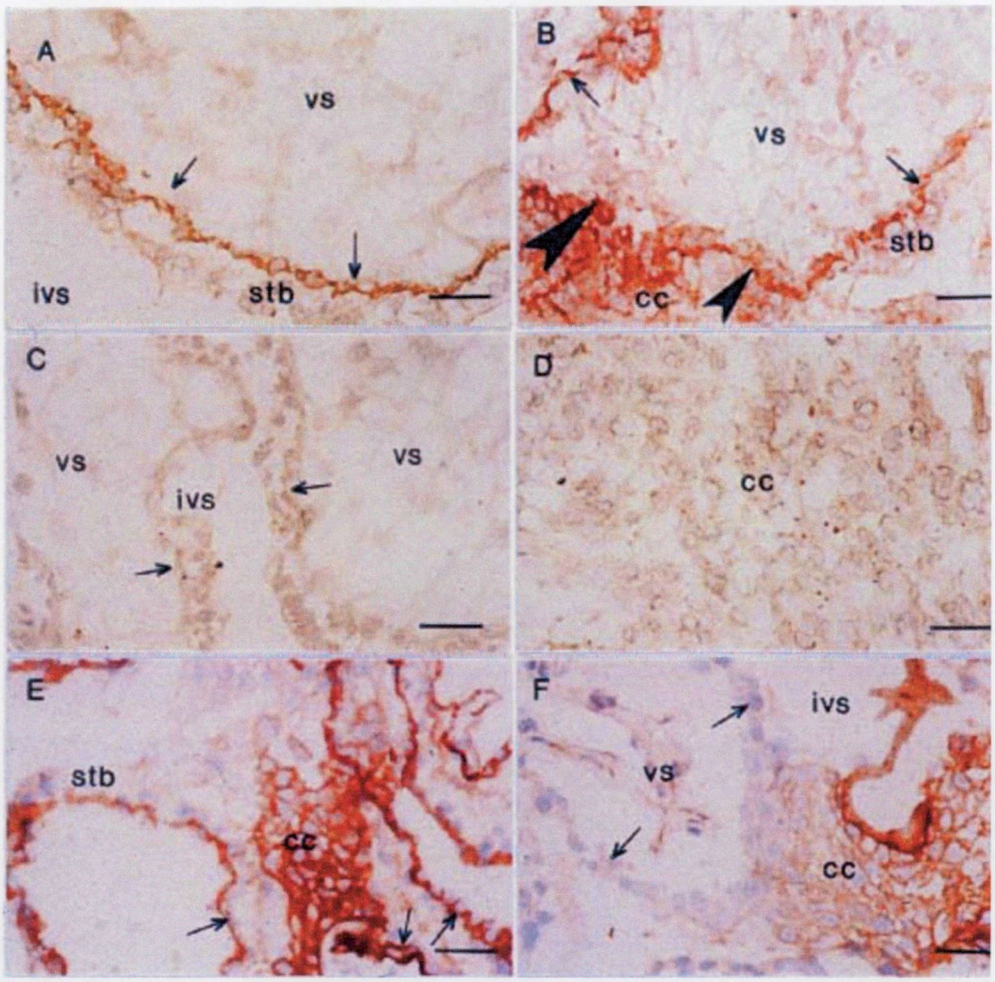


Figure 12. (**A**) Frozen section of placental villi stained with monoclonal antibody (Mab) to IL-10 receptor. The IL-10 receptor is strongly expressed by villous cytotrophoblast (arrows) but the overlying villous syncytiotrophoblast (stb) is negative (vs, villous stroma; ivs, intervillous space). (**B**) Frozen section of placental villi stained with Mab to IL-10 receptor showing an anchoring villous. The IL-10 receptor is strongly expressed by cytotrophoblast cells anchored to the basement membrane covering the villous tip (arrowheads) and by villous cytotrophoblasts (arrows). Cytotrophoblast cell column emerging from the villous tip (cc) and syncytiotrophoblast (stb) stain much more weakly if at all. Cells in the villous stroma (vs) are negative. (**C**) Frozen section of placental villi stained with non-immune class matched mouse IgG showing that the villous cytotrophoblasts and syncytiotrophoblasts (arrows) are negative (vs, villous stroma; ivs, intervillous space). (**D**) Frozen section of placental villi stained with non-immune class matched mouse IgG showing a part of the invasive cytotrophoblast cell column (cc). (**E**) Frozen section of placental villi stained with Mab to the α_6 integrin subunit. The α_6 integrin subunit is strongly expressed by villous cytotrophoblasts (arrows) but the villous syncytiotrophoblasts (stb) are negative. There is a reduction in staining intensity for α_6 in the distal part of the cytotrophoblast cell column (cc). (**F**) Frozen section of placental villi stained with Mab to the α_5 integrin subunit. Villous cytotrophoblast and syncytiotrophoblast cells do not stain (arrows). Expression of α_5 integrin increases from the proximal towards the distal part of the cytotrophoblast cell column (cc). (vs, villous stroma; ivs, intervillous space) Bar = 50 μm.

4.6 The functional characterization of the IL-10R: the role of IL-10 in placental growth control through the regulation of CTB proliferation

Immunohistochemical analysis of placental explants from N=8 different placentas indicated that IL-10 reduces dramatically the number of the Ki-67 positive CTB nuclei within first trimester placental villi, whereas the proliferation of other cell types remained unaffected (Fig. 13.). We did not observe any difference in the Ki-67 staining pattern of chorionic villi treated with different concentrations of IL-10.

In order to assess quantitative changes and dose-dependence which might not be revealed by immunohistochemical staining, in parallel we performed immunoblot analysis to clarify whether the proliferation inhibiting effect of IL-10 on villous CTB cells was dose-dependent. The western blot analysis, similar to the immunohistochemical results demonstrated that the expression of the Ki-67 antigen is markedly suppressed upon treatment with IL-10 (Fig. 14.). This effect proved to be maximal even at the lowest concentration of IL-10 and was reversible by the IL-10R specific function blocking antibody, showing that the observed biological changes were mediated through specific cell-surface IL-10Rs.

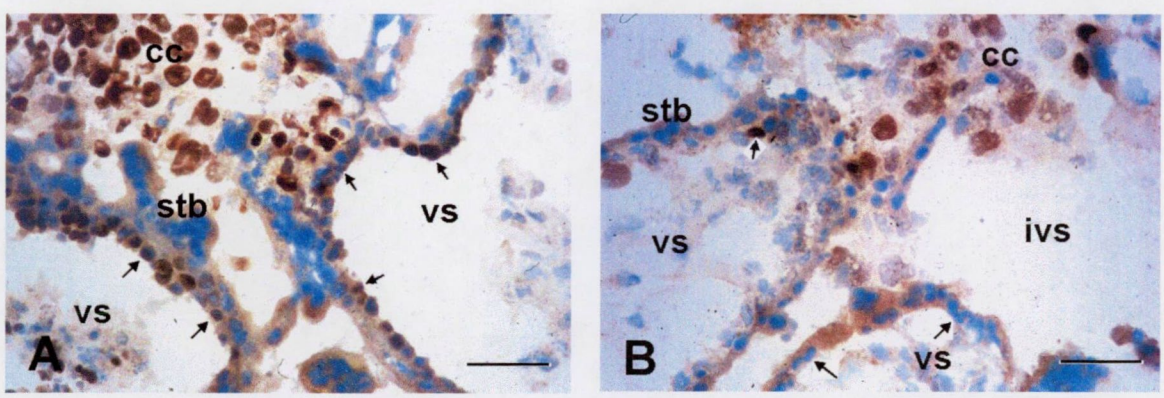


Figure 13. Frozen section of first trimester chorionic villi cultured for 24 hours (**A**) in Dulbecco's Modified Eagle Medium alone or (**B**) in the same medium supplemented with 10 ng/ml IL-10 and subsequently stained with a monoclonal mouse antibody to the S-phase marker Ki-67. Nuclear staining is confined to villous cytotrophoblast cells (arrows) and cytotrophoblasts of cell columns emerging from the villous tip (cc). In IL-10 treated chorionic villi the number of Ki-67 positive cytotrophoblast nuclei is markedly reduced. (vs, villous stroma; ivs, intervillous space) Bar= 100 μm.

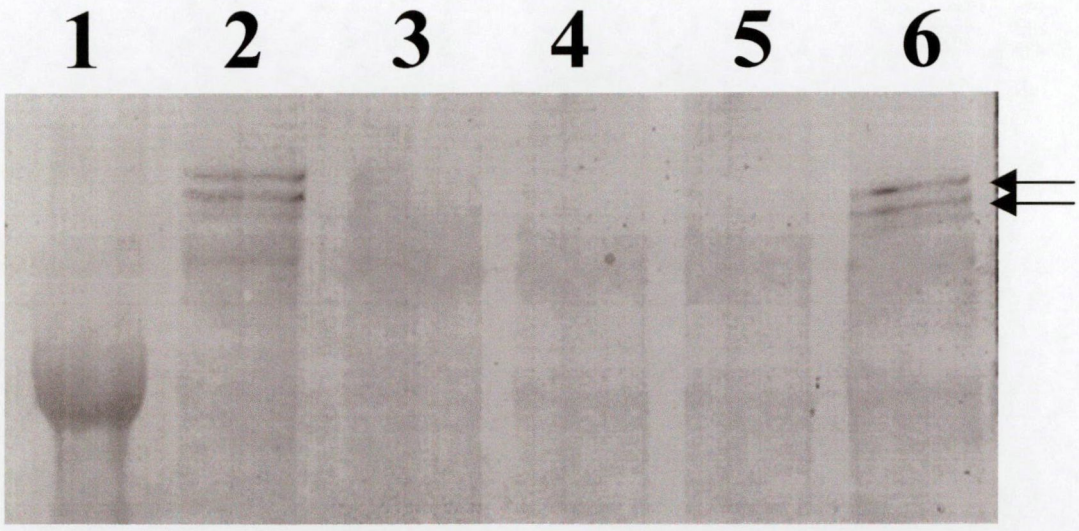
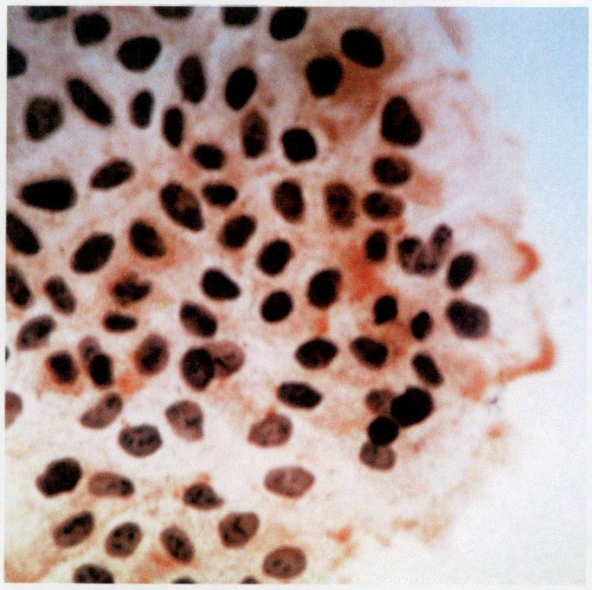


Figure 14. Western immunoblot analysis of Ki-67 antigen expressed by first trimester chorionic villi cultured *ex vivo* for 24 hours in serum-free Dulbecco`s Modified Eagle Medium. Lane 1, the position of the prestained 218 kD molecular weight standard. Lane 2, chorionic villi cultured for 24 hours in medium alone; lane 3, 4 and 5, chorionic villi treated with 0.1, 1, and 10 ng/ml IL-10, respectively. Lane 6, chorionic villi treated concomitantly with 10 μg/ml of an IL-10 receptor specific function blocking antibody and 10 ng/ml IL-10. The monoclonal mouse anti-Ki-67 antibody recognized two bands of 345 kD and 395 kD, respectively identical with the Ki-67 antigen (arrows).

4.7 IL-10 receptor expression at the protein level in HaCaT cells

Untreated cultured HaCaT cells were stained with a monoclonal antibody against human IL-10R. Relative to the isotype control the antibody clearly stained the cells. A clear membrane localisation was apparent and interestingly it seemed that cells localized at the edges of the colonies showed stronger expression. Beside the membrane staining there was some cytoplasmic staining as well (Fig. 15.). We have also performed immunocytochemistry on cultured HaCaT cells that were treated with dithranol. At all concentrations tested (0.1-0.5 μg/ml) the dithranol clearly had a cytostatic effect on the cells, that was apparent by the size of the cell colonies relative to the untreated control cultures. With the monoclonal antibody staining there was a slight indication for enhanced expression of IL-10R, predominantly on the cell membrane in the dithranol treated cultures, and this difference seemed to depend on the concentration (Fig. 16.).

A



B

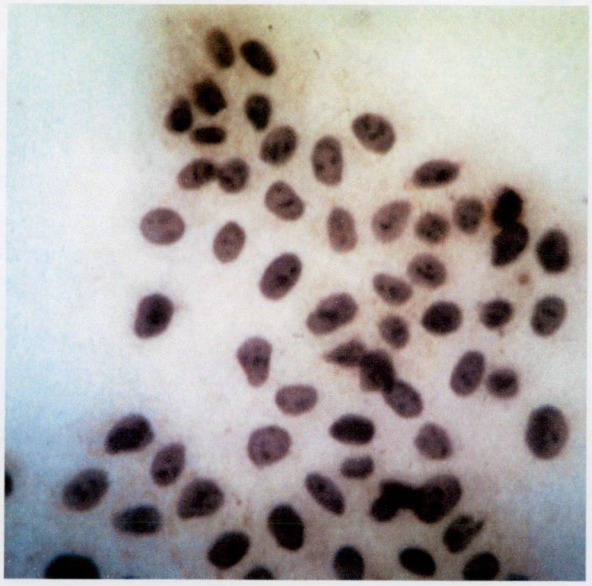
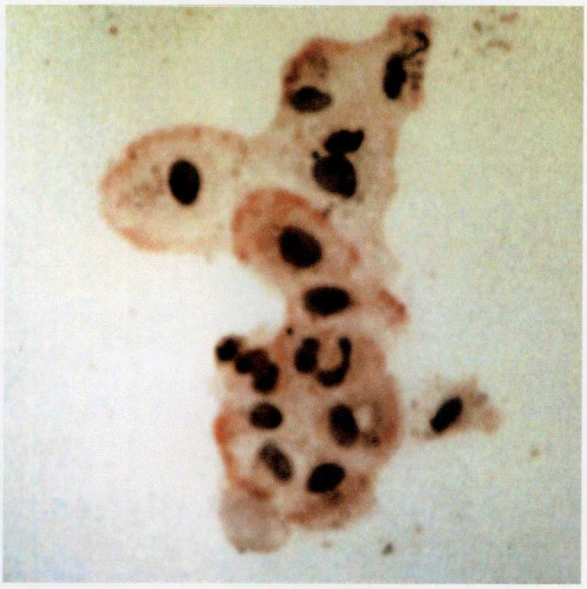


Figure 15. Immunocytochemistry with a monoclonal antibody against human IL-10R on cultured HaCaT cells. (**A**) HaCaT cells with clear membrane localization of the human IL-10 receptor. (**B**) Isotype control.

A



B

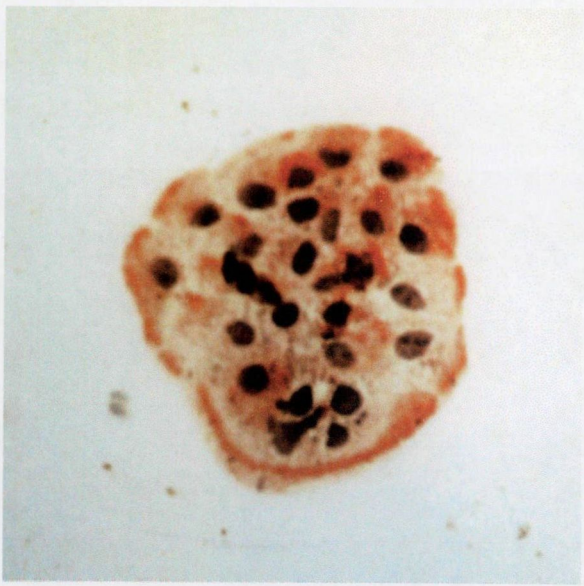


Figure 16. Immunocytochemistry with a monoclonal antibody against human IL-10R on cultured HaCaT cells that were treated with dithranol. (**A**) 0,1 m g/ml dithranol treated HaCaT cells. (**B**) 0,25 m g/ml dithranol treated HaCaT cells.

5 DISCUSSION

High purity isolation of living CTB cells represents an important step toward understanding the process of human embryo implantation since nidation is considerably morphologically different in humans than in experimental or domestic animals [157]. These morphologic variations presumably rely upon fundamental differences at the cellular and molecular levels [39]. For ethical reasons, *in vivo* human experimentation to study the steps of implantation is not feasible. However, elucidation of the mechanisms of human implantation is gaining strong importance, given the recent advances in assisted reproductive technologies and the search for new methods of contraception. In addition, very little is known about factors controling trophoblast proliferation, differentiation, and invasion, which explains the increasing attention recently paid by the scientific community to the study of cell biological and molecular events which govern these processes both *in vivo* and *in vitro*. Without any question, for *in vitro* characterization at the cell and molecular level, highly purified trophoblast cells are needed. To fulfill these needs, several attempts to isolate human trophoblast cells have been reported. In essence, these methods include:

- physical separation methods [82]
- physical and/or immunomagnetic separation methods [47]
- cell attachment procedures [99]
- sequential enzymatic digestion and Percoll gradient centrifugation [54, 82]
- selective culture conditions [97, 159]
- sedimentation methods [48, 81, 82, 116, 139]
- immunological or receptor-binding methods [18, 33, 38, 47, 98, 112, 133, 139, 149]
- selective disaggregation conditions [9, 27]
- immuno-flow cytometric cell sorting methods [21].

The difficulty in obtaining a preparation of pure trophoblast cells for culture can be appreciated by understanding the structure of the placenta. The outer surface of the chorionic villi is covered by the STB, underlying which is a single layer of CTB cells anchored to the villous basement membrane. A microvascular network connects this layer to the umbilical arteries and vein. The apical membrane of the STB is folded into numerous microvilli

forming a brush-border membrane. Disaggregation of this villous tissue results in a broken syncytial membrane, releasing not only CTB cells, but also other cell populations (macrophages, fibroblasts, giant cells, some adhering decidual cells, as well as endothelial cells) and DNA from syncytial nuclei. Separation of CTBs from this heterogeneous cell population has proven to be a challenge.

The present protocol to enrich a reasonable number of highly purified CTB cells from human first trimester placentae depends on the initial crude mechanical dissection of the chorionic villi from adhering decidual tissue fragments and fetal membranes, gentle enzymatic treatment to separate the CTB and STB layers from the mesenchyme, enrichment of CTB cells by Percoll gradient sedimentation and immunomagnetic purification using monoclonal antibodies to CD45 to remove bone marrow-derived cells or to various epitopes of the MHC molecules (HLA-ABC, HLA-DP, DQ, DR) to obtain a pure villous CTB population.

CD45 is a tyrosine phosphatase present on bone marrow-derived cells [35, 138], but absent on CTB cells [92]. Class I and class II MHC molecules are present only on contaminating cells but are absent on trophoblasts. HLA-ABC have been identified on invasive extravillous CTBs [19; for review see ref. 72]. Therefore, the elimination of HLA-ABC expressing cells by immunopurification yields a cell suspension consisting only of undifferentiated (*i.e.* villous) CTBs, whilst the CD45 depleted cells contain both villous and differentiating extravillous CTB populations [19].

The specific type and number of CTB cells isolated by this method is further determined by the age of the placenta and the time of enzymatic digestion.

Immunocytochemical studies of freshly isolated cells indicated that our Percoll-enriched CTBs still contain an appreciable and hardly controllable proportion of contaminating cells. The immunopurification step using either CD45 or HLA-ABC and HLA-DP, DQ, DR specific monoclonal antibodies decreased effectively the proportion of contaminating cells yielding a highly pure CTB population (92-98% CTBs). In addition, neither the immunopurification, nor the storage of cells in liquid nitrogen affected cell viability, which was always above 95%, as assessed by Trypan-blue exclusion.

On the basis of morphological criteria and hCG production, the CD45 depleted cells underwent rapid differentiation when cultured in serum supplemented media. Similar to

previously reported data by Fisher *et al.* [54], the analysis of substrate gels indicated that these cells secreted a single gelatinolytic enzyme with a molecular weight of approximately 90 kD, probably identical with MMP-9. All these data confirmed the trophoblastic origin of our cell preparations.

In human placenta at least two major morphologically and functionally distinct CTB populations can be identified. Villous CTBs are polarized immotile cells anchored to the basement membrane of chorionic villi. They differentiate by fusion to form the overlying syncytium which is in direct contact with maternal blood, mediating nutrient and gas exchange for the developing fetus, and representing the major endocrine component of human placenta. Extravillous, invasive CTB cells rise from CTBs that rest on the basement membrane surrounding the tip of anchoring villi. These cells differentiate by leaving their basement membrane and form non-polarized cellular aggregates (*i.e.* CTB cell columns). They give rise to the invasive CTB population, which invades the endometrium, its arterial system, thus connects the developing fetus to the maternal circulation. No relevant differences were reported between basement membrane-anchored cells giving rise to the extravillous trophoblast and those underlying the villous syncytial trophoblast, therefore it is very likely that both kinds of cells represent a uniform cell population, which can equally differentiate in either of the two major pathways already mentioned [72].

Previous immunohistochemical [4, 20, 41] and functional [29] studies have shown that CTB cells modulate their integrin repertoire during invasion of the endometrium. These authors have demonstrated that villous CTB cells, resting on a laminin rich basement membrane, express the integrin $\alpha_6\beta_4$ (a laminin receptor) in a clustered manner towards the basement membrane. When leaving their basement membrane at the tip of anchoring villi to form cell columns, they continue to express the integrin $\alpha_6\beta_4$, but in an unclustered way. CTBs located deeper in the placental bed have lost their capacity to express the integrin $\alpha_6\beta_4$, and instead express $\alpha_5\beta_1$ integrin, the major fibronectin receptor. Therefore, as trophoblast cells are gradually transformed from polarized villous epithelial layer into non-polarized extravillous population, a down-regulation of $\alpha_6\beta_4$ integrin expression with a reciprocal up-regulation of $\alpha_5\beta_1$ integrin occurs. Our immunohistological findings regarding the placental localization of α_6 and α_5 subunit expressing CTB subpopulations fit these reports perfectly. This gradual switch from the basal lamina receptor $\alpha_6\beta_4$ to interstitial receptors

such as $\alpha_5\beta_1$, was regarded as a mechanism by which invasive CTB cells adapt to their successive environments [15, 16]. These authors have shown, that this integrin switch is paralleled by significant changes in the invasive behaviour of CTBs [19]. If villous STB and deeply invasive extravillous CTB cells are considered terminally differentiated forms of the same α_6 integrin-positive CTB stem cell, the above mentioned integrin switch might equally be an expression of cellular differentiation.

Another important conclusion which derives from these immunohistochemical results is that α_5 and α_6 integrin subunit expression may be useful to further analyze the proportion of undifferentiated villous and differentiating extravillous cells in the isolated CTB population. Our immunocytochemical studies performed on cytospin smears obtained from CD45-depleted CTBs confirmed the presumption that these CTBs are a mixture of villous and extravillous cells.

The presence of IL-10R mRNA in human placental CTB cells has been demonstrated recently [129]. However they have not offered any direct evidence either for the presence of IL-10R protein on these cells *in vitro*, or for placental localization of IL-10R expressing CTBs, *in vivo*. Our flow cytometric analyses clearly show the ability of CTB cells to bind biotin-labelled IL-10. However, freshly isolated cells which had been exposed recently to the proteolytic activity of trypsin during the dissociation of placental villi, as well as cultured cells treated with trypsin to remove them from the culture plates, failed to bind IL-10. Binding was only seen when the cells were incubated overnight either in solution, on agarose or on a rocking platform in order to prevent their attachment to the culture dish. Throughout culture the use of FCS which might promote both attachment and differentiation was avoided. Under these conditions, CTBs remained floating in the medium, but at least some of them formed small aggregates. Based on these observations we can not exclude the possibility that for the recovery of IL-10R CTBs have to make contact with each other. According to our best knowledge, these experiments provide the first clear-cut evidence for IL-10R expression by CTB cells *in vitro*.

Our immunohistochemical studies demonstrate that IL-10R protein is expressed by early human CTBs not only *in vitro*, but *in vivo* as well. The most intense staining for IL-10Rs occurred on the polarized villous CTB cells, and on the very proximal cells, resting on the basal lamina of the villous tip of CTB cell columns. The remaining invasive cells

stained much more weakly if at all, and villous STB did not stain. Our results also show that positive staining for IL-10Rs and that for α_6 integrin subunits largely overlap. In contrast, α_5 integrin expressing cells seem to be devoid of receptors for IL-10. Therefore recent findings [129] could be interpreted as a mechanism by which basement membrane anchored α_6 integrin expressing CTB cells, including villous CTBs, suppress their own potentially invasive behaviour. It has not been clearly elucidated whether villous CTB cells produce gelatinases or possess gelatinase coding mRNA *in vivo*, but logically they have no reason to be invasive. The up-regulation of MMP-9 production by CTBs has been reported to occur after a 12 h culture period, at the time when IL-10 production of these cells is ceased. [129, 130]. Since IL-10 is a potent inhibitor of trophoblastic MMP-9 synthesis *in vitro*, one could speculate, that the gelatinase secreting activity of CTB cells starts later, when the cells escape from the suppressive effect of endogenous IL-10. Recent findings [129] provide the first clear-cut evidence for the molecular mechanism which might be responsible for this sequence of events *in vitro*, whereas our results identify the target cell population for this IL-10-mediated autoregulatory mechanism *in vivo*.

It was interesting to note that the expression pattern of IL-10R in early human placenta mirrors the proliferation marker Ki-67 expression reported previously [114]. Therefore it was tempting to speculate that IL-10 might act as an autocrine factor in regulating placental growth either by promoting or by inhibiting cellular proliferation within the human trophoblast lineages. Reports providing evidence for either the proliferation-promoting or the antiproliferative effect of this cytokine in other cell types were likely to support this hypothesis [65, 105, 137, 154, 161]. Taken together these studies suggest that the IL-10-dependent regulation of cellular proliferation might be cell-type-specific. However, signals involved by the IL-10/IL-10R system in the regulation of cell cycle progression that might give a comprehensive explanation for these presumably cell-type-specific differences are poorly understood. Since it was rather difficult to predict exactly how IL-10 would affect trophoblast proliferation we set out to examine the possible effect of IL-10 on CTB proliferation. The balance between proliferation and differentiation ultimately determines the structure and function of the developing placenta and is related to its "pseudomalignant" properties, thus the functional characterization of the IL-10/IL-10R system of undifferentiated CTBs as a possible autocrin/paracrin proliferation-regulating

factor was of particular interest for us. In accordance with previously reported data [91], our preliminary experiments indicated that CTB cells do not proliferate *in vitro* (data not shown), suggesting that these cells are not suited to study CTB cell proliferation at the feto-maternal interface. These findings indicate that either the process of tissue dissociation, when obtaining isolated trophoblast cells, does impair the cell or the loss of the mesodermal core of the placental villi as a source of growth factors is responsible for the inability of isolated CTB cells to proliferate *ex vivo* [8]. Thus, the only possibility which remained was to investigate CTB proliferation *in situ*, using short term cultures of chorionic villi. The localization of IL-10Rs on the latero-apical surface of proliferating CTB stem cells and previously reported works conducted on similar experimental models [100] made this experimental setting likely to be successful. As indicated by our immunohistochemical and immunoblotting results, IL-10 effectively blocked the proliferation of placental CTBs. The reversibility of this effect by the addition to the serum-free culture media of an IL-10R specific function-blocking antibody indicates that the proliferation-inhibiting effect of IL-10 was mediated through cell-surface IL-10Rs. Ki-67 positivity of CTB nuclei in control cultures confirmed that the viability of our villous explants was not altered by the culture conditions. This is the first study showing that IL-10 is a potent inhibitor of CTB proliferation.

The profound down-regulation of the IL-10R as soon as α_5 integrin expression is turned on might suggest that IL-10 could also affect the production of the integrin family adhesion molecules which, in turn, impacts both CTB proliferation and invasiveness. Another possibility is that IL-10 might play a role in CTB differentiation. However, the possible involvement of IL-10 in these processes would have interesting implications from the perspective of the pregnancy-specific disorder preeclampsia, in which enhanced trophoblast proliferation, decreased invasiveness and the inadequate regulation of adhesion molecules have been reported [93, 163, 164].

Near term and during parturition the effects of IL-10 are likely to be profoundly modulated by the pro-inflammatory mediators released in increasing concentrations by gestational tissues. A recent report [45] demonstrates that the basal production of MCP-1, IL-8 and RANTES by explants of fetal membranes, decidua and placenta at term greatly exceeds that of IL-10. Furthermore, PGE2, which might rise near term has been able to further augment this basal placental production of MCP-1 and IL-8 thus promoting a local

inflammatory reaction and favouring parturition. PGE2 has also been shown to stimulate release of IL-10 by perfused placental cotyledons thereby probably enhancing local immunosuppression and protecting the fetal allograft during the 'high-risk' inflammatory process of parturition. Although the pattern of IL-10R expression by various gestational tissues in the late pregnancy is not yet elucidated it seems logical to suppose that, as in keratinocytes, the overproduced IL-8 would suppress the expression of IL-10Rs by placental CTBs making them unresponsive to IL-10 [105]. One of the multiple consequences this unresponsiveness might have is the possible elevation of MMP-9 production, which might contribute to the remodelling of the uterus and placenta in parallel with the accelerated growth of the fetus in the second half of pregnancy. A role favouring the separation of the placenta from the uterine wall after delivery is also possible.

Therefore IL-10 appears to serve important functions throughout pregnancy as well as during parturition. Beside its role in placental immune protection of the fetal allograft and in the regulation of CTB invasiveness IL-10 seems to be involved in basic reproductive events such as placental growth, CTB differentiation and tissue remodelling.

The overall conclusion of our immunohistochemical and cytological studies is that first trimester CTB cells express IL-10Rs both *in vivo* and *in vitro*. IL-10R expression is localized mainly to the α_6 integrin subunit-expressing CTB cells resting on the basal lamina of both floating and anchoring villi, whereas differentiated trophoblasts (*i.e.* villous STB and α_5 integrin expressing invasive cells) seem to be devoid of receptors. On the basis of our results we propose that one major autocrine role of IL-10 *in vivo* is to suppress CTB proliferation, thereby interfering with mechanisms of placental growth control during early pregnancy. Another interesting possibility is that IL-10 could suppress the invasive potential of basement membrane-anchored CTB cells thereby preserving their uninvasive state and/or might be an autocrin regulator of CTB differentiation.

In psoriatic lesional skin the genes for proinflammatory factors as the IL8/IL-8R system are overexpressed [107]. IL-8 is chemotactic for neutrophils [103], T lymphocytes [87], basophils [90] and keratinocytes [108], it stimulates neutrophil degradation and oxidative burst activity [148]. It promotes keratinocyte proliferation [150] and induces HLA-DR expression in keratinocytes [79]. The effects of IL-8 on keratinocytes are mediated

through specific receptors, which may be upregulated by IL-1 and TNF-α [74, 75]. In lesional psoriatic epidermis IL-8R specific mRNA level was found to be 10-fold higher compared with the uninvolved skin [23]. Earlier results showed that several antipsoriatic compounds as cyclosporine, calcitriol, calcipotriol and dithranol have inhibitory effects on IL-8 binding to cultured keratinocytes [77, 78, 107]. The IL8/IL8R system can be downregulated when epidermal cells are treated with the antipsoriatic drug tacrolimus (FK-506) *in vitro* [106, 136].

IL-10 has gained increasing importance in psoriasis as a negative modulator of inflammatory processes [7]. IL-10 was firstly described in Th2 cells and characterized by its ability to inhibit the typical cytokine response of Th1 lymphocytes [52]. Besides Th2 cells monocytes/macrophages, B cells, eosinophils and mast cells are able to produce IL-10 [43, 95, 115, 140]. In human skin macrophages are the main source of IL-10 [71]. The IL-10 synthesis of keratinocytes is controversially discussed in the literature [62, 67, 118, 147]. IL-10 suppresses proinflammatory cytokine production and antigen-presenting capacity of monocytes/macrophages [43, 44, 53], dendritic cells [109], Langerhans cells [12, 49] and keratinocytes [10]. Earlier results demonstrated that in cultured keratinocytes IFN-γ induced HLA-DR expression is inhibited by IL-10 and it has a moderate but statistically significant and dose-dependent inhibitory effect on the keratinocyte growth rate [105].

The effect of IL-10 is mediated through IL-10R, which shares structural homologies with the receptor for IFN-γ [65]. Previous studies showed that human keratinocytes express IL-10R and this expression is decreased in psoriatic epidermis [105].

Although HaCaT cells are frequently used in experiments investigating the effect on keratinocyte physiology of various drugs currently applied in the treatment of psoriatic patients as well as in studies concerning keratinocyte proliferation and differentiation [56, 88, 121, 122], the expression of IL-10Rs on these cells has not yet been demonstrated. Our immunocytochemical studies performed on subconfluent HaCaT cell cultures provide the first clear-cut evidence for the expression of IL-10R by these cells *in vitro*. Therefore, HaCaT cells are a good experimental model for the study of IL-10 induced effects on keratinocyte physiology.

Dithranol is a highly effective antipsoriatic compound. Its mode of action is still not completely understood. Dithranol inhibits polymorphonuclear leukocyte function [135] and

modulates the arachidonic acid metabolism [11, 134]. It has a direct inhibitory effect on keratinocyte proliferation [126]. It is also capable of modulating cell-surface receptors of epidermal cells [73, 76].

Our immunocytochemical data also suggested that IL-10R expression is upregulated by HaCaT cells upon treatment with dithranol. The definitive evidence demonstrating that this effect of the antipsoriatic compound dithranol was due to the induction of the IL-10R gene was provided by the investigation of IL-10R expression at mRNA level using RT-PCR (these results, however, are not the subject of the present thesis). Thus, it can be concluded that IL-10R expression of HaCaT keratinocytes can be pharmacologically modulated. Since the IL-10R gene is downregulated in psoriatic epidermis [105] the pharmacological modulation of the receptor in our model system may be an important target in the future for the therapy of psoriasis.

6 CONCLUSIONS AND PERSPECTIVES

6.1. Developments in methods of isolation and culture of human placental trophoblast cells have opened a new era in the study of placental function and of the role of trophoblast both in the normal physiology and the pathology of human reproduction. As a result of these developments, rapid advances are being made in the area of trophoblast-endometrial interactions in implantation, reproductive immunology, placental endocrinology, metabolism, and pathology, trophoblast function as well as basic mechanisms of cell differentiation. This work reports the successful isolation and culture of highly purified CTB cells from normal first trimester placentae. The isolation of purified living placental CTB cells should allow further characterization of their structural, morphologic, and functional differentiation at the cellular and molecular level. The presented method of CTB isolation should facilitate ongoing *in vitro* studies of trophoblast adhesion, differentiation, migration, proteolytic activity, and invasion, and clarify how these events differ from those of malignant cells. Moreover, the study of adhesive interactions between trophoblast and decidual cells and their role in embryo implantation and development of the placenta would be a step toward outlining the events leading to the successful establishment and

maintenance of pregnancy. The elucidation of these processes may facilitate the further development of assisted reproductive techniques as well as of new contraceptive strategies. Furthermore, advances in understanding the process of implantation and placental development will also lend insights into clinically important trophoblast-related disorders of both first trimester pregnancy such as embryonic mortality, spontaneous abortion, abnormal placentation or gestational trophoblastic disease, and late pregnancy complications such as preeclampsia and/or intrauterine growth restriction, whose striking histopathologic feature is the abnormally shallow trophoblast invasion of the uterine wall, leading to placental maldevelopment.

6.2. The elucidation of the role of cytokines and growth factors in the regulation of implantation and placentation has received considerable attention in recent years because placenta has proven to be a rich source of cytokines and growth factors. Despite considerable efforts have been made to elucidate the role of different cytokines in the regulation of critical reproductive events like implantation and placentation, the complex autocrine, paracrine and juxtacrine regulatory mechanisms involved are still far from being completely understood, because it is still not clear which cell type will respond to which cytokine. Both animal and human studies pointed to the importance of the Th2 cytokine IL-10 in the establishment and maintenance of pregnancy. As the necessary initial step in the investigation of cytokine function is to define the potential responsive targets by defining the cell populations which express relevant cytokine receptors, we have demonstrated that in early human placenta IL-10Rs are expressed only by basement membrane anchored, undifferentiated CTB cells. The down-regulation of IL-10R expression which parallels the differentiation of these cells, as indicated by the loss of the receptor from differentiating cells, might indicate that, at least during early pregnancy, IL-10 might be important in the regulation of CTB differentiation. IL-10Rs were expressed not only *in vivo,* but also *in vitro,* which makes isolated CTBs a useful model to study the role of IL-10 in trophoblast biology.

6.3. The highly regulated balance between trophoblast proliferation and differentiation determines the 'pseudomalignant' characteristic of the human placenta. The presence

of IL-10Rs on proliferating CTBs raised the possibility, that this cytokine might interfere with placental growth control through the autocrine regulation of CTB proliferation. Thus the functional analysis of IL-10Rs expressed by placental CTBs aimed at elucidating this question. We could clearly demonstrate that IL-10 is a potent inhibitor of CTB proliferation. We consider that this finding is of particular importance especially from the perspective of the pregnancy disorder preeclampsia which is characterized both by a disregulation of trophoblast proliferation and a hyperproliferation of CTB cells associated with an abnormally shallow uterine invasion as a result of a seemingly defective trophoblast differentiation. Since the mechanisms of cell cycle control which are triggered by IL-10 seem to be cell-type-specific, the elucidation of these mechanisms in the particular case of CTBs is important not only from a general cell biological point of view, but also from the perspective of tumor biology.

6.4. Whilst the trophoblast layer of chorionic villi is the epithelium which during intrauterine life both connects to and protects the developing conceptus from its *milieu exterieur* represented at that time by maternal blood, and the mother's immune system, the epidermis, the superficial epithelial layer of the skin, has a somewhat similar role after birth. Recently, the role of cytokines in keratinocyte biology has received much attention. The identification of IL-10Rs on HaCaT cells opens the possibility to use this cell line as a model system for experiments designed to elucidate the role of IL-10 in keratinocyte physiology.

6.5. Demonstrating that Dithranol upregulates IL-10R expression on HaCaT keratinocytes is an important step toward the elucidation of the mechanism by which this potent antipsoriatic compound exerts its therapeutic effect. At the same time the pharmacological modulation of relevant cytokine receptors in our model system may be an important target in the future for the therapy of psoriasis.

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9 ANNEX