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ISOLATION AND CHARACTERIZATION OF HUMAN CYTOTROPHOBLAST CELLS FROM FIRST TRIMESTER PLACENTA

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1 INTRODUCTION

Embryo implantation and haemochorial placentation in humans are complex processes involving trophoblast interaction with the endometrial stroma and the vasculature (Aplin, 1996). They represent a biological paradox which cannot be explained easily with our present knowledge of cell biology (Denker, 1993). They involve formation of trophoblast, motility and proliferation states, changes in adhesive properties and differentiation processes (Aplin, 1991).

The objective of placentation in mammals with the haemochorial type of placenta is to bring fetal and maternal circulations into close proximity to each other. Placental development starts with the process of implantation, which involves a series of events (Schlafke and Enders, 1975). The first stage involves the establishment of position of the blastocyst within the uterus, or attachment. This includes appositional and adhesional events in which the blastocyst first "finds" its implantation site and then anchors itself to the apical surface of the epithelium. The human blastocyst usually attaches via its embryonic pole to the posterior wall of the uterus on the sixth day after fertilization (reviewed in Moore, 1988). In the following step, the blastocyst penetrates and displaces the uterine epithelium. After this stage has been accomplished, the trophoblast pauses at the residual basal lamina of the displaced uterine luminal epithelium before progressing into the endometrial stroma (Schlafke and Enders, 1975). After penetration of the basal lamina, the trophoblast cells invade the uterine stroma and finally breach the wall of maternal blood vessels and uterine glands (Figure 1).

The trophoblast represents extraembryonic fetal tissue derived from the trophectoderm of the blastocyst, which in turn is the earliest epithelium. Trophoblast can be found forming a variety of different structures, e.g., placental villi and fetal membranes (Benirschke and Kaufmann, 1995).
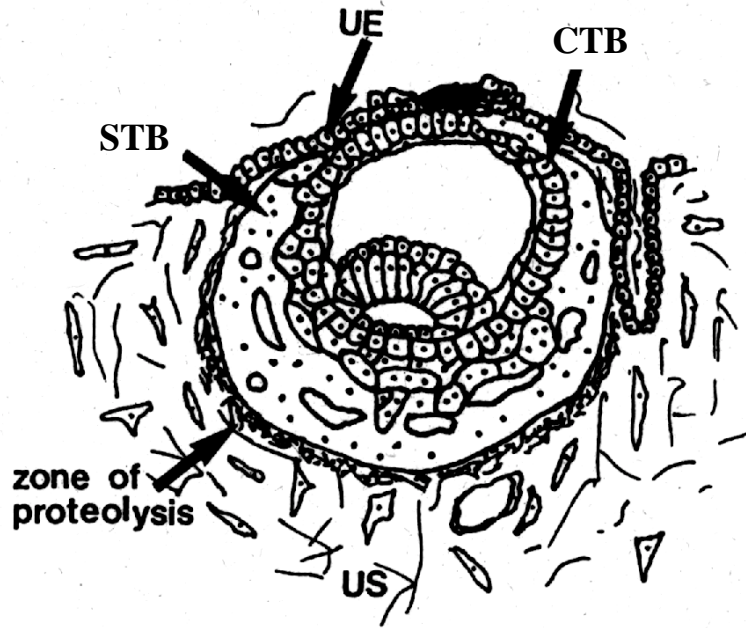
Figure 1 Schematic representation of a human implantation site at approximately nine days after conception (adapted from Aplin, 1991). The outer layer of the embryo consists of STB. Adjacent to the outer STB surface, at its interface with the maternal uterine stroma, is a zone of tissue degradation. Beneath the syncytium is the CTB layer.

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

As implantation in the human proceeds (Figure 2), the trophoblast cells surrounding the blastocyst differentiate into a peripheral layer of syncytiotrophoblast and an inner layer of cytotrophoblast (Hertig and Rock, 1945). Cells from the cytotrophoblast contribute to the syncytial mass by proliferation and fusion. Trabeculae of proliferating cytotrophoblast cells between fluid-filled and later maternal blood-filled spaces or lacunae form the primary villi, which become secondary villi after they are penetrated by fetally derived extraembryonic mesoderm. These are transformed into tertiary villi after angiogenesis has taken place within the mesodermal core of the villi (Figure 2).
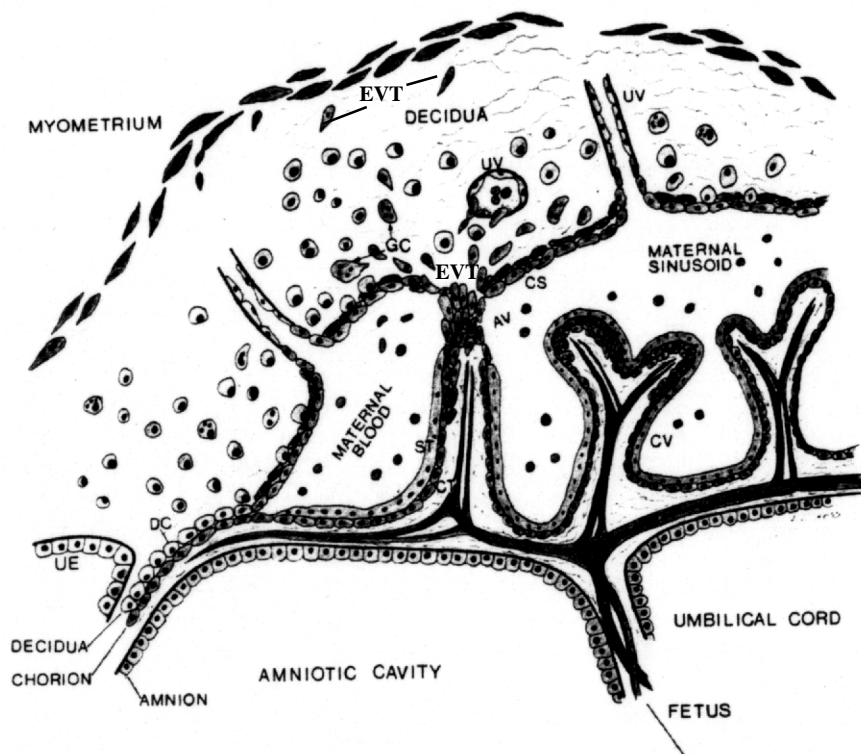
Figure 2 Schematic diagram of the placenta at the end of first trimester showing various trophoblast subpopulations (adapted from Lala and Hamilton, 1996). Anchoring villi serve to maintain the attachment of the placenta to the uterine wall. Extravillous trophoblast cells migrate out of the tips of anchoring 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

Three main trophoblast populations are present during placentation: cytotrophoblast stem cells and two differentiated derivative cell types - the syncytiotrophoblast and the extravillous cytotrophoblast (Vicovac and Aplin, 1996). The undifferentiated trophoblastic stem cell of the placenta, the cytotrophoblast cell, is the first fetal cell type arising during embryogenesis. In primates, it undergoes multistep differentiation to form villous (noninvasive) and extravillous (invasive) trophoblast cell populations (Yeh and Kurman, 1989; Zdravkovic et al., 1999) (Figure 3).

In the first half of pregnancy most of the invasive processes of the trophoblast are performed by the extravillous trophoblast, whereas the villous trophoblast does not show invasive behavior, being mainly involved in the maternofetal transfer mechanisms and synthetic processes (Benirschke and Kaufmann, 1995).

The villous tree undergoes stromal alterations and remodeling leading to the formation of different types of villi throughout pregnancy (Castellucci et al., 1990; Benirschke and Kaufmann, 1995). The mature villi (Figure 4) consist of an outer layer syncytium, an inner layer of cytotrophoblast (villous trophoblast) surrounding a fetal stroma (Boyd and Hamilton, 1970).

In the first trimester, during the early stage of placentation, cytotrophoblast stem cells reside in two different types of chorionic villi: floating and anchoring villi (Boyd and Hamilton, 1970; Benirschke and Kaufmann, 1995).

The floating villi are covered by an inner polarized, highly proliferative, mononuclear cytotrophoblast cell monolayer anchored to a basement membrane, and a superficial multinuclear syncytiotrophoblast cell layer, formed through proliferation and differentiation by fusion of the underlying cytotrophoblast. They are freely floating in the intervillous space, being in direct contact with the maternal blood. They are thus not in contact with the maternal uterine wall. Floating villi play a role in gas and nutrient exchange to the developing embryo.

The anchoring villi are covered by an inner cell layer of primitive mononuclear cytotrophoblast, dividing and fusing with the overlying syncytiotrophoblast layer. They are in contact with the uterine wall (Figure 5).
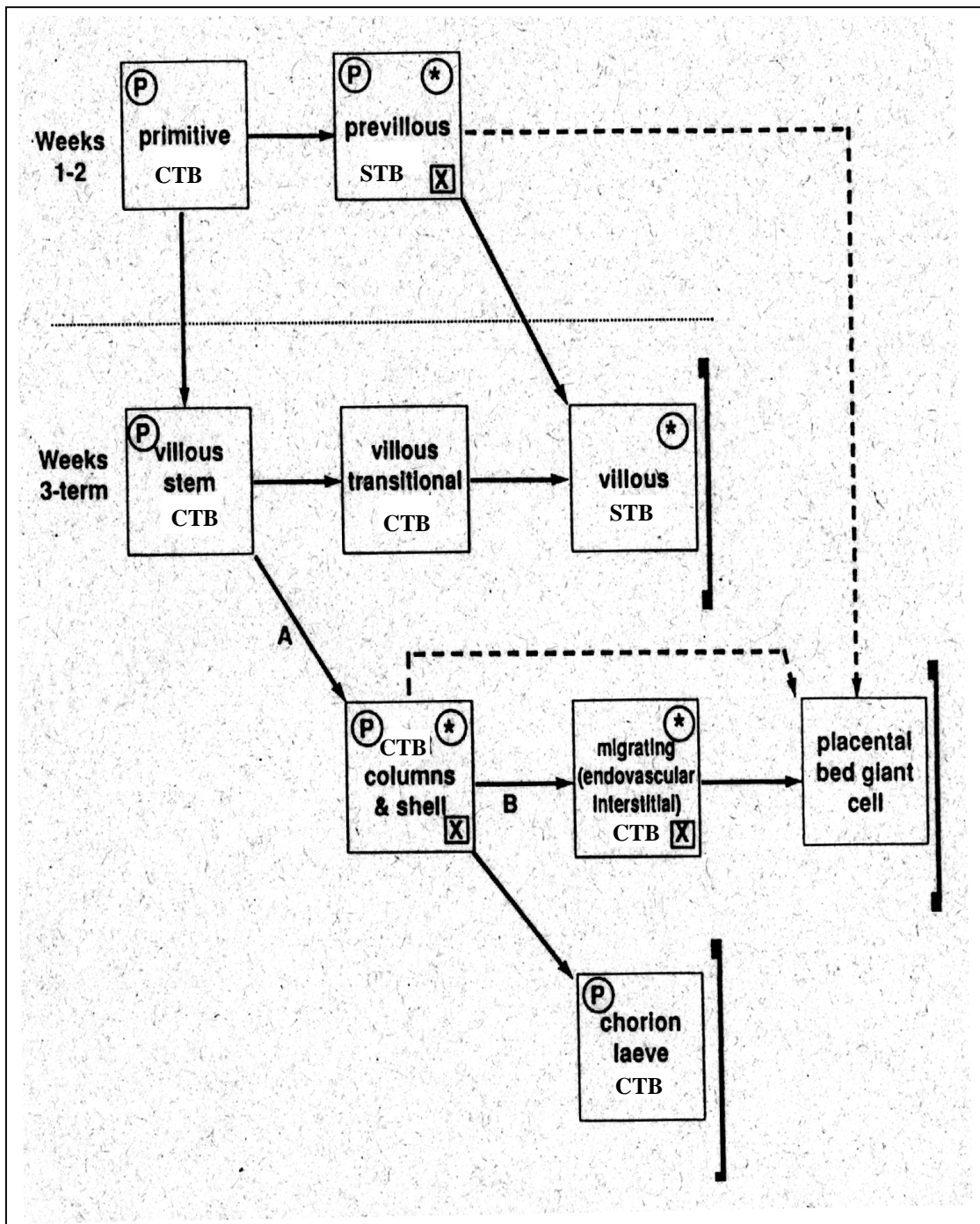
Figure 3 Scheme of human trophoblast differentiation (adapted from Aplin, 1991)

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
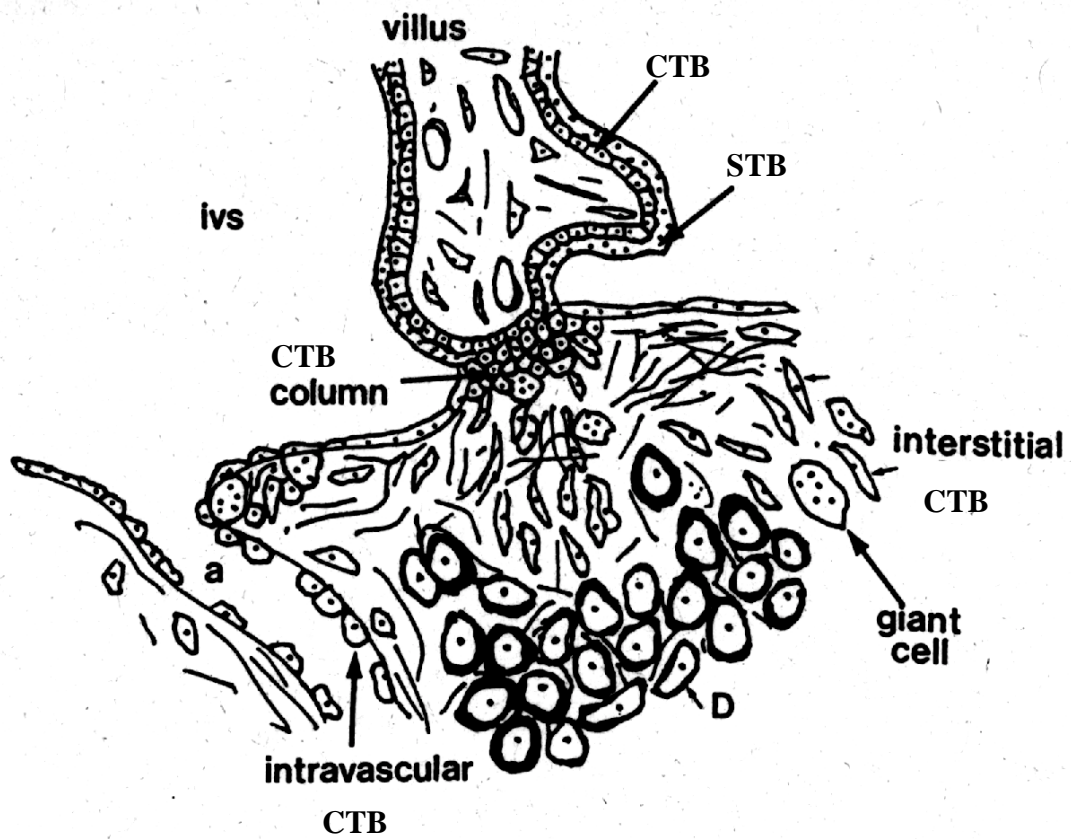
Figure 4 Schematic representation of the maternofetal interface approximately four weeks after fertilisation, showing the tip of a placental anchoring villus and the adjacent placental bed (adapted from Aplin, 1991)

Legend: CTB - cytotrophoblast; D - decidua; ivs - intervillous space; STB - syncytiotrophoblast
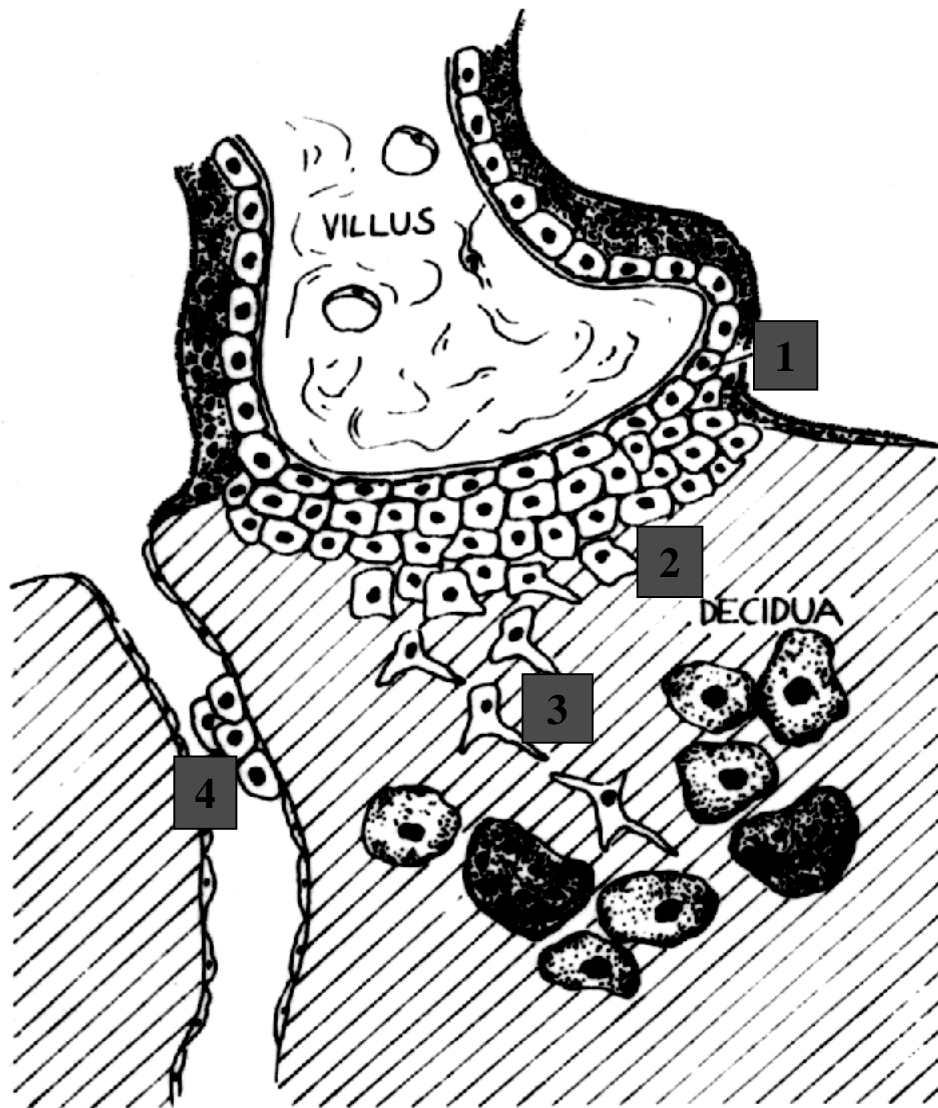
Figure 5 The anchoring villus model of the human placenta (adapted from Denker, 1993)
1   cytotrophoblast cells in contact with the basal membrane (integrin $\alpha6\beta4$, polarized)
2   detaching extravillous cytotrophoblast (intermediate cytotrophoblast; integrin $\alpha6\beta4$, unpolarized)
3   invasive extravillous cytotrophoblast (integrins $\alpha5\beta1$ and $\alpha1\beta1$)
4   extravillous cytotrophoblast within maternal spiral artery (integrin $\alpha5\beta1$)

The cytotrophoblast cells at the tip of the anchoring tertiary villi proliferate during the third post-ovulatory week and penetrate the syncytium, becoming nonpolarized cells, losing their basement membrane, forming the cytotrophoblast multilayered cell columns and making contact with the underlying maternal decidual tissue (Figure 6); this cytotrophoblast is termed the extravillous or intermediate cytotrophoblastic shell or lineage

(Kurman et al., 1984; Yeh and Kurman, 1989), a highly migratory and invasive trophoblast population. Extravillous trophoblast populations include:

- the cytotrophoblastic shell;
- the residual trophoblastic elements within the chorion laeve;
- the endovascular trophoblast (Pijnenborg, 1994; Benirschke and Kaufmann, 1995), invading the inner walls of the maternal spiral arteries up to their myometrial segments, by replacing the endothelial and smooth muscle cells (Boyd and Hamilton, 1967; Aplin, 1991; Foidart et al., 1992; Kaufmann and Castelluci, 1997);
- the interstitial mononuclear and multinucleated trophoblast cells, which penetrate the maternal decidual interstitium (decidual trophoblast) and the inner third of the myometrium wall (interstitial trophoblast) (Loke, 1990; Aplin, 1991);
- large, multinucleated trophoblast cells in the extravillous locations (placental bed) are designated placental bed giant cells (Loke, 1990; Graham et al., 1992); they are presumably noninvasive and remain essentially nonproductive with respect to hormones (hCG, hPL) (Loke, 1990).

The controled invasion of the extravillous trophoblast into the endometrium is an essential part of embryo implantation and resembles the invasion of malignant tumor cells (Lala and Graham, 1990; Librach et al., 1991; Bischof and Campana, 1996; Khoo et al., 1998). However, unlike tumor invasion, trophoblast invasion is precisely developmentally regulated (Lala and Graham, 1990; Graham and Lala, 1991), 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) (Fisher et al., 1989; Aplin, 1991). The mechanism regulating trophoblast invasion, proliferation and differentiation, leading to the successful establishment of pregnancy, are still not well understood.
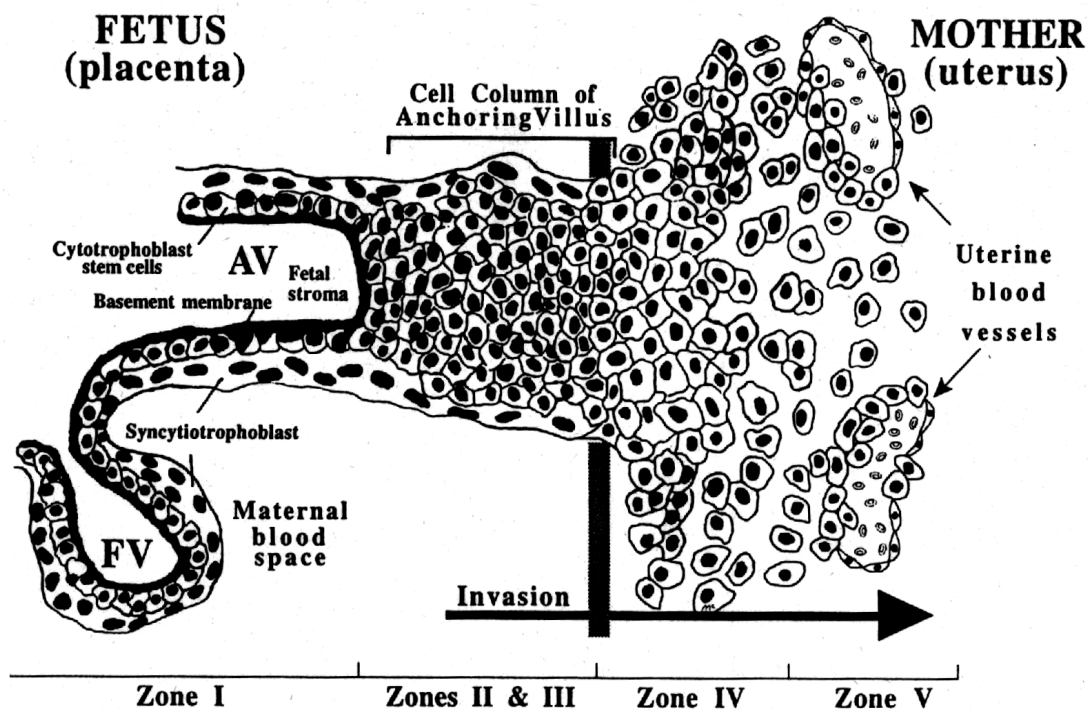
Figure 6 Diagram of a longitudinal section of an anchoring chorionic villus at the fetal-maternal interface at approximately 10 weeks gestational age (adapted from Zhou et al., 1997). The anchoring villus functions as a bridge between the fetal and maternal compartments, whereas floating villi are suspended 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

For the isolation of cytotrophoblast cells from human placenta, various methods using different techniques have been reported (reviewed in Bloxam et al., 1997). Criteria for a successful model for the study of implantation include substantially pure trophoblast cells from placental villous tissue. All current methods of isolating CTB, the precursor of STB, derive from the original tissue trypsinization method of Thiede (1960).

All the reported methods only resulted in an enriched but rather heterogeneous trophoblast cell population. Contaminating cells include placental macrophages (Hofbauer cells), lymphocytes, monocytes, granulocytes, and other blood elements, endothelial cells, fibroblasts, giant cells, and decidual cells. Therefore, reported cytotrophoblast cell purity

varies from 40 to 95 %, depending on the isolation procedure applied (Douglas and King, 1989).

The objective of this thesis is to report on a reproducible method for simultaneous isolation of human villous and extravillous cytotrophoblast cells from first trimester placenta, applying mechanical and enzymatic dissociation, Percoll gradient centrifugation, and immunomagnetic separation. The availability of highly purified, competent cytotrophoblast cells is greatly increasing the possibility of studying placenta development and the factors controling trophoblast cell invasion into the uterine wall.

2 MATERIALS AND METHODS

2.1   Chemicals

BSA fraction V: Serva, Boehringer Ingelheim Bioproducts, Heidelberg, Germany

Collagenase type IV: Sigma, Munich, Germany

Cytospin slides: Shandon, Pittsburgh, USA

Density marker beads: Pharmacia Biotech, Uppsala, Sweden

DMEM H-21: Gibco Life Technology, Paisley, UK

DNAse I type IV: Sigma, Munich, Germany

Dynabeads M-450 CD45: Dynal, Oslo, Norway

EDTA: Sigma, Munich, Germany

Ethanol: Merck, Darmstadt, Germany

FCS: Gibco Life Technology, Paisley, UK

Fix & Perm Cell Permeabilization Kit: Caltag Laboratories, San Francisco, USA

Gentamycin: Seromed, Berlin, Germany

HBSS: Gibco Life Technology, Paisley, UK

Hyaluronidase type I-S: Sigma, Munich, Germany

Percoll: Pharmacia Biotech AB, Uppsala, Sweden

PFA: Serva, Boehringer Ingelheim Bioproducts, Heidelberg, Germany

Propidium iodide: Sigma, Munich, Germany

Sodium azide (NaN$_3$): Merck, Darmstadt, Germany

Trypsin type XIII: Sigma, Munich, Germany

2.2   Isolation of human cytotrophoblast cells

2.2.1   Materials

Phosphate - buffered saline (PBS)

81.8 g NaCl

13.8 g $NaH_2PO_4 \bullet H_2O$

Filling up with bidistilled water to one liter (stock solution)

Dilution 1:10 with bidistilled water (use solution)

Setting to pH 7.3 with 2N NaOH

(Reagents from Merck, Darmstadt, Germany)

Erythrocyte lysis buffer

8.29 g NH_4Cl

1 g Na_2CO_3

0.038 g EDTA (Titriplex III)

Filling up with bidistilled water to one liter

Setting to pH 7.2 with 2N NaOH

(Reagents from Merck, Darmstadt, Germany)

Discontinuous Percoll gradient

The six layer Percoll gradient (22 – 78 %) was prepared in Hanks' balanced salt solution (HBSS, pH 7.4) as follows:

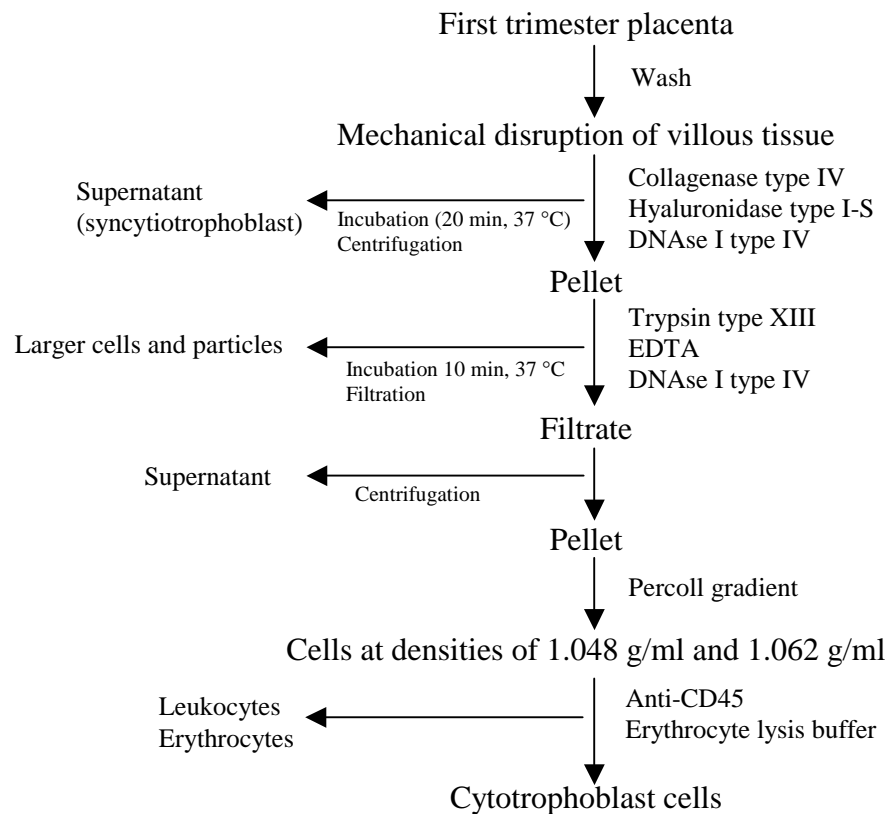| Layer	Percoll 90 % [Percoll:HBSS-H2, 10:1 (v/v)]	HBSS-H 1
1	3107 ml	893 ml
2	2667 ml	1333 ml
3	2227 ml	1773 ml
4	1773 ml	2227 ml
5	1333 ml	2667 ml
6	893 ml	3107 ml

(Reagents: Percoll from Pharmacia Biotech AB, Uppsala, Sweden; HBSS from Gibco Life Technology, Paisley, UK)

2.2.2 Protocol

First trimester trophoblast subpopulations were isolated from placentas (6 to 14 weeks of gestation, n = 15) obtained immediately after the legal termination of pregnancy (uterine currettage or vacuum aspiration). Written informed consent was obtained from the patients prior to the operation. The study project was approved by the Ethical Committee of the Technische Universität München, Klinikum rechts der Isar. Gestational ages were assessed by ultrasound and confirmed by histology. Placental tissue was immediately transported in ice cold 0.9 % NaCl to the research laboratory. It is important to use the placenta as soon as possible because of the protease-rich nature of the tissue.

Villous tissue was dissected from decidual tissue, amniotic membranes, and blood clots, washed in 50 ml of PBS (pH 7.4), and centrifuged (1,600 rpm, 10 °C, 5 min) until the supernatant was visually free of blood (Figure 7).

```
                        First trimester placenta
                                  │
                                  │ Wash
                                  ▼
                   Mechanical disruption of villous tissue
                                                  Collagenase type IV
  Supernatant          ◄───────────────────┐     Hyaluronidase type I-S
(syncytiotrophoblast)   Incubation (20 min, 37 °C) │  DNAse I type IV
                        Centrifugation         │
                                               ▼
                                            Pellet
                                                  Trypsin type XIII
  Larger cells and particles ◄──────────────┐     EDTA
                        Incubation 10 min, 37 °C │  DNAse I type IV
                        Filtration            │
                                              ▼
                                           Filtrate
  Supernatant          ◄───────────────────┐
                        Centrifugation       │
                                             ▼
                                          Pellet
                                             │
                                             │ Percoll gradient
                                             ▼
           Cells at densities of 1.048 g/ml and 1.062 g/ml
                                                  Anti-CD45
  Leukocytes           ◄───────────────────┐     Erythrocyte lysis buffer
  Erythrocytes                              │
                                            ▼
                              Cytotrophoblast cells
```

Figure 7 Schematic description of the cytotrophoblast isolation protocol

After the washing procedure, villous tissue was rigorously minced with scissors, washed twice by centrifugation (1,600 rpm, 20 °C, 5 min) in DMEM H-21 (pH 7.4) enriched with 10 % FCS and 50 µl/ml gentamycin. After having removed the supernatant, wet weight of the tissue was determined.

The washed villous tissue pellets were incubated for 20 min in a 37 °C shaking water bath in PBS containing 500 U/ml collagenase type IV, 200 U/ml hyaluronidase type I-S, 0.2 mg/ml DNAse I type IV and 0.1 % BSA fraction V.

After incubation, tissue pieces were allowed to settle for 2 min, then the supernatant containing the syncytiotrophoblast cells was removed and the pellet centrifuged (1,600 rpm, 20 °C, 5 min). The supernatant was discarded and the pellet incubated further in a

shaking water bath at 37 °C for 10 min in PBS containing 0.25 % trypsin type XIII, 2 mM EDTA, and 0.2 mg/ml DNAse I type IV.

After incubation, the suspension of villous cores and dissociated cells was diluted with an equal volume of DMEM H-21 containing 10 % FCS to inhibit trypsin activity. The suspension was filtered through 50 μm gaze in order to remove larger tissue fragments, the filtrate was then centrifuged (1,600 rpm, 20 °C, 5 min) and the supernatant removed. The cell pellet was resuspended in 4 ml of DMEM H-21 containing 10 % FCS.

2×10^5 cells/ml were layered on top of a preformed Percoll gradient as described by Kliman et al. (1986), composed of six layers spanning 22 - 78 % of Percoll in Hanks' balanced salt solution.

The discontinuous gradient was established by centrifugation (25 min, 1,000 x g, room temperature). After centrifugation, three regions are visible (Figure 8):

1. Top region, containing connective tissue elements, small vessels, fibroblasts, and villous fragments.
2. Middle region, corresponding to densities of between 1.048 – 1.062 g/ml, containing trophoblast cells.
3. Bottom region, containing red blood cells and polymorphonuclear leukocytes.
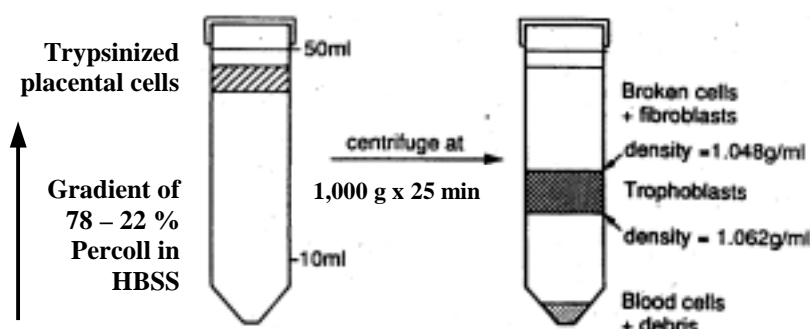
Figure 8 Percoll gradient centrifugation of the placental cell suspension

Cells at a density of 1.048 and 1.062 g/ml corresponding to bands 3 and 4, respectively, containing the cytotrophoblast cells, were precauciously collected using a Pasteur pipette and the cell suspension washed twice with PBS (1,600 rpm, 20 °C, 5 min). To lyse contaminating erythrocytes, the cell suspension was subjected to 5 ml of erythrocyte lysis buffer, centrifuged (1,600 rpm, 20 °C, 5 min) and the supernatant discarded.

96 - 98 % of the cells were alive, as tested by propidium iodide exclusion.

2.3 Cell fixation

2.3.1 Principle

Fixing cells in suspension and permeabilizing them gives antibodies access to intracellular structures and leaves the morphological scatter characteristics of the cells intact. Specific formulation of the reagents reduce background staining and allow simultaneous addition of permeabilization medium and fluorochrome labeled antibodies.

Cell fixation and permeabilization is an established technique designed for use in flow cytometry. They allow intracellular antigen analysis as easy as analysis of surface antigens. The only prerequisit is the availability of suitable antibody conjugates, because some determinants are sensitive to the fixation step.

2.3.2 Materials

Fix & Perm Cell Permeabilization Kit
FIX & PERM reagents are intended for fixing cells in suspension with Reagent A and then permeabilizing the cells with Reagent B:
1. addition of 50 µl of cell suspension in a 5 ml tube;
2. addition of 100 µl of Reagent A (Fixation Medium, stored at room temperature);
3. incubation for 15 minutes at room temperature;
4. addition of 5 ml PBS; centrifugation for 5 min at 300 g; removal of the supernatant;
5. addition of 100 µl of Reagent B (Permeabilization Medium) to cell pellet; vortex at low speed for 1-2 seconds;

6. incubation for 15 minutes at room temperature;

7. washing cells with PBS as described above;

8. removal of the supernatant and resuspension of cells in sheath fluid for immediate analysis or in 1 % PFA; storage of cells at 2-8 °C in the dark.

(Reagent: Caltag Laboratories, San Francisco, USA)

Paraformaldehyde (1 %)

10 g paraformaldehyde are solubilized in 200 ml bidistilled water with 2N NaOH on a warm plate. The solution is then cooled down to 4 °C. 25 ml PBS are added and the solution filled up to 250 ml with bidistilled water. The solution is filtered through filter paper. Freshly prepared solution is needed for each experiment.

(Reagents: PFA from Serva, Boehringer Ingelheim Bioproducts, Heidelberg, Germany)

2.3.3   Protocol

Cell suspensions were suspended in PBS, fixed and permeabilized for flow cytofluorometry by use of the Fix & Perm Cell Permeabilization Kit, according to the manufacturer's instructions. Cells were finally subjected to a second fixation procedure by resuspension in 1 % PFA and stored at 4 °C in the dark. If fixed cells were not analyzed within 24 hours, cell suspensions were resuspended in 0.01 % NaN_3 and stored at 4 °C in the dark.

2.4   Immunocytochemical staining of trophoblast cells

2.4.1   Principle of the hematoxylin – eosin staining technique

The hematoxylin – eosin staining technique is a relatively simple, reliable method using the acidic dye anyline. Eosin stains the cell cytoplasm red, while hematoxylin stains the cell nucleus blue after a washing procedure with distilled water and tap water (causing an alkaline pH change). Raising the pH to the alkaline range determines a permanent staining of the cell nucleus as well, because hematoxylin is not water soluble at neutral or alkaline pH. Destaining is performed using an acidic solution (2 % acetic acid). The sample is then

dehydrated by addition of increasing concentrations of alcohol:water, and finally embedded in paraffin.

2.4.2   Principle of immunocytochemical staining

The alkaline phosphatase anti-alkaline phosphatase (APAAP) method is one of the most sensitive and widely used immunologic – enzymatic staining techniques for light microscopy. The staining sequence is as follows: primary antibody – secondary bridging antibody – soluble APAAP complex – substrate solution. The primary antibody and the APAAP complex belong to the same animal species (e.g., mouse), in order to allow the secondary antibody to bind both.

The bridging antibody must fulfil two requirements:
1. it has to be directed toward immunoglobulins of the same species in which primary antibody and APAAP complex were produced;
2. an excess amount of this antibody has to be added in order to still have a free Fab fragment available for binding the APAAP complex, in case one of the Fab fragments bound the primary antibody.

A soluble enzyme – anti-enzyme immune complex (APAAP) is used, in which two molecules of alkaline phosphatase correspond to a molecule of antibody directed toward this enzyme. During incubation of the alkaline phosphatase substrate, the alkaline phosphatase hydrolyzes the naphtholphosphate-ester, resulting in the production of phenoles and phosphates. Phenoles combine to chromogenous diazonium-salts, yielding the production of soluble azo-dyes. Levamisol contained in the substrate solution blocks the endogenous alkaline phosphatase activity.

2.4.3   Protocol

Cells, at a concentration of 5×10^4 / ml PBS - 1 % BSA, were centrifuged onto single well slides at room temperature (500 rpm, 5 min). Cytospins were air-dried at room temperature and then fixed in 70 % ethanol. Hematoxylin - eosin staining was performed.

Cytospins were analyzed using an inverted microscope (Axiovert, Zeiss, Oberkochen, Germany) equipped with a 40 x objective. Photomicrographs were taken using the attached Contax 167 MT camera (Kyocera Corporation, Japan) with a Kodak Elite II day-light film, ISO 100 / 21° and a grey filter.

Placental tissue as well as minced tissue pieces and cell suspensions collected after first and second enzymatic digestion, and also cells isolated by gradient centrifugation and after immunomagnetic separation, were embedded in fibrin clots for immunocytochemical analysis as previously described by Luther et al. (1997). Cells embedded in fibrin clots were fixed in phosphate-buffered 3.7 % formalin (12 h, room temperature), and embedded in paraffin. Sections were cut (5 μm), deparaffinized, and rehydrated according to standard procedures (Luther et al., 1997). Primary antibodies were added for staining (Table 1).

Table 1 Primary antibodies used in immunocytochemistry (ICC)

Antibody	Concentration ($\mu g/\mu l$)	Source
anti-CK 8 (mAb, mouse IgG$_{2a}$)	0.5	Dako, Hamburg, Germany
anti-CK 18 (mAb, mouse IgG$_{2a}$)	0.5	Dako, Hamburg, Germany
anti-E-cadherin (mAb, mouse Ig)	0.25	Dianova, Hamburg, Germany
anti-β-hCG (pAb, rabbit Ig)	2.5	Dako, Hamburg, Germany
anti-hPL (pAb, rabbit Ig)	2.5	Dako, Hamburg, Germany
anti-CD45 (mAb, mouse IgG$_1$)	0.5	Dako, Hamburg, Germany

After incubation with the primary antibodies (1 h, room temperature), sections were washed three times (5 min each, room temperature) with TBS and processed by adding secondary antibodies (30 min, room temperature) and detection systems:

- for CK 8,18, β-hCG, hPL: Dako ChemMate detection-kit, APAAP, mouse (Dako Diagnostika, Hamburg, Germany);
- for CD45: Dako ChemMate detection-kit, alkaline phosphatase/RED, rabbit/mouse (Dako Diagnostika, Hamburg, Germany) and Vectastain Elite ABC-Kit (Camon, Wiesbaden, Germany);

- for E-cadherin: Vectastain Elite ABC-Kit.

Kit instructions were followed with regard to dilutions of reactants.

Cell nuclei were counterstained with hematoxylin. Positive stains gave a red color. Finally, slides were mounted on microscope slides with glycerol-gelatine and covered. The peroxidase activity was detected with the diaminobenzidine reaction. No antibody was evaluated on fewer than three samples.

Sections not exposed to primary antibodies or in which the primary antibody was replaced by irrelevant mouse immunoglobulin or normal rabbit serum served as control slides. None of the controls exhibited any significant staining.

2.5   Confocal Laser Scanning Microscopy (CLSM)

2.5.1   Principle

Cell-bound fluorescence can be assessed, among other techniques, by CLSM (Knebel et al., 1990; Schmitt et al., 1991). CLSM is a specific technique for the study of cell morphology, using antibodies directed to intracellular or membrane bound cell components. In order to reveal the binding site of the specific primary antibody, a second fluorescence-labeled antibody is added, which will bind to the first antibody. The fluorescent molecule on the second antibody is excited by light with a lower wave length, and subsequently emits light with a higher wave length. Special filters separate absorbance and emission lights, resulting in fluorescence of the labeled cell structures visualized by a fluorescence microscope. This technique is called indirect immunofluorescence technique because only the second antibody is fluorescence-labeled.

Many biological structures are complex and thick, resulting in superposition of the fluorescent components, thus impeding sample visualization in a single layer when using a conventional fluorescence microscope. The same problem is encountered when a three-dimensional sample is analyzed. CLSM allows emission identifying off the focus and is able to perform "optical slices" of a sample. It performs rapid scanning while moving the

cell in the z-axis in order to measure total cellular fluorescence and to minimize photo-bleaching of the fluorescent label (Schmitt et al., 1991).

The principle of CLSM can be explained as follows: The light source is a laser beam, focused by a lens and deviated by a scanning mirror in the microscope objective and the sample, respectively. The scanning mirror is turned in two axes. This arrangement considerably reduces the number of optical components, since no relaying optics for two-scanner mirrors are necessary. The high fluorescence yields a good image to be constructed from one scan. While passing through the sample, the laser beam excites fluorescent molecules to a certain wave length, resulting in a few nanoseconds in the emission of light with a higher wave length in all directions. The objective partially absorbs the emitted light and focuses it toward another mirror and a detector. The mirror is light splicing, i.e. it reflects light in a specific wave length range (e.g., blue light), while it is permissive for other wave lengths (e.g., yellow or green light). The detector in turn converts the light signal into an electrical signal.

The main advantage of CLSM resides in its high selectivity, i.e. only the labeled structures are visualized. High resolution of the technique is achieved by combining a fluorescence image (specific contrast image) with its transmission light image (unspecific phase interference contrast image). CLSM not only provides alternatives to the use of fluorescence-labeled ligands, but also allows real-time single cell analysis of living competent cells (Schmitt et al., 1991).

2.5.2   Protocol

Single cell-associated fluorescence was measured with the confocal laser scanning microscope. CLSM was performed on cells fixed with 1 % PFA in PBS. Cytospins (30,000 cells/slide) were prepared as described above.

Primary antibodies were applied for 30 min, at room temperature (Table 2).

Table 2 Primary antibodies used in confocal laser scanning microscopy (CLSM)

Antibody	Concentration (µg/µl)	Source
anti-CK 8,18 (mAb, mouse IgG$_{2a}$)	0.5	Medac, Hamburg, Germany
anti-β-hCG (pAb, rabbit Ig)	2.5	Dako, Glostrup, Denmark
anti-hPL (pAb, rabbit Ig)	2.5	Dako, Glostrup, Denmark
mouse IgG$_1$ pure	0.1	Caltag, San Francisco, USA
rabbit IgG (pAb)	0.1	Dianova, Hamburg, Germany
rat IgG chrompure	0.25	Dianova, Hamburg, Germany
anti-integrin α1 (pAb, rabbit Ig)	2.5	Boehringer Ingelheim, Heidelberg, Germany
anti-integrin α5 (mAb, mouse IgG$_{2bK}$)	2.5	Southern Biotechnology, Birmingham, USA
anti-integrin α6 (mAb, rat IgG$_{2a}$)	2.5	Boehringer Ingelheim, Heidelberg, Germany
anti-integrin αv (mAb, mouse IgG$_1$)	2.5	Boehringer Ingelheim, Heidelberg, Germany
anti-integrin β1 (mAb, mouse IgG$_1$)	2.5	Biomol, Hamburg, Germany
anti-integrin β3 (mAb, mouse IgG$_{1K}$)	2.5	Southern Biotechnology, Birmingham, USA
anti-integrin β4 (mAb, mouse IgG$_1$)	2.5	Boehringer Ingelheim, Heidelberg, Germany

After incubation, cytospins were washed twice with PBS (room temperature, 5 min each). FITC-conjugated secondary antibodies were added (30 min, room temperature, in the dark). The following antibodies were used:

- FITC-affini pure F(ab')$_2$ fragment rabbit anti-mouse IgG (H + L), (0.063 µg/ml; Dianova, Hamburg, Germany);

- FITC-swine anti-rabbit immunoglobulins (0.063 µg/ml; Dako, Glostrup, Denmark);

- FITC-mouse anti-rat immunoglobulins (1.5 µg/µl; Dianova, Hamburg, Germany).

Slides were washed again, mounted with 50 µl PBS (pH 7.4), a cover slip (18 x 18 mm, Menzel, Germany) added, which was sealed with nail polish, and immediately analyzed by confocal laser scanning microscopy (CLSM). The CLSM consists of an inverted microscope (Axiovert, Zeiss, Oberkochen, Germany) combined with a laser scanning detection unit (Leica, Heidelberg, Germany) equipped with an argon-krypton laser

allowing separate or simultaneous detection of fluorochromes excited at 488 nm and 568 nm. Transmission (differential interference contrast microscopy) and fluorescence images were recorded employing a 63 oil immersion objective.

Washing steps in each case were designed to be sufficient to eliminate background staining. Second antibody controls were performed in each run by using PBS in place of the first antibody and were negative in all cases.

2.6   Flow cytofluorometric analysis

2.6.1   Principle

Laser-based fluorometry has found wide application in cell biology and medicine to investigate structure – function relationships of ligands with their receptors on normal and malignant cells. Cell-bound fluorescence can be quantified, among other techniques, by flow cytofluorometry (Shapiro, 1988).

The principle of flow cytofluorometry resides in the simultaneous assessment of different physical characteristics of single fluorescence-labeled cells or particles, while passing through a directed laser beam (argon laser, wave length 488 nm). Fluorescence intensity and light deviation are measured using different detectors. Light deviation is attributed to differences in physical properties, e.g., cell size, cell shape, cell membrane, nuclear shape, number and type of intracellular components, and type of cell membrane surface. Thus, flow cytofluorometric analysis is a useful technique providing information relative to cell size, granularity, and antigenicity.

The laser beam induces emission of fluorescence by endogenous or exogenous molecules present either intracellularly or bound to the cell membrane. The forward light scatter (FSC) is associated with cell size and cell aggregation. Attenuation of the laser beam subsequent to cell passing is measured – transmission attenuation. The side scatter (SSC) is associated with cell density, cell granularity and cell membrane surface, and it represents the deviated right angle light signal. Fluorescence intensities can be assessed simultaneously for three different fluorescence emission spectra.

Immunologic pattern of cells can be assessed using specific fluorescence-labeled antibodies. Most cells show a certain degree of fluorescence even without being fluorescence-labeled; this characteristic is called autofluorescence, and it is attributed to fluorescent cell components. For this purpose, fluorescence of labeled cell samples is compared to the autofluorescence of unlabeled cells (negative control), above which cells can be considered to be positive for expressing the antigens tested.

Negative controls are considered:
- autofluorescence of unlabeled cells;
- fluorescence of the labeled monoclonal antibody (e.g., FITC);
- fluorescence of the unspecific antibody (e.g., mouse IgG_{2a});
- fluorescence of labeled monoclonal antibody plus unspecific antibody.

Fluorescence values above fluorescence of negative controls are considered positive and specific.

The fluorescent dyes are coupled to the Fc fragment of immunoglobulins. A fluorescent antibody can be directed either toward an antigenic cell structure or an unlabeled primary antibody. The latter method is preferred due to cost effectiveness.

The direction, intensity, and deviation of the emitted light by every single cell are recorded and analyzed by a computer system and represented graphically as histograms or dot plots, which can be eventually interpreted. The five parameters mentioned above (two light scatters and three fluorescence patterns) can be paired sequentially, resulting in a representation called dot plot.

The advantage of flow cytofluorometry compared to the classic fluorescence microscopy resides in the quality of cell analysis: different cell types in a sample are quantified separately, reliable, objective, and very fast (up to 3000 cells/second). However, no correlation to intracellular components is possible.

In the experiments presented following fluorescence intensities were used:

Fl_1 - green; fluorescent dye: FITC, 514 nm;

Fl_2 - red; fluorescent dyes: PE, 578 nm; and propidium iodide, 630 nm.

2.6.2 Protocol

Single cell-associated fluorescence was measured by flow cytofluorometry. 125,000 cells / 125 µl of PBS - 1 % BSA (30 min, room temperature) were reacted with primary antibodies (Table 3).

Table 3 Primary antibodies used in flow cytofluorometry (FACS)

Antibody	Concentration (µg/µl)	Source
anti-CK 8,18 (mAb, mouse IgG$_{2a}$)	0.5	Medac, Hamburg, Germany
anti-β-hCG (pAb, rabbit Ig)	2.5	Dako, Hamburg, Germany
anti-hPL (pAb, rabbit Ig)	2.5	Dako, Hamburg, Germany
anti-CD45 (mAb, mouse IgG$_1$)	0.3	Caltag, San Francisco, USA
anti-CD14 (mAb, mouse IgG$_1$)	0.25	Dako, Glostrup, Denmark
anti-vimentin (mAb, mouse IgG$_1$)	1	Sigma, Munich, Germany
mouse IgG$_1$ pure	0.1	Caltag, San Francisco, USA
rabbit IgG (pAb)	0.1	Dako, Glostrup, Denmark
rat IgG chrompure	0.25	Dianova, Hamburg, Germany
FITC-anti-CK 8,18 (mAb, mouse IgG$_{2a}$)	0.3	Becton Dickinson, Heidelberg, Germany
PE-anti-CD45 (mAb, mouse IgG$_1$)	0.5	Caltag, San Francisco, USA
FITC-mouse IgG$_{2a}$	0.32	Caltag, San Francisco, USA
PE-mouse IgG$_1$	0.02	Caltag, San Francisco, USA
anti-integrin α1 (pAb, rabbit Ig)	2.5	Boehringer Ingelheim, Heidelberg, Germany
anti-integrin α5 (mAb, mouse IgG$_{2bK}$)	2.5	Southern Biotechnology, Birmingham, USA
anti-integrin α6 (mAb, rat IgG$_{2a}$)	2.5	Boehringer Ingelheim, Heidelberg, Germany
anti-integrin αv (mAb, mouse IgG$_1$)	2.5	Boehringer Ingelheim, Heidelberg, Germany
anti-integrin β1 (mAb, mouse IgG$_1$)	2.5	Biomol, Hamburg, Germany
anti-integrin β3 (mAb, mouse IgG$_{1K}$)	2.5	Southern Biotechnology, Birmingham, USA
anti-integrin β4 (mAb, mouse IgG$_1$)	2.5	Boehringer Ingelheim, Heidelberg, Germany

In order to determine the appropriate antibody concentrations, preliminary antibody dilution experiments were performed and analyzed by flow cytofluorometry.

Cell suspensions were washed twice with PBS - 1 % BSA and, where applicable, the respective conjugated secondary antibody added (30 min, room temperature, in the dark).

The following FITC- or PE-conjugated antibodies were used:

- FITC-affini pure F(ab')$_2$ fragment rabbit anti-mouse IgG (H + L) (0.125 µg/ml; Dianova, Hamburg, Germany);
- FITC-swine anti-rabbit immunoglobulins (0.125 µg/ml; Dako, Glostrup, Denmark);
- FITC-mouse anti-rat immunoglobulins (1.5 µg/µl; Dianova, Hamburg, Germany);
- PE-mouse anti-human CD45 (0.25 µg/ml; Caltag Laboratories, San Francisco, USA).

Cell suspensions were washed with PBS - 1 % BSA, resuspended in 125 µl PBS - 0.1 % BSA and immediately analyzed by flow cytofluorometry using the FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with a 488 nm argon laser. Data were collected and analyzed by the Cell Quest Program (Becton Dickinson, Heidelberg, Germany). Cells were visualized by analyzing their light scatter properties (forward versus side scatter signal) and fluorescence signals. Twenty thousand cells were analyzed for each sample. "Negative" controls were used to set the detectors and marker settings on fluorescence histograms, above which cells were considered to be positive for expressing the antigens tested. Detector and amplifier were set as specified in the Results section.

Controls included:

(a) replacement of the primary antibody with native immunoglobulins of the species in which the first antibody was raised;

(b) omission of the primary antibody;

(c) untreated cells only for determination of cytotrophoblast cell autofluorescence.

All these "negative" controls provided fluorescence profiles indistinguishable from one another. The appropriate negative control value was always subtracted from the test sample before reporting on the percentage of positive cells labeled.

2.7 Cell testing for viability

2.7.1 Principle

2.7.1.1 Trypan Blue exclusion

Cell viability is determined by mixing two drops of the cell suspension with two drops of 0.5 % Trypan Blue and after 2 – 5 min, counted in a Neubauer chamber. The average number of cells that exclude the dye in each large square is multiplied by 2×10^4 to give the number of cells/ml. Those cells that take up the dye are dead cells.

2.7.1.2 DNA analysis

Flow cytofluorometric analysis can be performed on fixed cells (in PFA or ethanol) or on living cells. Analysis on living cells allows detection of cell membrane bound antigens only, while the fixation procedure allows detection of both intracellular and extracellular antigens. As the cytoplasmic membrane of dead cells becomes permeable to antibodies, it is necessary to exclude dead cells from the overall population of cells to be analyzed, in order to detect only extracellular antigens. For this purpose, cells are labeled first with propidium iodide, a fluorescent dye which binds to cellular DNA. Dead cells are subsequently excluded by selective gating of the cell population which does not emit fluorescence at the characteristic wave length for propidium iodide emission.

2.7.2 Protocol

The isolated cells were tested for viability by Trypan blue exclusion and by DNA analysis of freshly isolated, unfixed cells, labeled with propidium iodide; briefly, 8 µl of 1 mg/ml propidium iodide were added for 200 µl sample volume; samples were incubated on ice, in the dark, for 10 min, and then assessed by flow cytofluorometry.

3 RESULTS

3.1 Anti-CD45 immunomagnetic separation

In order to purify and characterize cytotrophoblast cells of first trimester placentas from normal pregnancies a protocol was developed which involves enzymatic dissection and immunomagnetic separation of trophoblast cells. The cytotrophoblast-enriched fraction harvested from the Percoll gradient was mixed with Dynabeads conjugated with a monoclonal antibody (mAb) to pan-leukocyte antigen CD45, in order to remove contaminating leukocytes.

Freshly dissected trophoblast cells were subjected to Percoll gradient centrifugation, bands 3 and 4 collected (as described in the Materials and Methods section) and then incubated with 62.5 µl (25 x 10^6) of Dynabeads M-450 CD45 in 1.25 ml of PBS. The number of Dynabeads per target cell was ten. The suspension was incubated at 4 °C for 30 min, with occasional gentle shaking. The test tube was placed on a magnetic particle concentrator (Dynal MPC-1, Dynal, Oslo, Norway) for 2 min. The cells that interacted with the Dynabeads (rosetted cells) and nonreacting Dynabeads were collected using the magnetic collector (Figure 9), and the nonrosetted cell fraction, containing trophoblast cells, collected. The trophoblast cell containing supernatant devoid of CD45-reactive cells, was kept for further analyses.
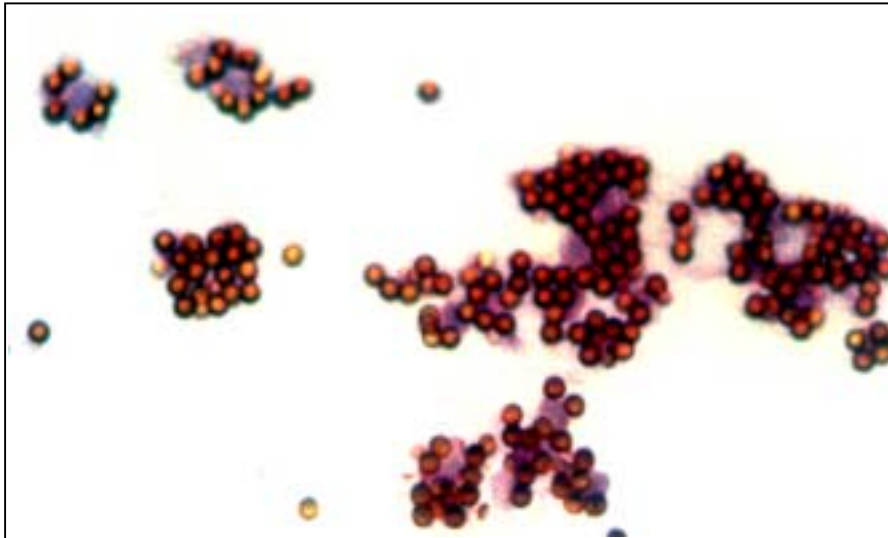
Figure 9 Immunocytochemical detection of cells that interact with the Dynabeads, conjugated with mAb to CD45 (rosetted cells)

The cell density gradient routinely yielded a cytotrophoblast cell suspension with 65 to 90 % purity before immunomagnetic separation, as judged by cytokeratin 8,18 and E-cadherin positivity. This cell population was then subjected to "negative" selection applying the described anti-CD45 immunomagnetic purification protocol. This additional purification step routinely yielded > 95 % CK 8,18-positive cells. In Figure 10 cell suspensions investigated by flow cytofluorometry before and after immunomagnetic separation are shown.
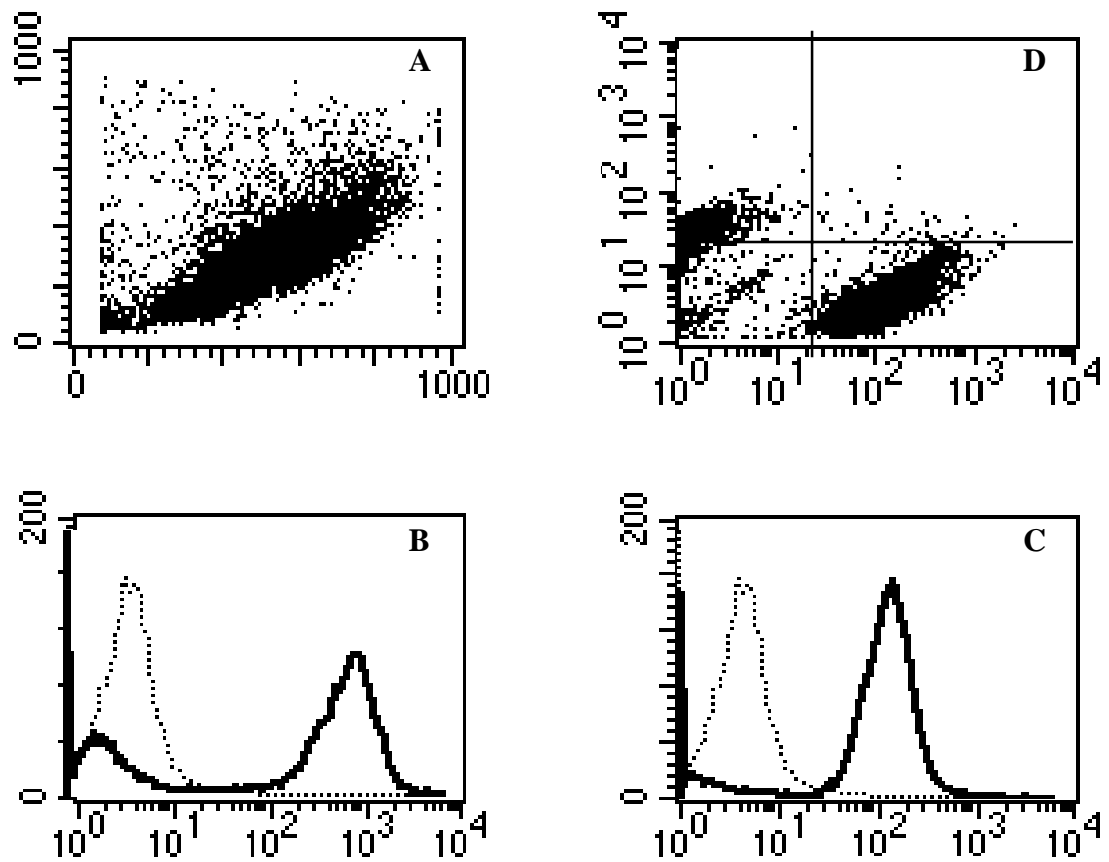
Figure 10 Flow cytofluorometric analysis of trophoblast cells from first trimester placenta before and after the immunomagnetic separation with Dynabeads M-450 CD45. **A** Light scatter image of the heterogeneous cell population before immunomagnetic separation (horizontal axis – FSC; vertical axis – SSC); **B** mAb (FITC-conjugated) to CK 8,18 reactive cell suspension before immunomagnetic separation (horizontal axis – log fluorescence; vertical axis – relative cell count); **C** mAb (FITC-conjugated) to CK 8,18 reactive cells after immunomagnetic separation with Dynabeads M-450 CD45 (horizontal axis – log fluorescence; vertical axis – relative cell count); **D** Dual fluorescence of the isolated cytotrophoblast cells after immunomagnetic separation with Dynabeads M-450 CD45, labeling of the nonreactive cell fraction with mAb (PE-conjugated) to CD45 – vertical axis, and mAb (FITC-conjugated) to CK 8,18 – horizontal axis

Legend: ▬▬▬ - CK 8,18 – FITC; ········· - control – mouse - FITC

In flow cytofluorometry, cells investigated were selected according to their light scatter properties (FSC versus SSC; data not shown). Cell debris and aggregated cells were not included in the analysis. Only living cells that did not stain with propidium iodide were selected. The gain settings and the detector amplification were recorded for various determinations and were the same for each experiment. The percentage of positive cells

was calculated from the number of events falling within a marker set on the negative controls.

The success rate of obtaining purities over 95 % cytokeratin-positive cells after negative selection for anti-CD45 was over 90 %. The present successful isolation of reasonable numbers of highly purified cytotrophoblast cells from human placentas depended on initial crude dissection of the chorionic villi, gentle trypsinization to cleave the cytotrophoblast and syncytiotrophoblast layers from the mesenchyme, and separation of the cytotrophoblast aggregates by sedimentation at 1,000 x g through a discontinuous Percoll gradient (Figure 11) and by immunomagnetic negative selection.

In the example shown, before immunopurification, only 73.5 % of the cytotrophoblast cells were CK 8,18 positive (Figure 10 B), whereas after immunomagnetic purification purity of 96 % (Figure 10 C) was achieved. Leukocytes accounted for 20 % of the contaminating cells (Figure 12 A) and became rather undetectable after the immunopurification step (Figure 12 B). Endothelial and/or fibroblast cells were not detected, neither before nor after purification (0 % vimentin positive cells; data not shown) whereas the immunomagnetic purification step did not affect the low (1.5 %) but consistent cell contamination (Figure 10 D, lower left quadrant). Only 0.47 % of all of the cells were positive for both CK 8,18 and CD45 (Figure 10 D, upper right quadrant). As shown by Trypan blue exclusion, and also by flow cytofluorometric analysis (propidium iodide exclusion), 98 - 99 % of the isolated cytotrophoblast cells retained viability (Figure 13). It is worth mentioning that syncytiotrophoblast cells appear to be very sensitive to mechanical and enzymatic treatment as the great majority of these cells were lysed during the enrichment steps, as confirmed histologically.

Figure 11 Photograph of a discontinuous Percoll gradient run with density marker beads
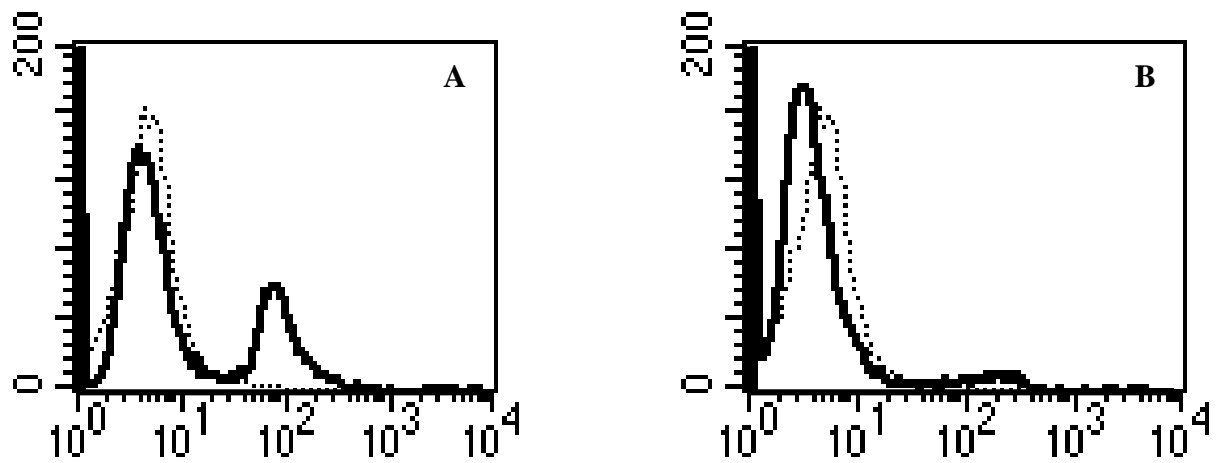
Figure 12 Flow cytofluorometric detection of contaminating cells before and after the immunomagnetic separation with Dynabeads M-450 CD45. **A** with mAb (FITC-conjugated) to CD45 labeled cell suspension before immunomagnetic separation; **B** with mAb (FITC-conjugated) to CD45 labeled cells after immunomagnetic separation. Horizontal axis – log fluorescence; vertical axis – relative cell count

Legend: ▬▬ - CD45 – FITC; ┈┈┈ - control – mouse - FITC
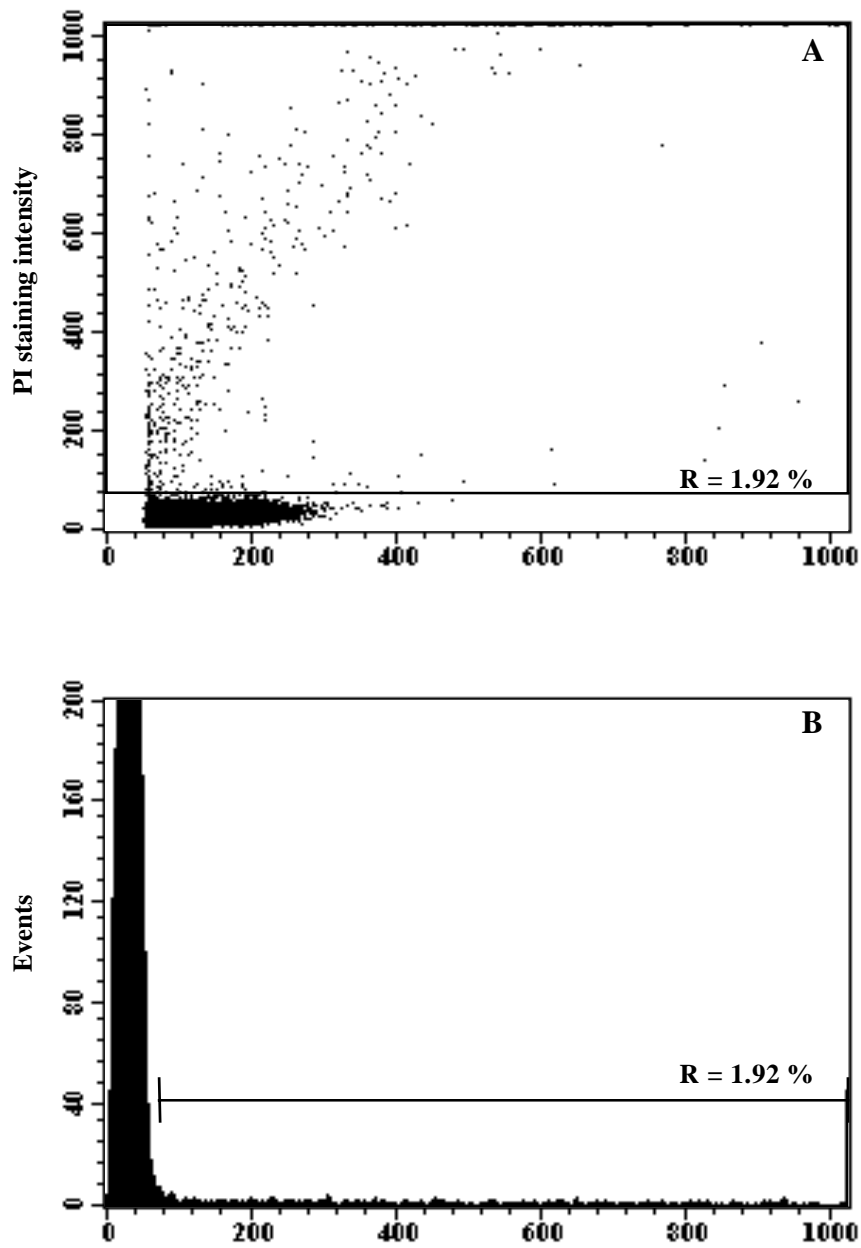
Figure 13 Absence of propidium iodide fluorescence staining in freshly isolated cytotrophoblast cells assessed by flow cytofluorometry indicate living cells. **A** Dot plot (horizontal axis - linear fluorescence; vertical axis - propidium iodide staining intensity); **B** Histogram (horizontal axis - linear fluorescence; vertical axis - relative cell count)

One of the difficulties associated with the use of "negative" selection procedure for targeting the CD45 antigen by antibodies to this epitope was the adhesion of some of the trophoblast cells to the immunomagnetic beads, although they did not bind to the antibody. This was demonstrated by control experiments using the two step immunomagnetic separation: first incubation with mouse mAb to CD45 (Caltag Laboratories, San Francisco, USA) for 30 min at room temperature, followed by a second incubation with Dynabeads M-450 sheep anti-mouse IgG (Dynal, Oslo, Norway) for 30 min at 4 °C. In almost all cases this resulted in the loss of some trophoblast cells during the immunomagnetic separation. Similar findings were reported by Aboagye-Mathiesen et al., 1996.

3.2   Characterization of isolated first trimester cytotrophoblast cells

The isolated cytotrophoblast cells were extensively characterized by routine histology, conventional immunocytochemistry, fluorescence confocal laser scanning microscopy, and flow cytofluorometry, showing that they express in situ markers of trophoblast cells: cytokeratin 8,18; E-cadherin; β-hCG; hPL (Zhou et al., 1997; Khoo et al., 1998; Hamilton et al., 1998); and integrin subunits α1, α5, α6, αv, β1, β3, β4 (Haynes et al., 1997; Douglas et al., 1999; Thirkill and Douglas, 1999).

To identify the isolated cells, we used two different monoclonal antibodies directed to trophoblast cells: antigens cytokeratin 8,18 (mAb CAM 5.2) and E-cadherin (mAb 120/80). Immunocytochemistry showed a strong positive cytoplasmic staining of cells reacting with mAb to CK 8,18 (Figure 14 A) and E-cadherin (Figure 15), respectively. Already in the cytotrophoblast epithelial monolayer obtained after first enzymatic dissociation, cytotrophoblast cells stained strongly for E-cadherin, showing a polarized pattern. Staining was even stronger on the surface of cytotrophoblast cells still in contact with each other and on their apical surface, and was absent at the basal surface of cytotrophoblast in contact with the basement membrane.
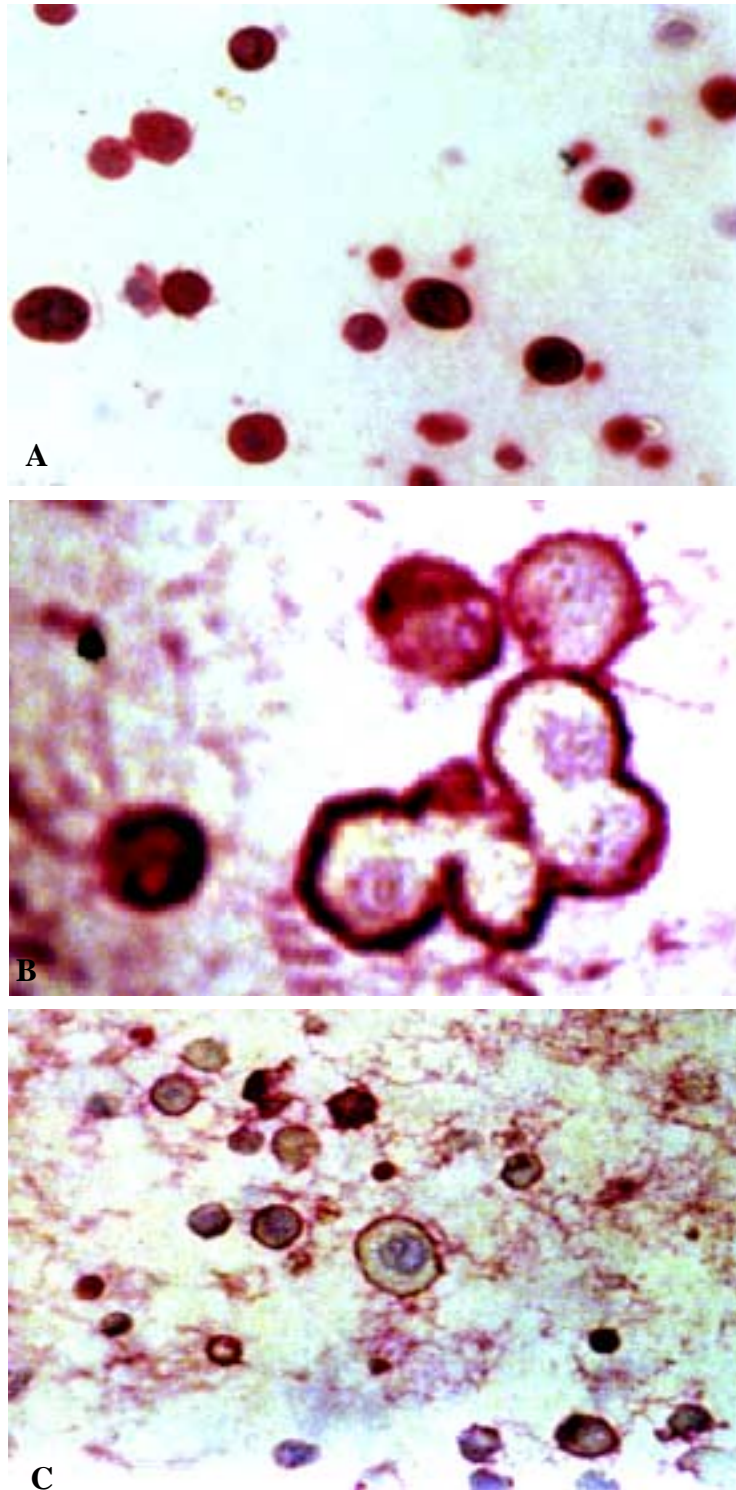
Figure 14 Immunocytochemical labeling of highly enriched first trimester cytotrophoblast cells stained with: **A** Anti-CK 8,18; **B** Anti-β-hCG; **C** Anti-hPL. Please, note the strong cytoplasmic staining of cells with antibodies to CK 8,18 (A) and the focal staining for β-hCG (B). There is a uniform but moderate to weak staining present in the plasma membrane with antibodies to hPL (C). Hematoxylin counterstain of nuclei (x 40)
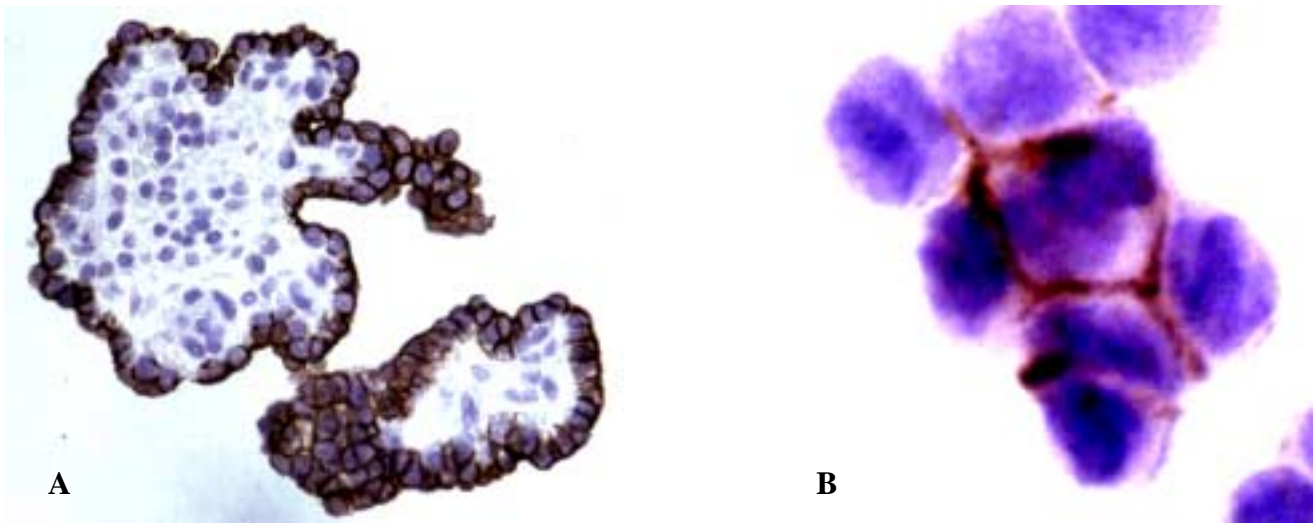
Figure 15 E-cadherin staining of freshly isolated cytotrophoblast cells. **A** microscopic magnification x 40; **B** microscopic magnification x 100. Please, note the polarized staining pattern with strong staining on the surface of cytotrophoblast cells in contact with each other and on their apical surface. Hematoxylin counterstain of nuclei (blue)

The purification of cytotrophoblast cells involves trypsinization, which to a certain degree destroys cell membrane proteins including the cell adhesion molecule E-cadherin. Villous cytotrophoblast cells are, however, relatively resistant to trypsinization, as demonstrated for the E-cadherin staining (Figure 15).

Three monoclonal antibodies to trophoblast cells were screened for their capacity to label isolated cells in flow cytofluorometry and the results compared with immunocytochemical labeling of cytospin preparations and tissue sections. For immunocytochemical staining and flow cytofluorometric analysis, monoclonal antibodies to cytokeratin 8,18 were used to monitor the extent of purification. Figure 16 shows CK 8,18-positive cytotrophoblast cells contaminated with CK 8,18-negative cells before the immunomagnetic anti-CD45 depletion step. Figure 14 A and Figure 17 A show CK 8,18-positive cells after anti-CD45 enrichment.
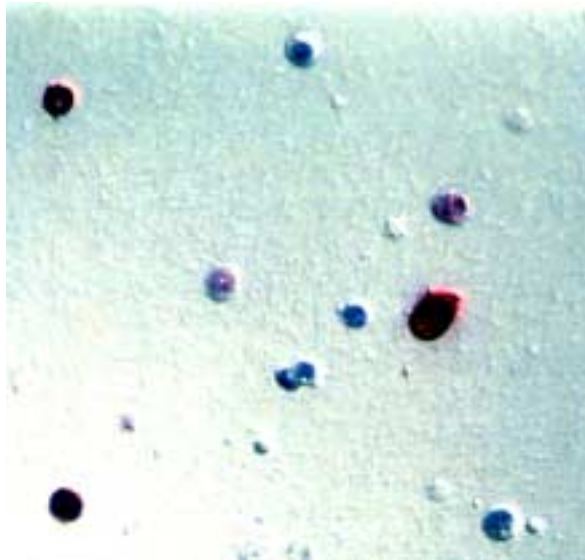
Figure 16 Mixture of cytotrophoblast (CK-positive) and nontrophoblast (CK-negative) cells before anti-CD45 immunomagnetic purification step of first trimester cytotrophoblast cells. Immunocytochemical staining (red), hematoxylin counterstain (blue) (x 40)

hCG and hPL, the other two established markers for differentiated trophoblast cells were also assessed. Immunoreactivity for hCG revealed a distinct focal pattern (Figure 14 B and Figure 17 B), that for hPL was weak but homogeneous (Figure 14 C and Figure 17 C).

Flow cytofluorometric and confocal laser scanning microscopic assessment of the isolated cytotrophoblast cells revealed a strong expression for integrin subunits $\alpha 6$, αv, $\beta 3$ and $\beta 4$ (Figure 18 C, D, F, G and Figure 19 C, D, F, G). Integrin subunits $\alpha 1$ and $\beta 1$ were expressed at low level (Figure 18 A, E and Figure 19 A, E). Expression of integrin subunit $\alpha 5$ was present, but weak (Figure 18 B and Figure 19 B).
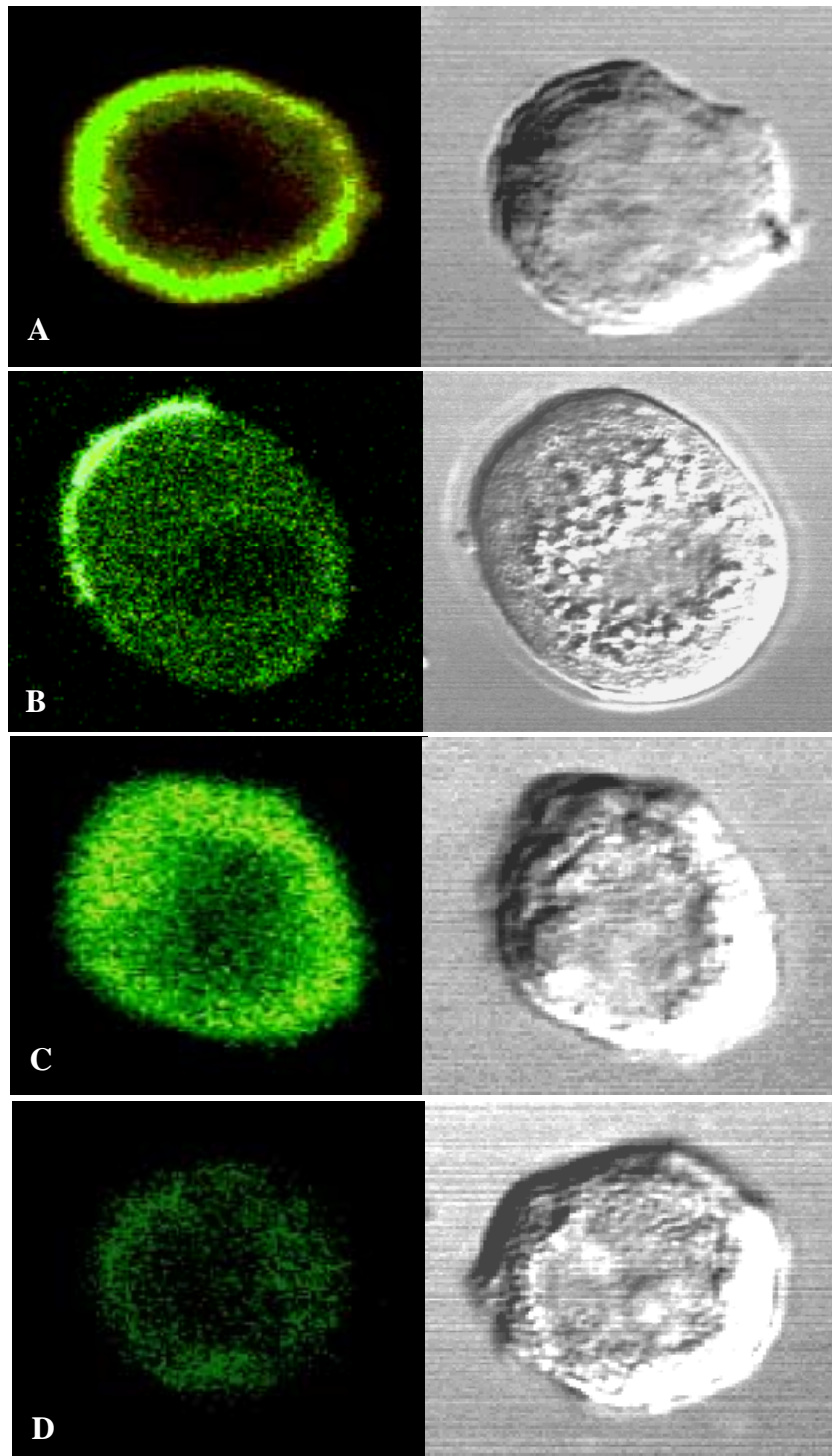
Figure 17 CLSM. Immunofluorescence of PFA-fixed cytotrophoblast cells stained with antibodies to: **A** CK 8,18; **B** β-hCG; **C** hPL; **D** Autofluorescence of cells. Please, note the strong fluorescence of the cells stained for CK 8,18 (A) and the focally positive fluorescence for β-hCG (B). There is a uniform moderate to weak fluorescence with anti-hPL (C). Left - fluorescence image; right - transmission light image (63 oil immersion objective)
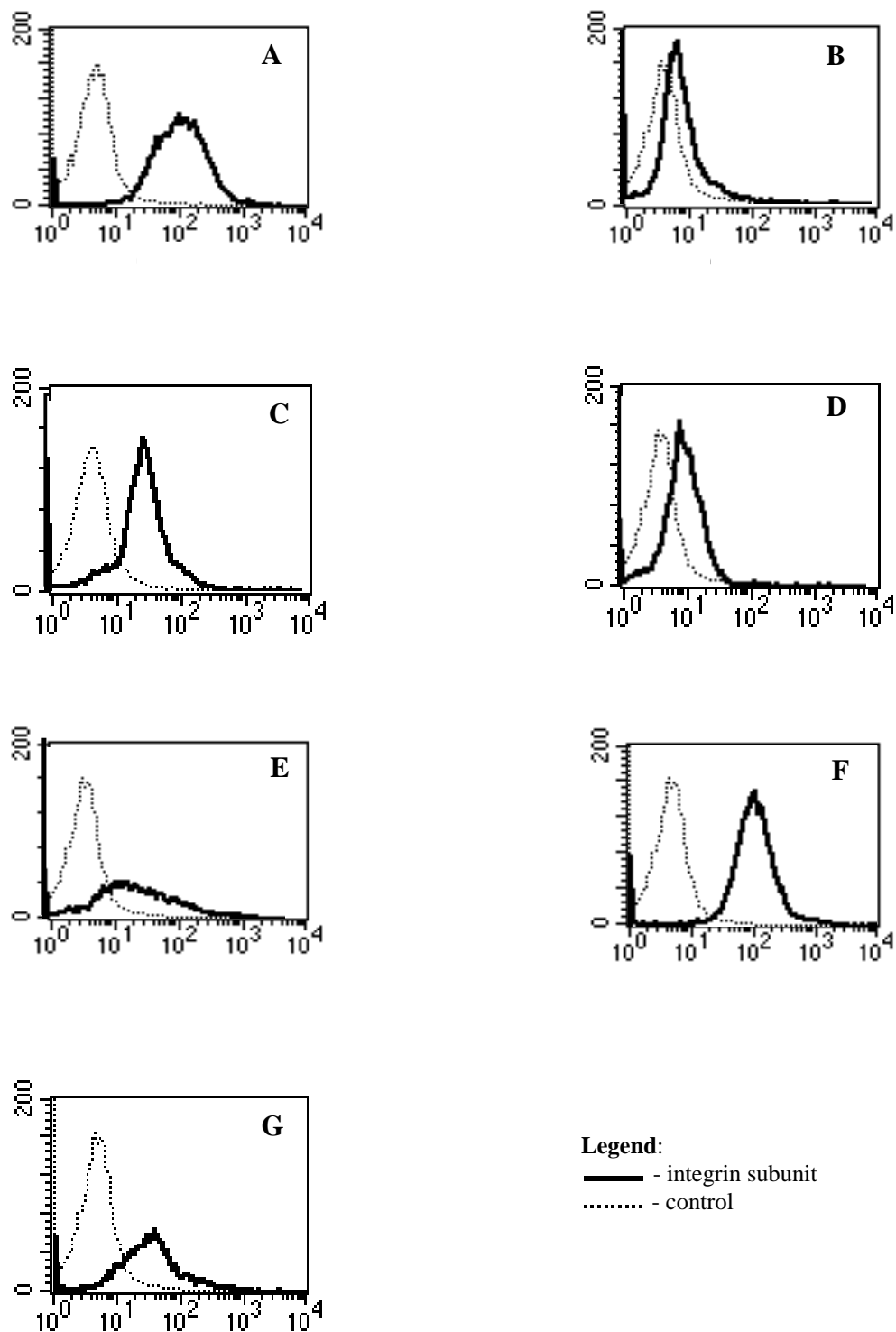
Figure 18 Flow cytofluorometric data for cell surface labeling of isolated CTB with antibodies to:
A Integrin subunit α1; **B** Integrin subunit α5; **C** Integrin subunit α6; **D** Integrin subunit αv; **E** Integrin subunit β1; **F** Integrin subunit β3; **G** Integrin subunit β4. Horizontal axis – log fluorescence; vertical axis – relative cell count
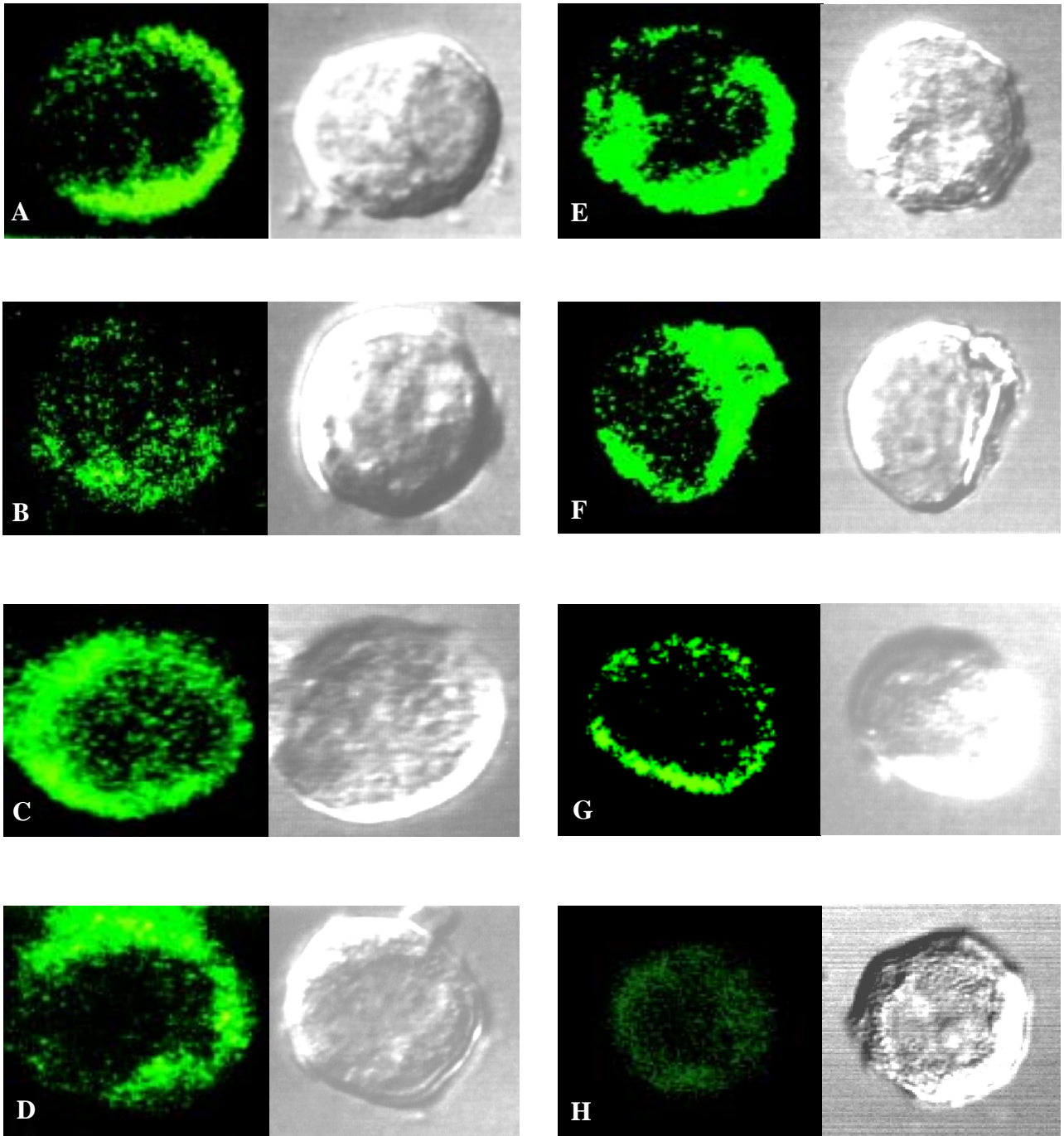
Figure 19 CLSM. Immunofluorescence of PFA-fixed cytotrophoblast cells stained with antibodies to: **A** Integrin subunit α1; **B** Integrin subunit α5; **C** Integrin subunit α6; **D** Integrin subunit αv; **E** Integrin subunit β1; **F** Integrin subunit β3; **G** Integrin subunit β4; **H** Autofluorescence of cells. Left - fluorescence image; right - transmission light image (63 oil immersion objective)

Immunofluorescence and immunocytochemical testing of the purified cytotrophoblast population revealed that cytotrophoblast cells do not react with antibodies to CD45, which among others is present on granulocytes, monocytes, B- and T-lymphocytes, and NK-cells. Anti-CD14 (monocyte and macrophage marker) was also nonreactive with cytotrophoblast cell preparation indicating that the isolated cells were free of leukocytes and other CD14-positive placental cells. All of the isolated cells were negative for vimentin, a marker for fibroblast cells.

Thus the isolated cells from human first trimester normal placenta express the phenotype of cytotrophoblast cells (Table 4), as outlined by Haynes et al. (1997), Zhou et al. (1997), Hamilton et al. (1998), Khoo et al. (1998), Douglas et al. (1999), Thirkill and Douglas (1999).

Table 4 Characteristics of isolated CTB cells from normal human first trimester placenta

Marker	Expression	Methods used
CK 8	+	ICC
CK 18	+	ICC
CK 8,18	+	FACS, CLSM
E-cadherin	+	ICC
β-hCG	+	ICC, FACS, CLSM
hPL	+	ICC, FACS, CLSM
integrin subunit α1	+	FACS, CLSM
integrin subunit α5	+	FACS, CLSM
integrin subunit α6	+	FACS, CLSM
integrin subunit αv	+	FACS, CLSM
integrin subunit β1	+	FACS, CLSM
integrin subunit β3	+	FACS, CLSM
integrin subunit β4	+	FACS, CLSM
CD45	-	ICC, FACS
CD14	-	FACS
vimentin	-	FACS

The cytomorphology of the isolated placenta cells showed classic criteria of cytotrophoblast cells (Boyd and Hamilton, 1970): irregularly ovoid, mononuclear, with large nuclei and a pale, faintly staining cytoplasm (Figure 17 D and Figure 20 C).

Morphological control studies were performed after each step of the isolation method, using light microscopy, in order to prove that each time the syncytiotrophoblast cells were removed and a mixture of cytotrophoblast cells and other placental cells was further analyzed (Figure 20). The chorionic villus syncytium was found to consist of a layer of basophilic syncytioplasm largely without cell boundaries, and large nuclei. The multinuclear syncytium is markedly vacuolated and has a foamy appearance (Figure 20 B).

This cytotrophoblast isolation procedure was applied on 15 different first trimester placentas and similar results have been obtained each time.
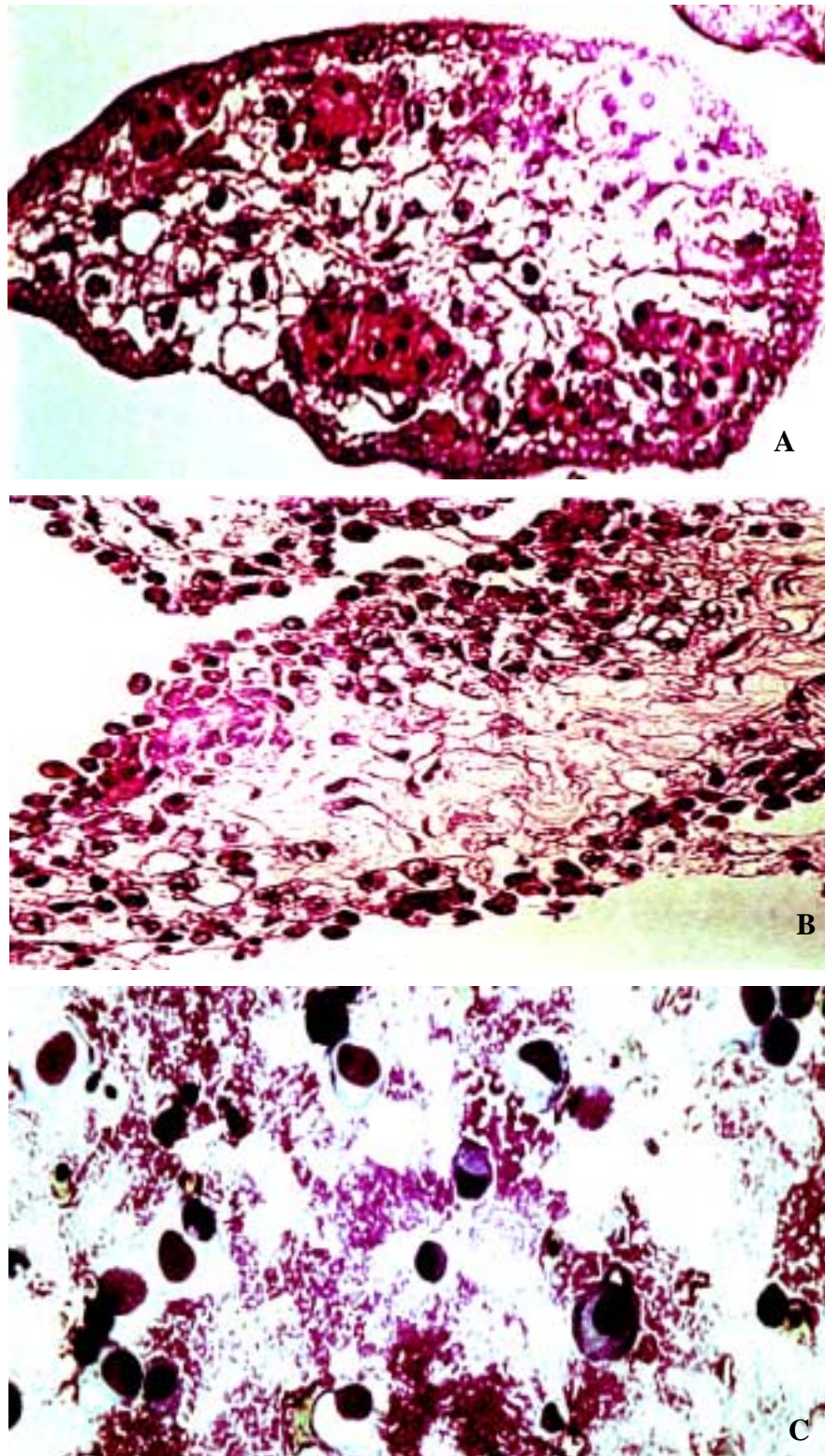
Figure 20 Transmission light microscopy of trophoblast cells. **A** Before enzymatic treatment of the chorionic villi; **B** After enzymatic treatment of the chorionic villi (before the immunopurification step); **C** Finally isolated cytotrophoblast cells . HE staining (x 40)

4 DISCUSSION

In recent years, *in vivo* and *in vitro* studies of trophoblast cells have gained increasing attention because very little is known about factors controling trophoblast proliferation, differentiation, and invasion. Without any question, for *in vitro* characterization at the cell and molecular level, highly purified trophoblast cells are needed. To fulfil these needs, several attempts to isolate human trophoblast cells have been reported. In essence, these methods include:

-   physical separation methods (Kliman et al., 1986);
-   physical and/or immunomagnetic separation methods (Douglas and King, 1989);
-   cell attachment procedures (Loke et al., 1989a);
-   sequential enzymatic digestion and Percoll gradient centrifugation (Kliman et al., 1986; Fisher et al., 1989);
-   selective culture conditions (Loke and Burland, 1988; Yeger et al., 1989);
-   sedimentation methods (Kliman et al., 1986; Douglas and King, 1990; Kliman and Feinberg, 1990; Nelson et al., 1990; Shorter et al., 1990);
-   immunological or receptor-binding methods (Contractor and Sooranna, 1988; Douglas and King, 1989; Loke et al., 1989b; Shorter et al., 1990; Schmon et al., 1991; Bischof et al., 1991; Morrish et al., 1991; Caulfield et al., 1992; Tse et al., 1994);
-   selective disaggregation conditions (Bax et al., 1989; Bullen et al., 1990);
-   immuno - flow cytometric cell sorting methods (Bloxam et al., 1997).

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 syncytiotrophoblast, underlying which is a single layer of cytotrophoblast cells that lies on the basement membrane. A microvascular network connects this cell layer to the umbilical arteries and vein. The apical membrane of the syncytiotrophoblast is folded into numerous microvilli, and this layer forms a syncytium. Disaggregation of this villous tissue results in a broken syncytial membrane, releasing not only the cytotrophoblast cells, but also the rest of the villous cell population (macrophages, fibroblasts, giant cells, some adhering decidual and endothelial cells) as well as DNA from

the nuclei of the syncytium. Separation of cytotrophoblast from this heterogeneous cell population has proven to be a challenge.

The present protocol to enrich a reasonable number of highly purified cytotrophoblast cells from human first trimester placenta depends on the initial crude mechanical dissection of the chorionic villi, gentle enzymatic treatment to separate the cytotrophoblast and syncytiotrophoblast layers from the mesenchyme, enrichment of the cytotrophoblast cells by Percoll gradient sedimentation and by an immunomagnetic separation step employing monoclonal antibodies to CD45 to remove blood cells.

CD45 is a tyrosine phosphatase present on bone marrow-derived cells (Charbonneau et al., 1989; Shimonovitz et al., 1998), but is absent on cytotrophoblast cells (Librach et al., 1991).

The specific type and number of cytotrophoblast cells isolated by this method depends on the age of the placenta and the time of enzymatic digestion. In the present study, incubation times necessary to remove the layer of syncytiotrophoblast cells covering the chorionic villi were assessed histologically for first trimester placentas of different ages and for every lot of enzymes. Thus the use of sequential enzymatic dissociation as the first step enabled the sequential dissociation of CTB and STB in a stepwise manner from the first trimester placenta.

Proteolytic enzymes other than trypsin used to digest the placental villous tissue have been tried, such as collagenase (Matsuzaki et al., 1992), collagenase with hyaluronidase (Fisher et al., 1990), hyaluronidase with trypsin (Ungar et al., 1987), collagenase and pronase (Morrish and Siy, 1986), without improvement or a reduction in the proportion of viable CTB extracted. On the other hand, protease XV (from *B. polymyxa*) (Richards et al., 1994) and dispase (from *B. polymyxa*) (Eis et al., 1995) appear to have been used successfully and may be more reliable than trypsin. Karl et al. (1992) have suggested that when a high yield of cells is required, or when an initial digestion with trypsin or dispase is unsatisfactory, serial digestion with dispase, collagenase and trypsin can overcome variable enzyme effectiveness.

hCG, hPL, and CK 8,18 as markers of the trophoblastic tissue is well documented in the literature (Kurman et al., 1984; Sabet et al., 1989; Khoo et al., 1998); immunocytological techniques have promoted many studies on diverse villous and/or extravillous trophoblast cell populations of uteroplacental tissues (Bulmer and Sunderland, 1984; Hsi et al., 1984; Redman et al., 1984; Wells et al., 1984; Bulmer and Johnson, 1985).

Cytokeratins are a family of intermediate filament proteins with molecular weights of 40 - 68 kD, among others expressed by epithelial cells. The type of cytokeratin synthesized depends on the origin of the epithelial cell and its stage of differentiation. In tissue sections it was shown that the villous cytotrophoblast cell staines heavily with hematoxylin - eosin, whereas syncytiotrophoblast cells are faintly labeled, only (Loke and Butterworth, 1987).

Cytokeratin 8,18 is a very sensitive and reliable marker for all types of trophoblastic tissues but not for villous stromal components (Fisher et al., 1989; Daya and Sabet, 1991; Hamilton et al., 1998). hCG and hPL are positive in differentiated trophoblast - syncytiotrophoblast and extravillous trophoblast (Daya and Sabet, 1991). These data correlate with our immunocytochemical findings: isolated cytotrophoblast cells were not uniform in their positivity regarding hCG and showed a low positivity for hPL.

E-cadherin is a calcium-dependent cell-cell adhesion molecule, e.g., expressed on differentiated epithelia, which, in the placenta, is specific for cytotrophoblasts. In the placenta, antibodies to E-cadherin stain trophoblast cells, only (Fisher et al., 1989; Coutifaris et al., 1991a) and in a polarized pattern (Zhou et al., 1997). In contrast, antibodies to CD45 stain granulocytes, monocytes, B- and T-lymphocytes, and NK-cells, but not trophoblast cells. All of the isolated cells were negative for vimentin, an intermediate filament protein present in endothelial cells and fibroblasts (Morgan et al., 1998).

Integrins are a subfamily of cell adhesion molecules regulating calcium-dependent cell – substrate interactions. They are widely expressed cell – surface adhesion receptors. They are all α β heterodimers.

Trophoblast cells reaching the endometrial basement membrane express integrins ($\alpha 6 \beta 4$), which anchor them into the basement membrane and induce the secretion of matrix

metalloproteinases (MMPs), in particular MMP-2 and MMP-9. MMPs digest the basement membrane allowing the embryo to make contact with the endometrial extracellular matrix. Further integrins (α5β1) anchor the embryo into the extracellular matrix and induce its secretion of collagenases (MMP-1 and MMP-8). These MMPs in turn digest the extracellular matrix allowing the embryo to nidate in the endometrium (Bischof and Campana, 1996).

Villous CTB expresses the laminin receptor integrin α6β4 clustered toward the basement membrane (Lala and Hamilton, 1996; Zhou et al., 1997) (Figure 21; see also Figure 6), and the extravillous CTB expresses the fibronectin receptor integrin α5β1 (Lala and Hamilton, 1996; Zhou et al., 1997). Endovascular trophoblast expresses the collagen receptor integrin α1β1. Integrin α6β4 is associated with invasive properties of CTB (Bischof and Campana, 1996; Zhou et al., 1997). Integrin αvβ5 is characteristic of villous CTB, αvβ6 is only expressed on cells at the base of the invasive cell columns, while αvβ3 characterizes the CTB of the placental bed (Zhou et al., 1997; Coutifaris et al., 1998). Expression of particular subsets of integrins has been associated with invasive cell behavior (Albelda, 1993; Douglas et al., 1999; Crowe and Shuler, 1999).
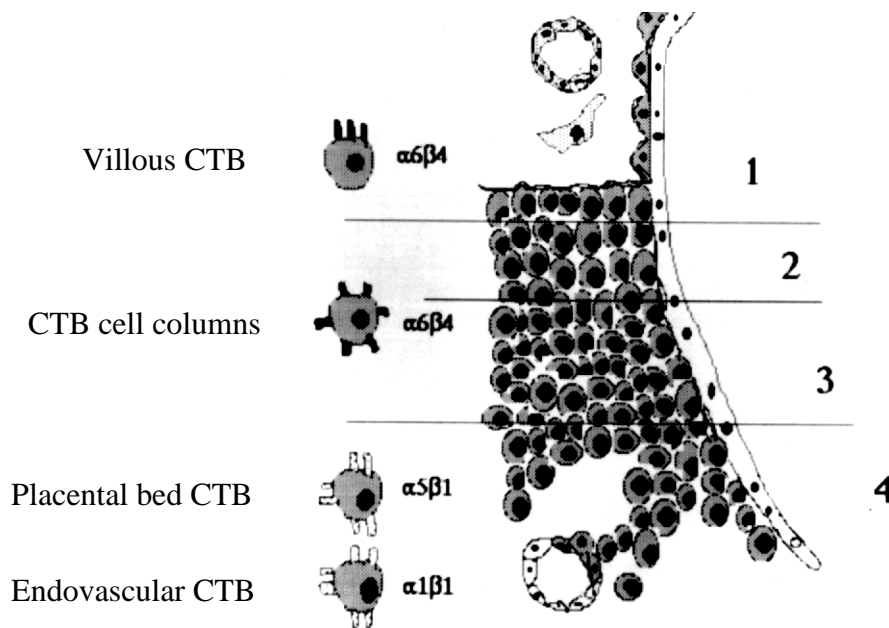
Figure 21 Patterns of integrins during trophoblast invasion (adapted from Bischof and Campana, 1996)

In the present study, integrin subunit α1 was found to be less expressed by the isolated normal CTB, probably due to the nature of the tissue examined (placental tissue without decidua or myometrium containing the spiral arteries with endovascular trophoblast). Integrin subunits α1, α5 and β1 are absent or very low in normal villous CTB, while upregulated in placental bed (Damsky et al., 1997).

The isolated CTB cells were characterized employing routine histology, conventional immunocytochemistry, fluorescence confocal laser scanning microscopy and flow cytofluorometry. Double labeling of cells with monoclonal antibodies to CK 8,18 (FITC-conjugated) and CD45 (PE-conjugated) revealed that viable cytokeratin-positive cytotrophoblast cells were isolated with sufficient purity (96 %) and yield (20 x 10^6 cells/ml) for potential use in functional studies.

CLSM and flow cytofluorometry allowed the exact location of cell-associated antigens by fluorescence on the surface or inside living or fixed cells, because these two techniques are

designed for rapid laser scanning of the cell and immediate data processing at real-time (Knebel et al., 1990). Both techniques are relatively easy to perform. Compared to transmission light microscopy, CLSM has the main advantage to achieve high resolution of cell structures (transmission and fluorescence light images) together with a powerful computer and image analysis system. The advantage of flow cytofluorometry on the other hand resides in the possibility of detection and quantitation of surface antigens. Moreover, using different fluorochromes (double labeling), simultaneous co-localization of various antigens (receptors) in the same cell is possible.

Isolated cytotrophoblast cells do not proliferate *in vitro* (Lewis et al., 1993), suggesting that these cells are not suited to study cytotrophoblast cell proliferation at the feto-maternal interface (Babawale et al., 1996). These findings indicate that either the process of tissue dissociation, when obtaining isolated trophoblast cells, does impair the cell or that the loss of the mesodermal core of the placental villi as a source of growth factors is responsible for the inability of isolated cytotrophoblast cells to proliferate *ex vivo* (Babawale et al., 1996).

5 CONCLUSION

High purity isolation of living cytotrophoblast 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 (Weitlauf, 1994). These morphologic variations presumably rely upon fundamental differences at the cellular and molecular levels (Coutifaris et al., 1991b). 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. Thus, the recent development of new techniques for isolation of purified living placental cytotrophoblast cells should allow further characterization of their structural, morphologic, and functional differentiation at the cellular and molecular level.

Advances in understanding the process of implantation and placental development will also lend insights into clinically important trophoblast-related disorders of first trimester pregnancy, such as embryonic mortality, spontaneous abortion, abnormal placentation, or gestational trophoblastic disease. The presented method of cytotrophoblast 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. The study of adhesive interactions between trophoblast cells and trophoblast and decidual cells and their role in embryo implantation and development of the placenta is a step toward outlining the events leading to the successful establishment and maintenance of early human pregnancy.

6 PERSPECTIVES

Developments in methods of isolation and culture of human placental trophoblast have opened up 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 areas of trophoblast - endometrial interactions in implantation, reproductive immunology, placental endocrinology, metabolism, and pathology, trophoblast function as well as basic mechanisms of cell differentiation. Another key area of placental function whose investigation should benefit from the ability to isolate pure placental trophoblast is maternal – fetal transfer of substances needed by the fetus for its development and growth, and, in the opposite direction, of substances for excretion or for use by the maternal organism.

7   ABSTRACT

A protocol for the preparation of highly purified human placental cytotrophoblast cells from normal first trimester placental villi is described. Cytotrophoblast cells were isolated from placentas (6 - 14 weeks of gestation) by a process of mincing, sequential two-step enzymatic treatment of placental villi by collagenase, hyaluronidase, DNAse, and trypsin, followed by discontinuous Percoll gradient centrifugation, and immunomagnetic separation employing antibodies to CD45 to remove contaminating leukocytes. A population of 96 % pure living mononuclear cytotrophoblast cells (identified by positive cytokeratin 8,18 and E-cadherin staining) was obtained at a density of 1.048 and 1.062 g/ml Percoll. The isolated cells were characterized employing routine histology, conventional immunocytochemistry, fluorescence confocal laser scanning microscopy and flow cytofluorometry for the cell antigens CK 8,18, β-hCG, hPL, E-cadherin, integrin subunits $\alpha 1$, $\alpha 5$, $\alpha 6$, αv, $\beta 1$, $\beta 3$, $\beta 4$, and possess the morphological and structural characteristics of cytotrophoblast cells. The presented method of cytotrophoblast 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.

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9   FIGURE LEGENDS

10 TABLE LEGENDS

11 ABBREVIATIONS

APAAP	Alkaline phosphatase anti-alkaline phosphatase
β-hCG	Beta - human chorionic gonadotropin
BSA	Bovine serum albumin
CK	Cytokeratin
CLSM	Confocal Laser Scanning Microscopy
CTB	Cytotrophoblast
DMEM	Dulbecco's modified eagle medium
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanat
FSC	Forward light scatter
HBSS	Hanks' balanced salt solution
HE	Hematoxylin – eosin
hPL	Human placental lactogen
ICC	Immunocytochemistry
Ig	Immunoglobulin
log	Logarithmic
mAb	Monoclonal antibody
MMP	Matrixmetalloproteinase
pAb	Polyclonal antibody
PBS	Phosphate - buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium iodide
SSC	Side scatter
STB	Syncytiotrophoblast
TBS	Tris - buffered saline

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14 CURRICULUM VITAE

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Education

Date attended	Institution and area of study
1982 - 1986	German Highschool of Bucharest
June 1986	International Baccalaureate
	Graduation Grade: 9.69 (out of 10)
1986 - 1992	Study of Medicine at the Faculty of Medicine - Carol Davila University of Medicine and Pharmacy Bucharest
	Graduation Grade: 9.73 (out of 10)
September 1992	Graduation Exam, Diploma thesis entitled: "Pregnancy and Viral Hepatitis – Clinical and Management Principles"
	Mark: 10 (out of 10)
March 1993	Assistant Professor at the Carol Davila University of Medicine and Pharmacy Bucharest, Clinic of Obstetrics and Gynecology, Dr. I. Cantacuzino Clinical Hospital
November 1993	Resident Physician Obstetrics and Gynecology - Clinic of Obstetrics and Gynecology, Dr. I. Cantacuzino Clinical Hospital Bucharest
October 1996	Specialist Physician Obstetrics and Gynecology - Dr. I. Cantacuzino Clinic of Obstetrics and Gynecology, Clinical Hospital Bucharest

1.10.1996. - 31.07.1997.	DAAD (Deutscher Akademischer Austauschdienst) Annual Grant for clinical and scientific research at the Frauenklinik und Poliklinik der Technischen Universität Munich, Germany - Klinikum rechts der Isar; MD PhD student; MD PhD thesis entitled: "Isolation and characterization of human cytotrophoblast cells from first trimester placenta".
March 1999	Ultrasound in obstetrics and gynecology – proficiency achieved Mark: 8 (out of 10)
1.10.1999. – 31.12.1999.	UICC (Union Internationale contre le Cancer) ICRETT Grant for clinical and scientific research at the Frauenklinik und Poliklinik der Technischen Universität Munich, Germany - Klinikum rechts der Isar

Dominant Study Fields

1. Fetal Assessment
2. Ultrasound in obstetrics and gynecology
3. Diabetes mellitus and pregnancy
4. Menopause
5. Gynecologic Oncology

Working Experience

Dates	Hospitals and Duties
currently - October 1996	Carol Davila University for Medicine and Pharmacy Bucharest, Dr. I. Cantacuzino Clinical Hospital, Clinic of Obstetrics and Gynecology – MD, Assistant Professor, Specialist Physician Obstetrics and Gynecology
1.10.1999. – 31.12.1999.	UICC ICRETT Grant for clinical and scientific research at the Frauenklinik und Poliklinik der Technischen Universität Munich, Germany - Klinikum rechts der Isar

1.10.1996. – 31.07.1997.	DAAD Annual Grant for clinical and scientific research at the Frauenklinik und Poliklinik der Technischen Universität Munich, Germany - Klinikum rechts der Isar
March 1993 – Sept. 1996	Carol Davila University of Medicine and Pharmacy Bucharest, Dr. I. Cantacuzino Clinical Hospital, Clinic of Obstetrics and Gynecology - MD, Assistant Professor, Resident Physician Obstetrics and Gynecology
December 1992 – March 1993	Polizu Clinical Hospital of Obstetrics and Gynecology of the Carol Davila University of Medicine and Pharmacy Bucharest - Physician internship
May 1990	Study visit at the Philips University Marburg – Germany

Publications

1. **Hera, R.**, Balteanu, M., Esanu, S., Banceanu, G. **(1992)** Pregnancy and viral hepatitis - clinical and management principles. Obstetrica Ginecologia, 12, 25-27.

2. Hudita, D., Russu, M., Popescu, M., Grama, M., **Hera, R. (1993)** Maternal complications in pregnancy induced hypertension. Obstetrica Ginecologia, 61, 61-65.

3. Hudita, D., Russu, M., Grama, M., **Hera, R.**, Constantin, A., Gheorghiu, M. **(1993)** Fetal risk in pregnancy induced hypertension. Obstetrica Ginecologia, 61, 66-69.

4. Hudita, D., Russu, M., **Hera, R.**, Ciulcu, P., Condor, A., Ghinda, E., Grama, M., Popescu, M., Sgarbura, Z., Subtirelu, G.P., Tufan, A., Constantin, A., Gheorghiu, M., Târlea, A**. (1993)** Pelvic suppurative trombophlebitis - a postpartum complication. Proceedings of the XIth National Congress on Obstetrics and Gynecology, Timisoara, Romania, September 22-24, 1993.

5. Hudita, D., Popa, P.M., Russu, M., Vaduva, A., Dumitrescu, R., Marinescu, S., **Hera, R. (1994)** Proposal for prenatal assessment file. Proceedings of the IIIrd Edition "Medical Days V. Dobrovici", Suceava, Romania, September 29-30, 1994.

6. Hudita, D., Irimia, V., Russu, M., **Hera, R.,** Dumitrescu, R. **(1994)** Clinical considerations on incidence and results of cesarean sections in diabetic pregnancies. Proceedings of the IIIrd Edition "Medical Days V. Dobrovici", Suceava, Romania, September 29-30, 1994. Proceedings of the Annual Session of the National Mother and Child Institute, Bucharest, Romania, November 11, 1994.

7. Hudita, D., Russu, M., Irimia, V., **Hera, R.,** Dumitrescu, R., Dima, A.M. **(1994)** Indications for cesarean section in premature gestations. Proceedings of the IIIrd Edition "Medical Days V. Dobrovici", Suceava, Romania, September 29-30, 1994.

8. Hudita, D., Irimia, V., **Hera, R. (1994)** A retrospective study on cesarean sections performed during 1989 - 1994 in the Clinic of Obstetrics and Gynecology Dr. I. Cantacuzino Bucharest. Proceedings of the IIIrd Edition "Medical Days V. Dobrovici", Suceava, Romania, September 29-30, 1994.

9. Hudita, D., Russu, M., **Hera, R.**, Enescu, M. **(1994)** Congenital malformations with clinical manifestation in the neonate and their influence on the perinatal mortality rate. Proceedings of the IIIrd Edition "Medical Days V. Dobrovici", Suceava, Romania, September 29-30, 1994.

10. Hudita, D., Subtirelu, G.P., Popescu, M., Russu, M., **Hera, R.**, Popa, P.M., Irimia, V. **(1995)** Correlation radiotherapy - surgery to the anatomical and clinical stage of cervical neoplasia. Proceedings of the IVth Edition "Medical Days V. Dobrovici", Iasi, Romania, June 1-3, 1995.

11. Hudita, D., Russu, M., **Hera, R.**, Dumitrescu, R., Popescu, M., Ciuca, S. **(1995)** A retrospective study on total vaginal hysterectomies among vaginal operations. Proceedings of the IVth Edition "Medical Days V. Dobrovici", Iasi, Romania, June 1-3, 1995.

12. Hudita, D., Subtirelu, G.P., Popescu, M., Russu, M., **Hera, R.**, Popa, P.M. **(1995)** Carcinoma of the residual cervix - a diagnostic and therapeutical error. Proceedings of the IVth Edition "Medical Days V. Dobrovici", Iasi, Romania, June 1-3, 1995.

13. Hudita, D., Russu, M., **Hera, R.**, Duta, M. **(1995)** Bacterial vaginosis and premature birth. Proceedings of the First National Conference of the Romanian Association of Perinatal Medicine, Baia Mare, Romania, September 21-23, 1995. Proceedings of the Romanian - German Symposium on Obstetrics and Gynecology, Suceava, Romania, September 28-29, 1995.

14. Hudita, D., Constantinescu, L., Rosca, A., Russu, M., **Hera, R. (1995**) The third degree premature neonate - an obstetrical and neonatological analysis. Obstetrica Ginecologia, 63, 85-87.

15. Hudita, D., Russu, M., Constantinescu, L., Rosca, A., **Hera, R. (1995**) Congenital anomalies with clinical neonatal manifestation. Obstetrica Ginecologia, 63, 88-89.

16. Hudita, D., Russu, M., **Hera, R.**, Dumitrescu, R., Secara, D., Draghici, M. **(1995)** Family planning desire in puerperium. Proceedings of the Romanian - German

Symposium on Obstetrics and Gynecology, Suceava, Romania, September 28-29, 1995.

17. **Hera, R. (1995)** Genital herpes - a sexually transmitted disease. Spitalul, 3, 48-51.

18. Hudita, D., Russu, M., **Hera, R. (1996)** Pregnancy induced hypertension in pregnant women with diabetes mellitus – a clinical study. Proceedings of the Session of the Romanian Society of Obstetrics and Gynecology, Bucharest, February 27, 1996.

19. Hudita, D., Russu, M., **Hera, R. (1996)** Clinical study of hypertensive disorders in diabetic pregnancies. Proceedings of the 28th International Congress on Pathophysiology of Pregnancy, Timisoara, Romania, May 22-25, 1996.

20. Hudita, D., Russu, M., **Hera, R. (1996)** Hypertensive disorders in diabetic pregnancies. Proceedings of the 28th International Congress on Pathophysiology of Pregnancy, Timisoara, Romania, May 22-25, 1996.

21. Hudita, D., Russu, M., **Hera, R.**, Dumitrescu, R. **(1996)** Pregnancy induced hypertension and fetal risk. Proceedings of the 28th International Congress on Pathophysiology of Pregnancy, Timisoara, Romania, May 22-25, 1996.

22. Hudita, D., Russu, M., **Hera, R.**, Dumitrescu, R. **(1996)** Pregnancy induced hypertension and maternal complications. Proceedings of the 28th International Congress on Pathophysiology of Pregnancy, Timisoara, Romania, May 22-25, 1996.

23. Thieme, B., **Hera, R.**, Koop, F., Harbeck, N., Kolben, M., Schneider, K.T.M., Graeff, H., Schmitt, M. **(1997)** Flow cytofluorometric characterization of isolated human trophoblast cells from normal and preeclamptic placental tissue. Proceedings of the XIth Workshop on Applications of Flow Cytofluorometry in Clinical Cell Diagnosis, Regensburg, Germany, March 13-14, 1997.

24. Hudita, D., Russu, M., Dumitrescu, R., **Hera, R.**, Ceausu, I., Posea, C., Filipescu, A. **(1998)** A retrospective study on endometrial cancer in the Clinic of Obstetrics and Gynecology – Clinical Hospital Dr. I. Cantacuzino. Proceedings of the XIIth National Congress on Obstetrics and Gynecology, Iasi, Romania, May 27-30, 1998.

25. Hudita, D., Irimia, V., Russu, M., **Hera, R.**, Ceausu, I., Malincenco, M., Vaduva, A., Popa, P.M. **(1998)** On the actual obstetrical risk of the diabetic pregnant woman. Proceedings of the XIIth National Congress on Obstetrics and Gynecology, Iasi, Romania, May 27-30, 1998.

26. Hudita, D., Russu, M., **Hera, R. (1998)** Hormone replacement therapy in menopause: experience of the Clinic of Obstetrics and Gynecology – Clinical Hospital Dr. I. Cantacuzino. Proceedings of the Symposium "From Puberty to Postmenopause" and

the First National Conference of the Romanian Society of Gynecological Endocrinology, Sinaia, Romania, September 18-20, 1998.

27. Hudita, D., **Hera, R.**, Svasta, S. **(1998)** A retrospective study on indications for cesarean section in the Clinic of Obstetrics and Gynecology – Clinical Hospital Dr. I. Cantacuzino Bucharest. Proceedings of the Romanian – Hungarian Days of Obstetrics and Gynecology, Arad, October 29-31, 1998.

28. Hudita, D., **Hera, R.**, Toma, A. **(1998)** Vaginal birth after cesarean section: a retrospective study in the Clinic of Obstetrics and Gynecology – Clinical Hospital Dr. I. Cantacuzino Bucharest, and review of the literature. Proceedings of the Romanian – Hungarian Days of Obstetrics and Gynecology, Arad, October 29-31, 1998.

29. Hudita, D., Russu M.C., **Hera, R.**, Ceausu, I. **(1999)** Shoulder dystocia – a retrospective clinical study, and review of the literature. Proceedings of the 3rd National Conference on Perinatal Medicine, Timisoara, October 7-9, 1999.

Languages: Romanian – native language

German – native proficiency achieved

English – fluent

French – good

Member of

Romanian Society of Obstetrics and Gynecology

Romanian Society of Menopause (founding member)

Romanian Society of Perinatal Medicine

FIGO, UPIGO, EAGO, UEMS / Ob. Gyn.

Munich, 8.12.1999.

Raluca Hera, MD, Ass. Prof.