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Isolation and Characterization of Human Placental Trophoblast Subpopulations from First-Trimester Chorionic Villi

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A method for the simultaneous preparation of highly enriched human placental trophoblast populations (villous and extravillous) from first-trimester placental villi (5 to 12 weeks) by using sequential trypsinization, percoll gradient centrifugation, and negative selection with anti-CD9 immunomagnetic separation is described. The purification method resulted in the isolation of four distinct trophoblast populations identified on the basis of morphology and phenotyping: (i) mononuclear villous cytotrophoblast cells which, through differentiation, become committed to syncytium formation; (ii) an extravillous trophoblast population which appeared as a "crazy pavement" and, with subsequent subculturing, differentiated morphologically to mononuclear cells; (iii) an extravillous trophoblast fraction which fused to form multinucleated trophoblast giant cells; and (iv) floating intermediate extravillous trophoblast cells which fused together to form cell clumps and which further differentiated to a mononuclear anchoring intermediate extravillous trophoblast. Short-term cultures of the freshly isolated cell fractions consisted of heterogeneous trophoblasts at different differentiation stages as determined by their varied biochemical and morphological properties. All the isolated trophoblast populations expressed the cytokeratin intermediate filament and the epithelium-specific cell-cell adhesion molecule E-cadherin. The isolated villous trophoblasts in culture expressed integrins α6 and β4 and reduced levels of β1 subunits, whereas the proliferating extravillous trophoblast cultures expressed α1, α3, and α5 and high levels of β1 integrin subunits, vitronectin receptor (αVβ3/β5), and major histocompatibility complex class 1 molecules. Furthermore, the isolated trophoblast populations secreted metalloproteases (such as type IV collagenases [mainly 72- and 92-kDa enzymes, i.e., gelatinases A and B]) and urokinase plasminogen activator, as evaluated by substrate gel zymography. This method of isolation should facilitate in vitro studies of trophoblast proliferation, differentiation, invasion, virus interactions, cytokenesis, and immunology.

The cytotrophoblast is the first fetal cell type to arise during embryogenesis and undergoes multistep differentiation pathways to form villous and extravillous trophoblasts of the primate placenta. In the human placenta, the cytotrophoblast exists in both the floating villi, which are not in contact with the maternal uterine wall but are bathed by the maternal blood and which play a role in gas and nutrient exchange to the developing embryo, and anchoring villi, which are in contact with the uterine wall. The cytotrophoblast in the floating villi exists mainly as polarized and highly proliferative mononuclear cells anchored to a basement membrane. These cells differentiate exclusively by fusion to form a multinuclear syncytial cell layer that surrounds the villus. In the anchoring villi, the cytotrophoblast undergoes an additional pathway to form nonpolarized cells that penetrate the syncytium, lose their basement membrane, and become a highly migratory and invasive trophoblast population sometimes termed the "intermediate trophoblast." Their destinations are in the endometrium (decidual), myometrium wall (interstitial), and the uterine spiral arteries (endovascular). Multinucleated trophoblast cells in the extravillous locations (placental bed) are designated placental bed giant cells (11).

The invasion of the extravillous trophoblast into the endometrium is an essential part of embryo implantation and resembles the invasion of malignant tumors. However, unlike tumor invasion, trophoblast invasion is precisely regulated, is confined spatially to specific areas in the uterus, and is confined temporally to early pregnancy. Several factors, including cytokines (10), growth factors (2), and integrins (5, 12), have been reported to play critical roles in trophoblast differentiation, migration, and invasion. Furthermore, the placenta represents an allogeneic tissue graft that is intimately exposed to maternal immunocompetent cells but that does not elicit an effective allograft reaction. The detailed mechanisms regulating trophoblast proliferation and differentiation as well as invasion and the immunological acceptance of the antigenically foreign conceptus leading to the successful establishment of pregnancy are not well understood.

The basic requirement for the detailed study of the individual trophoblast cell populations and their possible functions during pregnancy is the development of reliable methods for the isolation of these cells to a high degree of purity. Numerous methods that have used physical (14) and/or immunomagnetic (7) separation and cell attachment procedures (23) have been reported. In vitro studies of the physiological, biochemical, and endocrine functions of trophoblasts have been performed by using organ cultures or isolated trophoblast cells by methods which result in an enriched trophoblast population of one type. Therefore, the ability to isolate, purify, and culture human trophoblast subpopulations will facilitate in vitro studies of the mechanisms which regulate trophoblast functions.

Here we report a method for the simultaneous isolation of human villous and extravillous trophoblast subpopulations from first-trimester placentae. Furthermore, the morphological and biochemical differentiation of the trophoblast cell subsets in vitro are also reported.

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MATERIALS AND METHODS

Isolation of trophoblast subpopulations. First-trimester trophoblast subpopulations were isolated from placentas (5 to 12 weeks) derived by the legal termination of pregnancy. Briefly, 200 g of tissue was washed thoroughly in 50 ml of cold sterile phosphate-buffered saline (s-PBS) until the supernatant was nearly free of blood. Areas rich in chorionic villi were selected and were minced between scalpel blades into small pieces. The minced tissues were washed in s-PBS and were subjected to three sequential 10-min treatments with 0.125% trypsin and 0.2 mg of DNase I (Boehringer Mannheim GmbH, Mannheim, Germany) per ml in s-PBS containing 5 mM $MgCl_2$. At every 10-min step, the cells that were released were pooled and filtered through two layers of muslin, and the trypsin activity was inactivated with fetal calf serum (FCS). The filtrates were centrifuged, and the cell pellets were resuspended in s-PBS. Cells from each 10-min step were resuspended in 70% percoll (Pharmacia, Uppsala, Sweden) at a density of 2×10^5 cells per ml, and the mixture was layered under 20 ml of 25% percoll. A total of 10 ml of s-PBS was put on top of the 25% percoll, and a gradient was established by centrifuging at 2,000 rpm for 20 min. Cells from the middle band (density, 1.048 to 1.062 g/ml) of the gradient were pooled, washed, and resuspended in s-PBS at a density of 20×10^6 cells per ml. In most cases the isolated cells were seeded at a density of 10^6 cells per ml of keratinocyte growth medium (KGM) supplemented with 10% FCS and were tested for purity by cytokeratin staining. Third-trimester villous trophoblast cells, which were used for comparison, were isolated from term placentas by the method of Douglas and King (7) immediately after spontaneous vaginal delivery.

Anti-CD9 immunomagnetic separation. A total of 40 μl of anti-CD9 antibody (The Binding Site, Birmingham, United Kingdom) was added to 20×10^6 freshly isolated or cultured cells, and the mixture was incubated for 30 min on ice with gentle agitation. A total of 4×10^6 Dynabeads M-450 (sheep anti-mouse immunoglobulin G) were mixed with the cell suspension, and the mixture was kept on ice for 1 h with gentle rotation. The cells that adsorbed to the magnetic beads were collected with a magnetic collector, and the unadsorbed cells containing trophoblasts were collected, washed three times, and seeded at a density of 10^6/ml of KGM (Clonetics Corporation, San Diego, Calif.) supplemented with 10% FCS.

Characterization of isolated trophoblast populations. The isolated trophoblast cells were phenotyped by flow cytometry and immunocytochemical staining with monoclonal mouse antibodies to human cytokeratins (Dako-CK, MNF 116, and 35BH11; Dako A/S, Glostrup, Denmark), which stain only the trophoblasts within the placental villi; antibody to the epithelium-specific cell-cell adhesion molecule E-cadherin (HECD-1; Takara Shuzo Co., Shiga, Japan), which also stains only trophoblasts in the placenta (8); anti-CD9, which stains placental mesenchymal cells; placental macrophages, and blood monocytes but not trophoblasts (26, 34); and antibody to porcine vimentin and anti-CD68 (a macrophage marker). The trophoblast cultures were further characterized by their secretion of chorionic gonadotropin hormone and placental lactogen.

To further characterize the cells isolated from the different trophoblast populations, cultured cells were stained or analyzed for the expression of integrins by Western blotting (immunoblotting) with antibodies to integrins α1 (VLA-1), α3, and α5 and the vitronectin receptor (integrin αVβ3/β5) (Gibco BRL, Life Technologies Inc., Gaithersburg, Md.) and also anti-human HLA class 1 antibodies (W6/32; Dako A/S) which are expressed by extravillous trophoblasts but not villous trophoblasts. Villous trophoblast and syncytiotrophoblast cultures were further characterized by their expression of α6 and β4 integrin subunits (1, 15) and NDOG1 (4), respectively.

Immunocytochemical staining of trophoblast cells. Cultured trophoblast cells (cells that were used 15 to 20 h after isolation or that were established for 5 to 10 passages) that were tested for their positivity to cytokeratin were washed three times with s-PBS and were fixed in 99.9% ethanol for 10 min at room temperature. Nonspecific immunoglobulin binding sites were blocked by a 20-min preincubation with rabbit serum diluted in s-PBS. The fixed cultures were preincubated in 3% hydrogen peroxide in s-PBS for 10 min to quench endogenous peroxidase activity, washed three times with s-PBS, and incubated for 1 h at room temperature with the following primary antibodies: anti-cytokeratin (Dako-CK, MNF 116, and 35BH11; 1:100; Dako A/S), anti-E-cadherin (1:100), anti-vimentin (1:20; Dako A/S), anti-major histocompatibility complex (anti-MHC) class 1, W6/32 (1:50; Dako A/S), NDOG1 (1:10; Serotec, Kidlington, United Kingdom), (anti-human placental lactogen (anti-hPL; 1:10; Dako A/S), antibody to the integrin α6 subunit (1:50; VLA-6, clone GoH3, CD49f; Serotec), and anti-CD9 (1:100). The cultures were washed three times with s-PBS and were incubated in peroxidase-labelled anti-mouse or anti-rat antibodies (1:40 dilution in s-PBS) for 30 min at room temperature. The reaction was visualized by using diaminobenzidine solution containing hydrogen peroxide in PBS for 10 min and was stopped by washing several times with sterile water. Positive stains gave a red color, and photographs were taken with an Olympus inverted photomicroscope.

Western blot analysis. In order to confirm the identification of the isolated cells as trophoblasts and to classify them as villous or extravillous subpopulations, in addition to immunocytochemical staining with cytokeratin, E-cadherin, and integrins, we further performed Western immunoblot analysis on the respective samples, as follows.

(i) Cytokeratin. Trophoblast cells were removed from culture bottles by lightly scraping, and the cells were washed three times with s-PBS. Proteins were extracted from cultures of individual trophoblast populations by boiling for 10 min in lysis buffer (50 mM Tris-HCl [pH 7.4], 4 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 mg of antipain [Sigma Chemical Co., St. Louis, Mo.], per ml, 5 mg of pepstatin [Sigma] per ml, 10 mM dithiothreitol, 1% sodium dodecyl sulfate [SDS]) and the resultant suspensions were centrifuged at $10,000 \times g$ for 20 min at 4°C. A total of 20 μl of each supernatant fraction was mixed with sample buffer, and each fraction was separated by SDS-polyacrylamide gel electrophoresis (PAGE; 12% acrylamide) as described by Laemmli (18). The separated proteins were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked in 5% nonfat milk in PBS for 1 h. After incubation of the membrane with a 1:200 dilution of anticytokeratin antibody (clones 116, 35βH11, and OV-TL 12/30; Dako A/S) for 1 h, the membrane was then washed three times, incubated for 1 h with a 1:10,000 dilution of goat anti-mouse immunoglobulin G biotinylated whole antibody, and further incubated for 45 min with a 1:10,000 dilution of streptavidin-biotinylated peroxidase complex. The bands were visualized by enhanced chemiluminescence detection (Amersham) according to the manufacturer's instructions.

(ii) E-cadherin. Trophoblast cells, which were scraped from the culture bottles, were washed twice in s-PBS and were dissolved in SDS-PAGE sample buffer, boiled for 10 min, and centrifuged at 14,000 rpm for 20 min. The resulting cell lysates were fractionated by SDS-PAGE (8% acrylamide). After electrophoresis, the proteins were transferred onto a PVDF membrane, the membrane was incubated with anti-E-cadherin (HECD-1; 1 μg/ml), and the bands were detected by enhanced chemiluminescence detection (Amersham) as described above.

(iii) Integrins. Trophoblast cells, identified by their positivity to cytokeratin and E-cadherin, were gently removed by scraping them from the culture bottles and were extracted for 20 min at 4°C with 0.5% Triton X-100 in 25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mg of leupeptin per ml, 4 mg of pepstatin per ml, and 0.1 trypsin inhibitory unit of aprotinin per ml (all from Sigma). After centrifugation at $14,000 \times g$ for 20 min, 20 μl from each soluble cell extract was mixed with PAGE sample buffer without β-mercaptoethanol and the mixture was fractionated by SDS-PAGE (8% acrylamide) by the method described by Laemmli (18) under nonreducing conditions. The separated proteins were transferred onto PVDF membranes and were incubated with the respective antibodies (integrin α1, α3, α5, α6, β1, or β4 or human vitronectin receptor [integrin αVβ3/β5; Gibco BRL, Life Technologies]), and the bands were visualized by enhanced chemiluminescence detection (Amersham) as described above.

Hormone assays. Human chorionic gonadotropin (hCG) was measured in the culture supernatant by using the Guildhay hCG enzyme-linked immunosorbent assay kit (Guildhay, Guildford, United Kingdom). Briefly, 50 μl of serial dilutions of the culture supernatants or standards (5 to 500 IU/liter), calibrated against the third IRP 75/537, was added in duplicate to precoated microtiter plates. After 15 min of incubation at room temperature with the samples, the plate was washed four times, 100 ml of alkaline phosphate-labelled monoclonal antibody to hCG was added, and the contents were mixed gently. After an additional 15 min of incubation at room temperature, the plate was washed again to remove unbound labelled antibody, 100 ml of substrate reagent containing phenolphthalein monophosphate and coenzymes in diethanolamine was added to each well, and the plate was incubated for 15 min. The A_{550} was read after stopping the reaction by the addition of sodium hydroxide and a chelating agent in a diethanolamine buffer.

Substrate gel zymography. Isolated trophoblast cells were cultured in KGM supplemented with 10% FCS for 24 h. The media were removed, and the cultures were washed three times with s-PBS and were cultured for 48 h in KGM without serum. After 48 h, the media were harvested, the numbers of trophoblast cells were counted, and the volumes of the supernatants were normalized for cell number. The proteases secreted into the culture supernatants were analyzed by zymography. Briefly, a 12% polyacrylamide gel in 0.1% SDS containing 1 mg of gelatin (Merck, Darmstadt, Germany) per ml or 2 mg of α-casein per ml and 5 mg of plasminogen (Sigma) per ml were cast at a final dimension of 80 by 85 by 0.8 mm. Cell culture supernatants (25 μl) were reconstituted in SDS-PAGE sample buffer lacking 2-mercaptoethanol. The samples were separated on the substrate-impregnated gels at 150 V for 2.5 h. After electrophoresis, the gels were washed twice for 15 min in a solution of 2.5% Triton X-100 to remove the SDS to reactivate the proteases, rinsed briefly in water, and incubated for 16 h in PBS containing 5 mM $CaCl_2$ at 37°C. After incubation, the gels were stained for 30 min with Coomassie brilliant blue R-250 in 10% acetic acid–40% methanol and were destained in 10% acetic acid–40% methanol. The gels were then dried and photographed.

RESULTS

Isolation and culture of trophoblast subpopulations. The isolation of trophoblast subpopulations at the different trypsinization times (10-min intervals) and then percoll gradient centrifugation in most cases yielded 65 to 95% cytokeratin-positive cells before seeding. However, the success rate of culturing the isolated trophoblast cell populations with con-

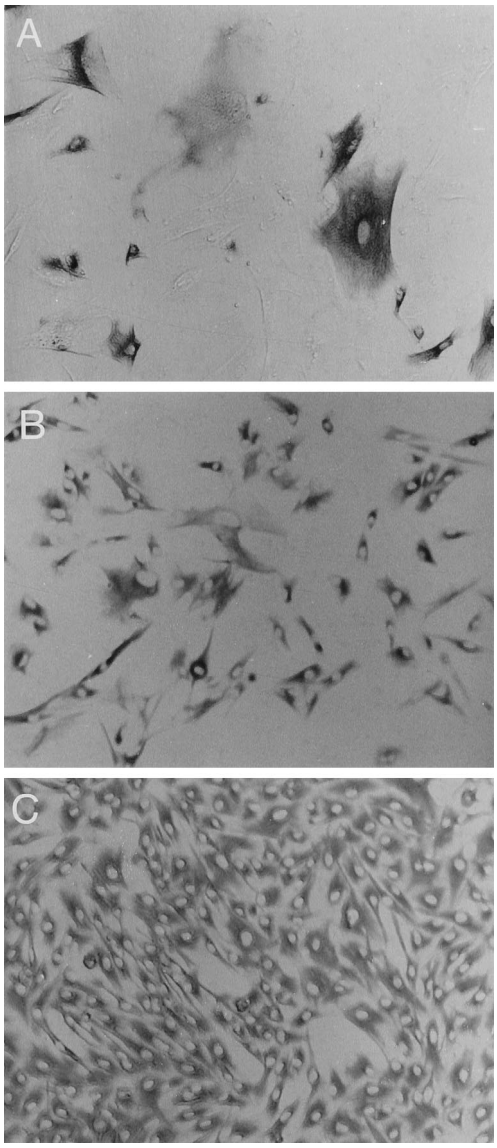

FIG. 1. Anti-CD9 immunomagnetic purification of first-trimester trophoblast cells. (A) Mixture of trophoblast (cytokeratin positive) and nontrophoblast (cytokeratin negative) cells in culture at passage 5 before the purification. (B and C) Cytokeratin-stained trophoblast cells in culture after the negative selection for anti-CD9.

firmed trophoblastic characteristics (that is cytokeratin and E-cadherin positivity) ranged between 40 and 55% after percoll gradient centrifugation and then the use of selective KGM. Cultures that proved to contain <60% cytokeratin-positive cells after three to five passages were discarded. However, cultures that yielded 60 to 95% cytokeratin-positive cells after three to five passages were subjected to negative selection by anti-CD9 immunomagnetic purification. This purification in most cases yielded >99% cytokeratin-positive cells after five additional passages in KGM supplemented with 10% FCS. The success rate of obtaining purities of >99% cytokeratin-positive cells after negative selection for anti-CD9 was >70%. The minor problem associated with the use of negative selection for anti-CD9 was the adhesion of some of the trophoblast cells to the immunomagnetic beads, although they did not bind to the antibody. In most cases this resulted in the loss of some trophoblast cells during the anti-CD9 step. Isolated trophoblast cells were identified by their expression of the epithelium-specific antigens cytokeratin and E-cadherin.

Cytokeratin staining was used to monitor the method of purification. For example, Fig. 1A shows a mixture of partially isolated trophoblast cells contaminated with cytokeratin-negative cells before using negative selection for anti-CD9 to obtain trophoblast cells. Figure 1B and C show cytokeratin-positive cells after anti-CD9 purification and culture for three and five passages, respectively. Immunofluorescence and immunocytochemical staining of the isolated trophoblast populations revealed the cultures to be negative for anti-CD68 (macrophage marker) and anti-CD9 (mesenchymal cell marker), indicating that the cultures were free of macrophages and other placental cells.

Morphologic differentiation of trophoblast populations in culture. Cultures obtained from the 10-min and the 30-min trypsinization periods displayed distinct morphologies. The 20-min cell fractions consisted of cell types from both the 10-min and the 30-min fractions. Figure 2 shows the differentiation of the 10-min trophoblast cells in culture. After 12 h of seeding, all of the cells in the cultures were mononuclear and were attached to the culture flask (Fig. 2A). However, at 4 days in culture, the 10-min cell fractions appeared oval or round and detached from the culture flasks and formed cell clumps (Fig. 2B and C). When the cell clumps were removed and further cultured in a new culture flask, they differentiated and attached to the culture flask (Fig. 2D and E). Figure 2E shows cytokeratin staining of the trophoblast cells in the 10-min fraction when they were attached to the culture flask.

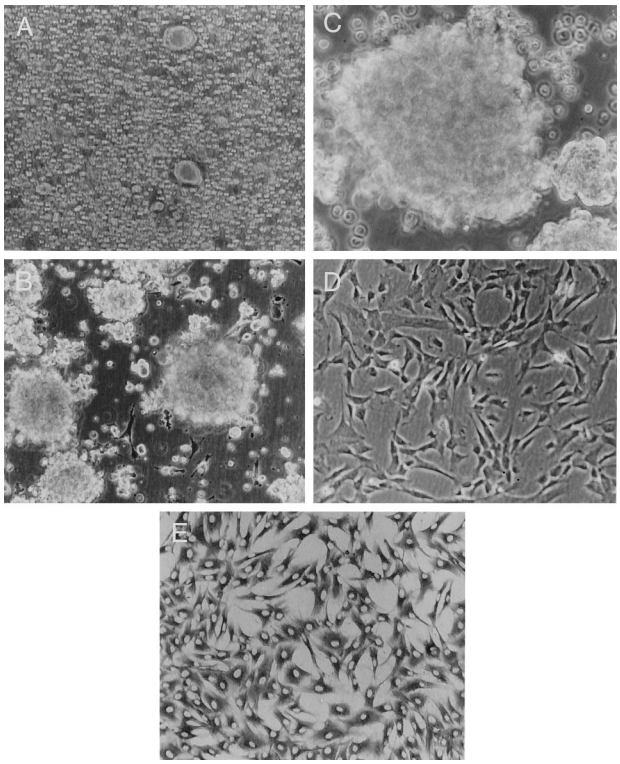

FIG. 2. Differentiation of trophoblast cells obtained from the 10-min preparation in culture. (A) Mononuclear trophoblast cells 12 to 20 h after seeding. (B and C) Floating cell clumps. (D) Differentiated and anchored trophoblast cells (passage 5). (E) Fixed and cytokeratin-stained anchored trophoblast cells (passage 5).

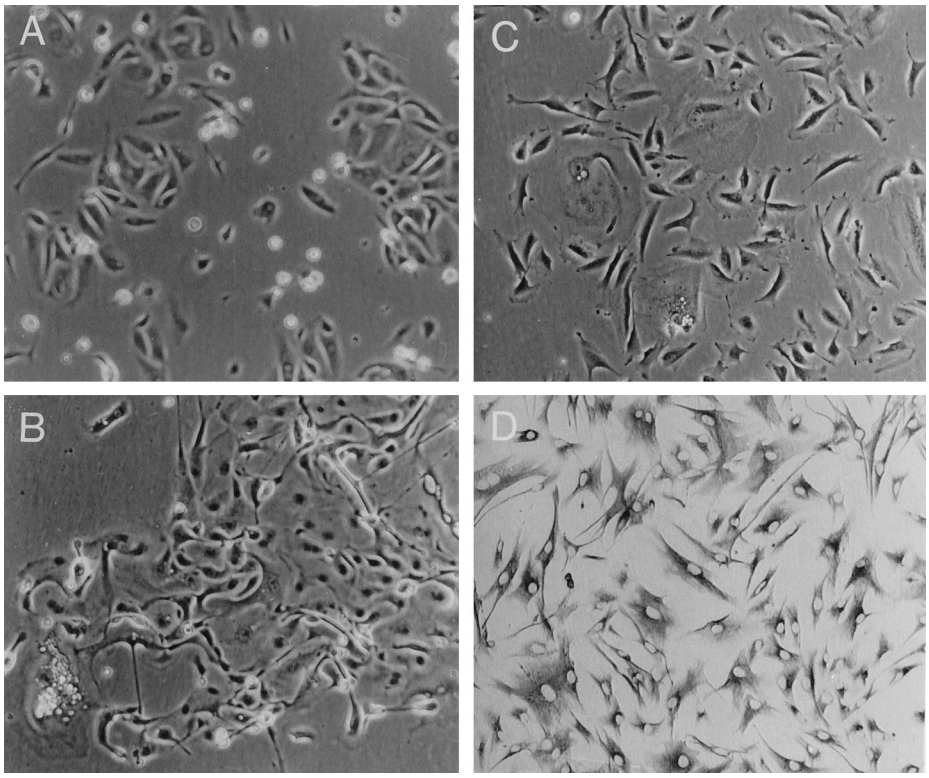

FIG. 3. Isolated trophoblast cells (30-min preparation) from young first-trimester placentae. The freshly isolated cells were mononuclear (A) but proliferated and fused to form pleomorphic interdigitating cells resembling crazy pavement (B) after 5 days in culture. Further subculturing resulted in the formation of mononuclear trophoblast cells (C) at passage 3. (D) Fixed and cytokeratin-stained trophoblast cells after passage 5 in culture.

When young placental villi (5 to 10 weeks) were used for isolation, two trophoblast subpopulations were obtained from the 30-min preparations. Usually, pleomorphic interdigitating cells resembling a "crazy pavement" contour as described previously by Loke et al. (22) were obtained (Fig. 3A and B). These interdigitating cells proliferated and fused together, and subculturing resulted in the elimination of the multinucleated cells, but a fraction of these interdigitating cells differentiated morphologically into mononuclear cells (Fig. 3C and D). Figure 3D shows cytokeratin-stained trophoblastic cells in culture obtained from the 30-min fractions of young placentae.

On a few occasions, when the young placental villi were used for the purification, trophoblast subpopulations with a flattened appearance and copious cytoplasm were also obtained (Fig. 4) from the 30-min preparation. These cells proliferate very slowly and differentiate in culture to form a multinucleated cell mass usually referred to as trophoblast giant cells. A few vacuolated trophoblasts (Fig. 4E, arrow) were also identified in such preparations. Figure 4E and F show the cytokeratin positivity of large and flattened trophoblast cells.

When villi from older placentae (>10 weeks) were used for isolation, mostly villous trophoblast cells were obtained in the 30-min fraction (Fig. 5). These villous trophoblast cultures (Fig. 5A and B) differentiated to form multinucleated syncytiotrophoblasts (Fig. 5C and D) with no ability to proliferate.

Characterization of isolated trophoblast cells. The cytokeratin positivity and E-cadherin expression by the isolated trophoblast cells from the immunocytochemical staining were confirmed by Western immunoblot analysis with mouse anti-cytokeratins (Dako-CK, clones MNF 116, 35BH11, and OV-TL 12/30) and anti-E-cadherin (HECD-1) antibodies. All of the isolated trophoblast cells expressed cytokeratins with molecular masses of between 45 and 54 kDa (Fig. 6, arrows) and the 120-kDa E-cadherin cell adhesion molecule (Fig. 7). How-

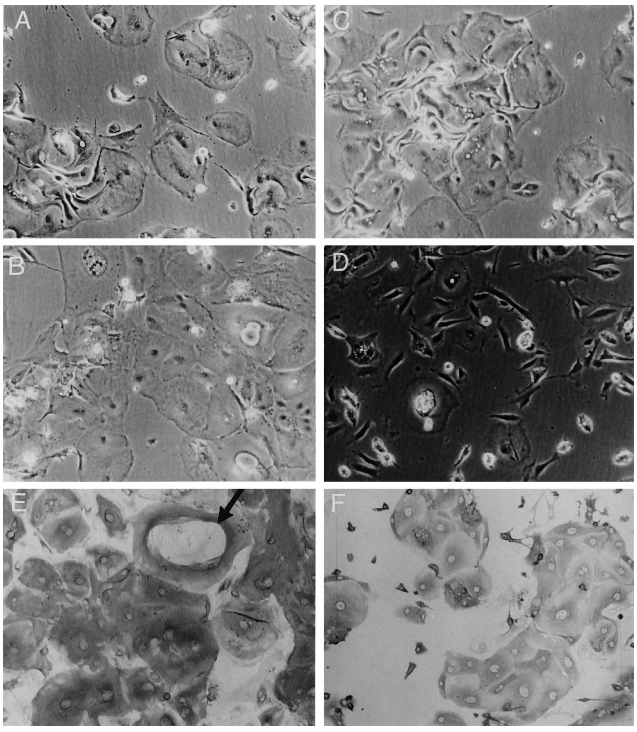

FIG. 4. Large and flattened cytotrophoblast cells sometimes obtained from the 30-min preparations from young first-trimester placentae. They appeared to be mononuclear (A) 1 to 2 days after seeding but differentiated by fusion to form a syncytial cell mass (B and C) 8 days after seeding. Further subculturing eliminated the syncytial cells, and mononuclear cells were obtained (D). (E and F) Fixed and cytokeratin-stained syncytial cell mass formed 8 days after seeding from two different preparations. (E) The arrow points to a large vacuolated trophoblast cell that was sometimes detected in the preparations.

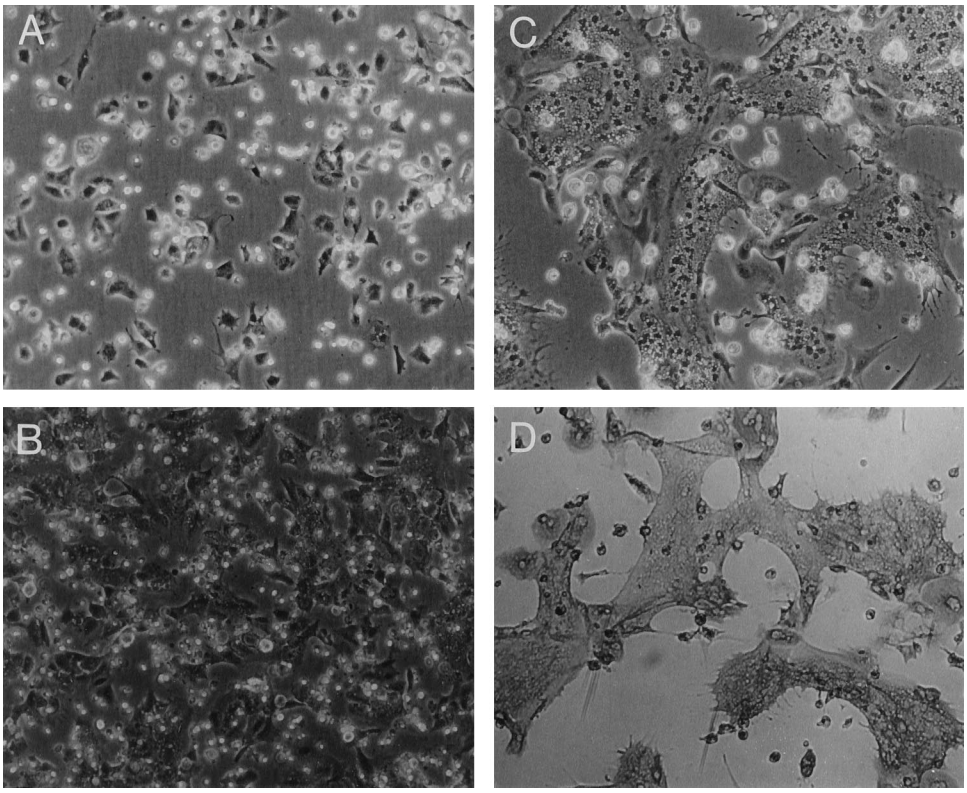

FIG. 5. Villous trophoblast cultures obtained from 30-min preparations when older first-trimester placentae were used for the isolation. Freshly isolated villous trophoblasts were mononuclear (A, 12 h after seeding; and B, 24 h after seeding) but differentiated by fusion to form syncytiotrophoblasts (C and D, 4 days after seeding). (D) Fixed and cytokeratin-stained syncytiotrophoblast cells.

ever, the extravillous trophoblast populations (as identified by additional markers; see below) coexpressed cytokeratin and vimentin, as determined by immunocytochemistry (data not shown). Other placental cells such as fibroblasts, endothelial cells and macrophages that usually contaminate the cultures were negative for cytokeratin and E-cadherin. Therefore, cytokeratin and E-cadherin proved to be the most reliable markers for the identification of isolated trophoblast cells.

The isolated trophoblast cultures were further characterized by their surface expression of MHC class 1 molecules, integrins, and NDOG1. The trophoblast population, obtained from the 10-min trypsinization (Fig. 2), stained positive for MHC class 1 molecules and expressed α1, α3, and α5 integrin subunits and the vitronectin receptor (Fig. 8), whereas the trophoblast population from the 30-min trypsinization, which fused to form syncytiotrophoblasts (Fig. 5), expressed integrin α6 and β4 subunits, as determined by Western immunoblot analysis (Fig. 9) and immunocytochemistry (Figure 10) methods. The villous trophoblasts, when differentiated to form syncytiotrophoblasts, stained strongly to NDOG1, whereas the extravillous trophoblast populations stained negative. The

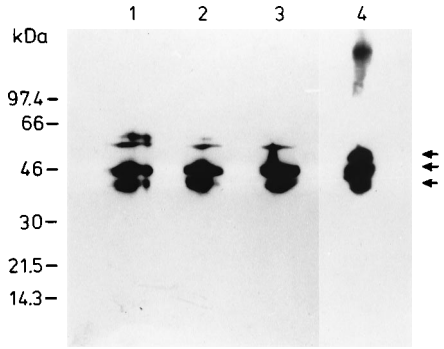

FIG. 6. Western immunoblot analysis of cytokeratins (arrows) expressed by isolated trophoblast populations. Lanes 1 and 2, cytokeratins expressed by extravillous trophoblasts from the cell clumps and the anchored trophoblast cells from the 10-min preparation, respectively (Fig. 2); lanes 3 and 4, the interdigitating trophoblast cells resembling crazy pavement (Fig. 3) and villous trophoblasts (Fig. 5), respectively. The positions of the prestained molecular mass standards are indicated by the bars on the left.

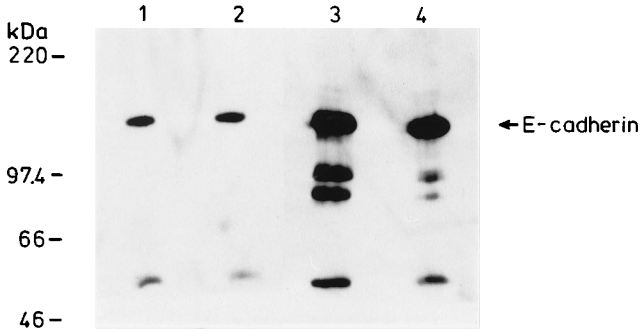

FIG. 7. Western immunoblot analysis of E-cadherin from isolated trophoblast cultures. Lane 1, expression of E-cadherin by extravillous trophoblast (10-min preparation; Fig. 2); lane 2, interdigitating trophoblast cells resembling crazy pavement (Fig. 3); lane 3, third-trimester villous trophoblast (positive control); lane 4, first-trimester villous trophoblast (Fig. 5). The positions of the prestained molecular mass standards are indicated by the bars on the left.

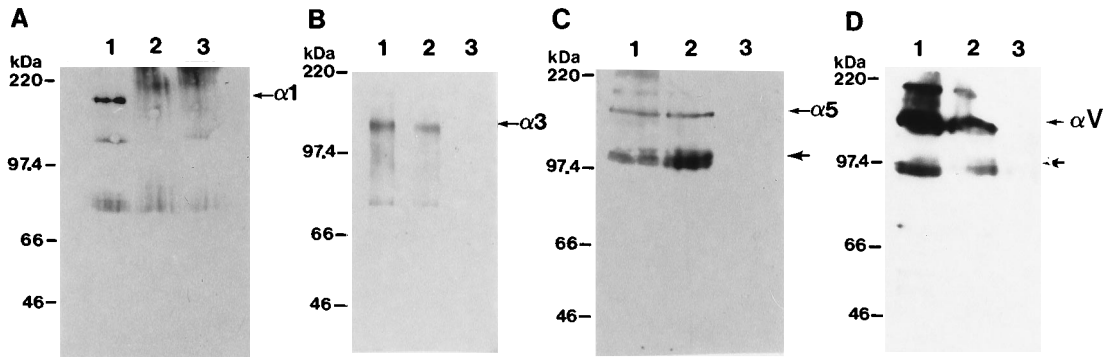

FIG. 8. Western immunoblot analysis of integrins α1 (A), α3 (B), and α5 and β1 (arrows) (C) and the vitronectin receptor (α_v and β_3; arrows) (D) by extravillous trophoblasts. Lanes 1, integrins expressed by extravillous trophoblasts (10-min preparation; Fig. 2); lanes 2, interdigitating trophoblast cells resembling crazy pavement (30-min preparation; Fig. 3); lanes 3, first-trimester villous trophoblasts (30-min preparation; Fig. 5). The positions of the prestained molecular mass standards are indicated by the bars on the left.

crazy pavement-type trophoblast cells (Fig. 3), although classified as extravillous by staining positive for MHC class 1 molecules, did not express the integrin α1 subunit but expressed the α3 and α5 subunit, the vitronectin receptor (Fig. 8), and very low levels of the α6 and β4 integrin subunits (Fig. 9) when they were analyzed by Western immunoblotting. The extravillous trophoblast from the 10- and 30-min (interdigitating trophoblast cells with the crazy pavement contour) expressed higher levels of the β1 integrin subunit than the villous trophoblast (Fig. 9). Table 1 provides a summary of the characteristics of the isolated trophoblast cells.

Hormone production by cultured trophoblast cells. The isolated trophoblast populations in culture secreted the pregnancy hormone hCG (Table 2). However, the levels of hCG secretion were different among the trophoblast populations. In short-term cultures, the intermediate extravillous trophoblast (10-min population) secreted higher levels of hCG than the villous trophoblasts (30-min population) and the contour-shaped trophoblasts (30-min population) (Table 2). When the extravillous trophoblast cells (10-min fractions) were cultured for 21 days after seeding, the level of hCG secretion was reduced significantly below 5% (data not shown).

Protease secretion by trophoblast populations. The isolated and cultured trophoblasts secreted gelatinases (with molecular masses of between 72 and 300 kDa) and plasminogen activator, as determined by substrate gel zymography in polyacrylamide gels impregnated with either gelatin or casein (in the presence of plasminogen). The first-trimester extravillous trophoblasts (from the 10-min preparation [Fig. 2]) secreted mostly the 72- and 92-kDa gelatin-degrading metalloproteases (Fig. 11A, lanes 1 and 2). The contour-shaped cells (from the 30-min preparation [Fig. 3]) and the villous trophoblast (from the 30-min preparation [Fig. 5]) secreted predominantly the 72-kDa gelatin-degrading metalloprotease and a minor amount of the 92-kDa gelatin-degrading metalloprotease (Fig. 11A, lanes 3 and 4). All of the trophoblast populations secreted plasminogen activator with a molecular mass of 52 kDa, as evaluated by gel zymography (Fig. 11B).

DISCUSSION

In recent years, much attention has been focused on the study of trophoblasts in vivo and in vitro, because little is yet known about the factors that control trophoblast proliferation, differentiation, immunology, virology, cytokenesis, and invasiveness. For in vitro studies, the use of highly purified trophoblast cells is a prerequisite, and therefore, several methods for the isolation and characterization of specific trophoblast pop-

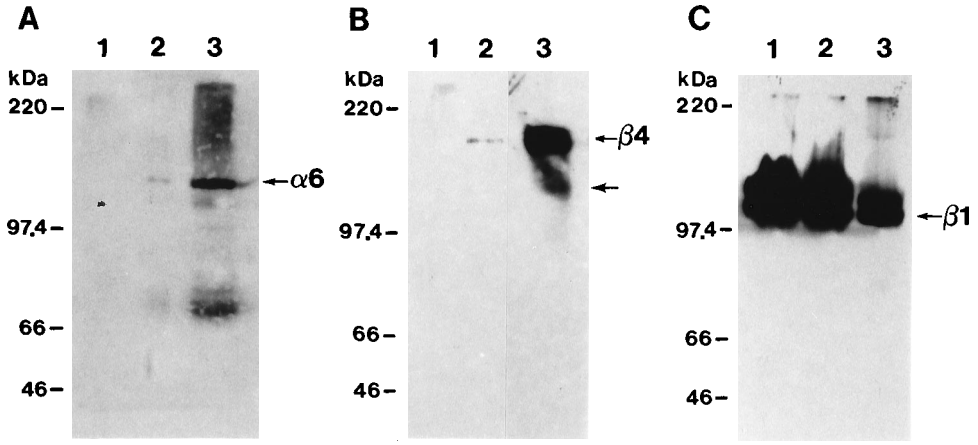

FIG. 9. Western immunoblot analysis of α6 (A), β4 and α6 (arrows) (B), and β1 (C) integrin subunits by first-trimester villous and extravillous trophoblasts. Lanes 1, integrins expressed by extravillous trophoblasts (10-min preparation; Fig. 2); lanes 2, interdigitating trophoblast cells resembling crazy pavement (30-min preparation; Fig. 3); lanes 3, first-trimester villous trophoblasts (30-min preparation; Fig. 5). The positions of the prestained molecular mass standards are indicated by the bars on the left.

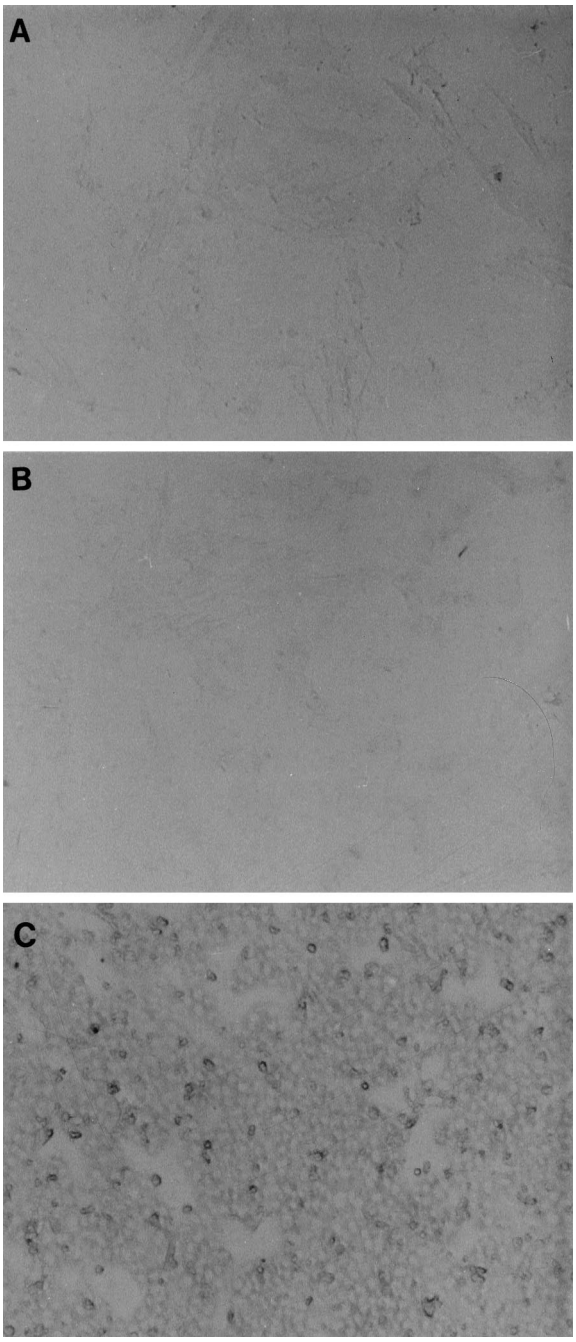

FIG. 10. Immunocytochemical staining of isolated trophoblast populations by using anti-rat α6 integrin (VLA-6, GoH3). (A and B) Negative staining for extravillous trophoblasts (from 10- and 30-min preparations). (C) Immunoperoxidase staining of first-trimester villous trophoblast.

TABLE 1. Summary of the markers used for identification and characterization of trophoblast populations

Marker	Marker presence[a]		
	10-min preparation (first-trimester extravillous trophoblast)	30-min preparation (first-trimester, crazy pavement-type trophoblast)	30-min preparation (first-trimester villous trophoblast)
Cytokeratin	++	++	++
E-cadherin (HECD-1)	+	+	++
NDOG1	−	−	+/++[b]
MHC class 1 (W6/32)	+	+	−
Vimentin	+	+	−
hCG	++[c]	++[c]	++
hPL	++	+	−/++[d]
Integrins			
α6 (clone GoH3)	−	−/+[e]	++
β4 (clone 3E1)	−	−/+[e]	++
β1 (clone P4C10)	++	++	+
α1	++	−	−
α3	++	++	−
α5 (clone p1D6)	++	++	−
Vitronectin receptor (αVβ3/β5)	++	++	−
CD68	−	−	−
CD9	−	−	−

[a] +, weakly positive; ++, strongly positive; −, negative.
[b] Mononuclear villous trophoblasts stained weak, whereas syncytiotrophoblasts stained very strong.
[c] hCG secretion in short-term cultures.
[d] Stained negative on mononuclear villous trophoblasts but stained strongly on syncytiotrophoblasts.
[e] Undetectable by immunocytochemistry, but a weak band was detected by Western immunoblot analysis.

ulation have been reported (13, 23, 33). The differential expression of MHC class 1 (7) and CD9 (26, 34) molecules on specific placental cells has been used to isolate and characterize villous trophoblasts from term placentas. The method reported here uses sequential enzymatic dissociation and, in some cases, sequential enzymatic dissociation together with negative selection with anti-CD9 coupled to magnetic beads for the isolation of trophoblast populations. The use of sequential enzymatic dissociation as the first step enables the sequential dissociation of villous and extravillous trophoblasts in a stepwise manner from the first-trimester placenta. However, the type of trophoblast populations isolated by this method depends on the age of the placenta and the time of trypsinization.

For the identification of isolated trophoblasts, we found cytokeratin and E-cadherin which are specifically expressed by the trophoblast cells in situ to be the best markers. However, the differentiation between villous and extravillous trophoblast populations was based on the fact that they vary in their levels of expression of cell surface proteins such as integrins, MHC class 1 molecules, and hyaluronic acid (NDOG1). Villous trophoblasts express high levels of α6 and β4 and low levels of β1 integrin subunits, whereas extravillous trophoblasts express high levels of α1, α3, α5, and β1 integrin subunits, vitronectin receptors, and MHC class 1 molecules on the cell surface. The

TABLE 2. hCG secretion by isolated first-trimester trophoblast populations

Day(s) in culture	hCG secretion (U/liter)[a]		
	10-min preparation	30-min preparation (crazy pavement type)	30-min preparation (villous)
1	2,226 ± 144	108 ± 16	228 ± 22
3	7,886 ± 224	654 ± 48	779 ± 36
5	11,150 ± 280	1,000 ± 64	1,370 ± 78

[a] hCG secretion per 10^7 cells in culture. Data are the means ± standard deviations (n = 3).

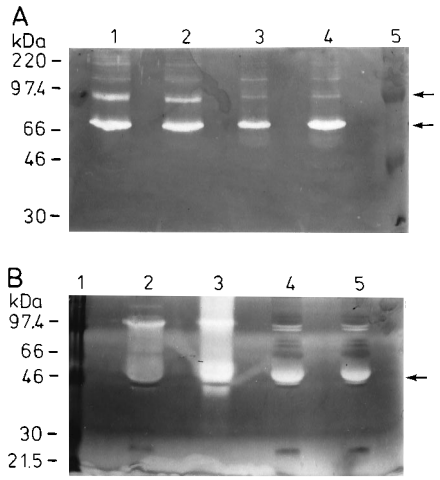

FIG. 11. Zymograms showing gelatinase-degrading metalloproteases (A) and plasminogen activator (B) secreted by cultured trophoblasts. (A) Lanes 1 and 2, gelatinases secreted into the culture medium by the cell clumps and anchored extravillous trophoblast (10-min preparation; Fig. 2), respectively; lanes 3 and 4, gelatinases secreted by the interdigitating trophoblast cells resembling crazy pavement (30-min preparation; Fig. 3) and first-trimester villous trophoblasts (30-min preparation; Fig. 5), respectively (the two arrows indicate the major gelatinases of 72 and 92 kDa secreted by the extravillous trophoblast population); lane 5, molecular mass markers. (B) Lane 1, molecular mass markers; lanes 2 and 3, plasminogen activator (arrow) secreted by the cell clumps and anchored extravillous trophoblasts (10-min preparation; Fig. 2), respectively; lanes 4 and 5, plasminogen activator (arrow) secreted by the interdigitating trophoblast cells resembling crazy pavement (30-min preparation; Fig. 3) and first-trimester villous trophoblasts (30-min preparation; Fig. 5), respectively.

transient expression of both classical HLA (HLA A, B, and C) as well as nonclassical HLA (HLA G) molecules on some extravillous trophoblast populations has been reported by others (29). The level of expression of the nonclassical HLA molecules on the isolated trophoblast populations remains to be determined. The use of hCG and hPL as markers for trophoblasts is well described in the literature (9, 16, 17, 28), but they are not uniform in their positivity with all trophoblast subpopulations in long-term cultures. Therefore, hCG, NDOG1, and hPL may be useful for the further characterization of individual trophoblast subpopulations.

Expression of the intermediate filament protein vimentin and reduced levels of or the loss of the cellular adhesion protein E-cadherin have been reported to be associated with the increased invasiveness of some cancer cell lines of epithelial origin in vivo and in vitro (3, 31). Villous trophoblasts express high levels of E-cadherin but do not express vimentin, whereas the extravillous trophoblast populations express vimentin and lower levels of E-cadherin in vitro, a characteristic similar to that of some invasive cancer cells of epithelial origin. It has also been reported that E-cadherin function is required for the formation of the trophectoderm. In situ staining has shown strong staining of E-cadherin in villous trophoblast cells (nonpolarized trophoblasts) and weak staining in the invading extravillous trophoblast cells (5, 6, 30). Our data showing the decreased levels of expression of E-cadherin in the isolated extravillous trophoblast cells is consistent with reports of reduced levels of E-cadherin expression in some invasive tumor cells of epithelial origin.

Coexpression of cytokeratin and vimentin expression by extravillous trophoblast populations was reported earlier by Loke and Butterworth (21). Furthermore, cytokeratin and vimentin expression has been reported in cultured epithelial cell lines (32) and also in human metastatic carcinoma cells (25, 27). It has therefore been proposed that vimentin expression occurs after a cell detaches from its epithelial sheets (19) and may influence both mitotic activity and motility and thus might affect the invasive property of a particular cell. Vimentin expression by the isolated extravillous trophoblast cells might therefore be significant with regard to their invasive properties.

The isolated trophoblast populations secreted metalloproteinases, such as the 72- and the 92-kDa gelatinases and the 52-kDa plasminogen activator. The extravillous trophoblast population (from the 10-min preparation) predominantly secreted the 92-kDa gelatinase, whereas the villous trophoblasts and the crazy pavement-type trophoblasts mainly secreted the 72-kDa gelatinase. Of these metalloproteinases, the 92-kDa gelatinase has been reported (20) to regulate the invasive properties of extravillous trophoblasts. The various levels of secretion of the 92-kDa gelatinase by the isolated trophoblast populations probably suggests a difference in the invasive capacities of the cells. Ongoing characterization may reveal such properties.

The acquisition of motility and invasiveness by epithelial cells have been reported (24) to correlate with dramatic changes in the state of differentiation: migrating cells acquire mesenchymal properties, for example, downregulation of $\alpha 6$ and $\beta 4$ subunits and upregulation of the expression of integrin $\alpha 1\beta 1$ and $\alpha 5\beta 1$ complexes. Such profound changes are also observed during cytotrophoblast differentiation to form the invasive phenotype. Our results showed that isolated villous trophoblasts in vitro express $\alpha 6$ and $\beta 4$ integrin subunits and reduced levels of $\beta 1$ integrin subunits, which is characteristic of polarized epithelial cells in contact with the basement membrane, whereas the extravillous trophoblast population expresses mesenchymal-type integrins $\alpha 1$, $\alpha 3$, and $\alpha 5$, high levels of $\beta 1$, and vitronectin receptors, as has also been reported by others (5, 13) and which has been postulated to regulate trophoblast migration and invasion (5, 12). The availability of the reported method for the simultaneous isolation of the individual trophoblast subpopulations should facilitate ongoing in vitro studies of trophoblast proliferation, differentiation, migration, and invasion and how these events differ from those of malignant cells.

REFERENCES

1. **Aplin, J. D.** 1993. Expression of integrin $\alpha_6\beta_4$ in human trophoblast and its loss from extravillous cells. Placenta **14:**203–215.
2. **Bass, K. E., D. Morrish, I. Roth, D. Bhardwaj, R. Taylor, Y. Zhou, and S. J. Fisher.** 1994. Human cytotrophoblast invasion is up-regulated by epidermal growth factor: evidence that paracrine factors modify this process. Dev. Biol. **164:**550–561.
3. **Birchmeier, W., K. M. Weidner, and J. Behrens.** 1993. Molecular mechanisms leading to loss of differentiation and gain of invasiveness in epithelial cells. J. Cell Sci. Suppl. **17:**159–164.
4. **Bulmer, J. N., W. D. Billington, and P. M. Johnson.** 1984. Immunohistologic identification of trophoblast populations in the early human pregnancy with the use of monoclonal antibodies. Am. J. Obstet. Gynecol. **148:**19–26.
5. **Damsky, C. H., C. Librach, K.-H. Lim, M. L. Fitzgerald, M. T. McMaster, M. Janatpour, Y. Zhou, S. K. Logan, and S. J. Fisher.** 1994. Integrin switching regulates normal trophoblast invasion. Development **120:**3657–3666.
6. **Damsky, C. H., A. E. Sutherland, and S. J. Fisher.** 1993. Adhesive interactions in early mammalian embryogenesis, implantation and placentation. FASEB J. **7:**1320–1329.
7. **Douglas, G. C., and B. F. King.** 1989. Isolation of pure villous cytotrophoblast from term human placenta using immunomagnetic microspheres. J. Immunol. Methods **119:**259–268.
8. **Fisher, S. J., T.-Y. Cui, L. Zhang, L. Hartman, K. Grahl, Z. Guo-Yang, J. Tarpey, and C. H. Damsky.** 1989. Adhesive and degradative properties of human placental cytotrophoblast cells *in vivo*. J. Cell Biol. **109:**891–902.
9. **Gosseye, S., and H. Fox.** 1984. An immunohistochemical comparison of the secretory capacity of villous and extravillous trophoblast in the human placenta. Placenta **5:**329–348.
10. **Graham, C. H., and P. K. Lala.** 1991. Mechanism of control of trophoblast invasion in situ. J. Cell. Physiol. **148:**228–234.

11. **Graham, C. H., J. J. Lysiak, K. R. McCrae, and P. K. Lala.** 1992. Localization of transforming growth factor-β at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biol. Reprod. **46:**561–572.
12. **Irving, J. A., and P. K. Lala.** 1995. Functional roles of cell surface integrins on human trophoblast cell migration: regulation by TGF-β, IGF-II and IGFDP-1. Exp. Cell Res. **217:**419–427.
13. **Irving, J. A., J. J. Lysiak, C.-H. Graham, V. K. M. Han, S. Hearn, and P. K. Lala.** Characteristics of trophoblast cells migrating from first trimester chorionic villous explants and propagated in culture. Placenta, in press.
14. **Kliman, H. J., J. E. Nestler, E. Sermasi, J. M. Sanger, and J. F. Strauss III.** 1986. Purification, characterization and in vitro differentiation of cytotrophoblasts from human placentae. Endocrinology **118:**1567–1582.
15. **Korhonen, M., J. Ylanne, L. Laitinen, H. M. Cooper, V. Quaranta, and I. Virtanten,** 1991. Distribution of the α1-α6 integrin subunits in human developing and term placenta. Lab. Invest. **65:**347–356.
16. **Kurman, R. J., C. S. Main, and H. C. Chen.** 1984. Intermediate trophoblast: a distinctive form of trophoblast with specific morphological biological and functional features. Placenta **5:**329–348.
17. **Kurman, R. J., R. H. Young, H. J. Norris, C. S. Main, M. D. Laurence, and R. E. Scully.** 1984. Immunocytochemical localization of placental lactogen and chorionic gonadotrophin in the normal placenta and trophoblastic tumours with emphasis on intermediate trophoblast and placental site trophoblastic tumour. Int. J. Gynaecol. Pathol. **3:**101–102.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
19. **Lane, E. B., B. L. M. Hogan, M. Kurkinen, and J. I. Garrels.** 1983. Coexpression of vimentin and cytokeratins in parietal endodermal cells of early mouse embryo. Nature (London) **303:**701–704.
20. **Librach, C., Z. Werb, M. Fitzgerald, K. Chiu, N. Corwin, R. Esteves, D. Grobelny, R. Galardy, C. Damsky, and S. Fisher.** 1991. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J. Cell Biol. **112:** 437–449.
21. **Loke, Y. W., and B. H. Butterworth.** 1987. Heterogeneity of human trophoblast populations, p. 197–209. *In* T. J. Gill and T. G. Wegmann (ed.), Immunoregulation and fetal survival. Oxford University Press, New York.
22. **Loke, Y. W., B. H. Butterworth, J. J. Margetts, and K. Burland.** 1986. Identification of cytotrophoblast colonies in cultures of human placental cells using monoclonal antibodies. Placenta **7:**221–231.
23. **Loke, Y. W., L. Gardner, and A. Grabowska.** 1989. Isolation of human extravillous trophoblast cells by attachment to laminin-cated magnetic beads. Placenta **10:**407–415.
24. **Matthay, M. A., J.-P. Thiery, F. Lafon, M. F. Stampfer, and B. Boyer.** 1993. Transient effect of epidermal growth factor on the motility of an immortalized mammary epithelial cell line. J. Cell Sci. **106:**869–878.
25. **McNutt, M. A., J. W. Bolen, A. M. Gown, S. P. Hammar, and A. M. Vogel.** 1985. Coexpression of intermediate filaments in human epithelial neoplasms. Ultrastruct. Pathol. **9:**31–43.
26. **Morrish, D. W., A. R. E. Shaw, J. Seehafer, D. Bhardwaj, and M. T. Paras.** 1991. Preparation of fibroblast-free cytotrophoblast cultures utilizing differential expression of the CD9 antigen. In Vitro Cell Dev. Biol. **27A:**303–306.
27. **Ramaekers, F. C. S., D. Haag, A. Kant, O. Moesker, P. H. K. Jap, and G. P. Vooijs.** 1983. Coexpression of keratin and vimentin-type intermediate filaments in human metastatic carcinoma cells. Proc. Natl. Acad. Sci. USA **80:**2618–2622.
28. **Sabet, L., D. Daya, R. Stead, H. Richmond, and C. L. Jimeneze.** 1989. Significance and value of immunohistochemical localization of pregnancy-specific proteins in feto-maternal tissue throughout pregnancy. Mod. Pathol. **2:**227–232.
29. **Shorter, S. C., P. M. Starkey, B. L. Ferry, L. M. Clover, I. L. Sargent, and C. W. Redman.** 1993. Antigenic heterogeneity of human cytotrophoblast and evidence for transient expression of MHC class 1 antigens distinct from HLA-G. Placenta **14:**571–582.
30. **Takeichi, M.** 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. Science **251:**1451–1455.
31. **Umbas, R., J. A. Schalken, T. W. Aalders, B. S. Carter, H. F. M. Karthaus, H. E. Schaafsma, F. M. J. Debruyne, and W. B. Isaacs.** 1992. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res. **52:**5104–5109.
32. **Virtanan, I., V.-P. Lehto, E. Lehtonen, T. Vartio, S. Stenman, P. Kurki, O. Wager, J. W. Small, D. Dahl, and R. A. Bradley.** 1981. Expression of intermediate filaments in cultured cells. J. Cell Sci. **50:**45–63.
33. **Yeger, H., L. D. Lines, P.-Y. Wong, and M. M. Silver.** 1989. Enzymatic isolation of human trophoblast and culture on various substrates: comparison of first trimester with term trophoblast. Placenta **10:**137–151.
34. **Yui, J., M. Garcia-Lloret, A. J. Brown, R. C. Berdan, D. W. Morrish, T. G. Wegmann, and L. J. Guilbert.** 1994. Functional, long term trophoblasts purified by column-elimination of CD9 expression cells. Placenta **15:**231–246.