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Isolation of Hofbauer Cells from Human Term Placentas with High Yield and Purity

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Abstract

Problem—Placental villus macrophages (i.e. Hofbauer cells, HBCs) were identified more than 100 years ago. Alterations in their numbers and characteristics are associated with several complications of pregnancy. Although HBCs have previously been isolated and cultured, there is no consensus methodology to obtain these cells with high yield and purity for *in vitro* studies.

Method of Study—HBCs were isolated from human term placentas using protocols in which cytotrophoblasts (CTs) and fibroblasts (FIBs), other major villous cell types, were isolated in parallel. Enzymatic digestion, Percoll gradients, and immunoselection were used to isolate the three cell types. Purity was assessed by morphology, flow cytometry, and in phagocytosis assays.

Results—HBCs were isolated with 98–99% purity and a yield of 130–200 × 10⁶ cells/80 to 100 g of tissue. HBCs exhibited a pleiomorphic and vacuolated appearance for at least 5 days in culture medium with and without serum. High levels of phagocytosis in HBCs, but not in CTs, or FIBs, confirmed macrophage function in HBCs. Phagocytotic activity was maintained across several days in culture.

Conclusion—HBCs were isolated from term placenta with high yield and purity using protocols in which CTs and FIBs were also obtained. This methodology will foster future studies which examine the role of HBCs in regulating villus function.

Keywords

Placenta; fetal macrophages; Hofbauer cells; cell isolation and culture

INTRODUCTION

The human placental villus is composed of syncytiotrophoblast (SCT), the outer cell layer lining the intervillous space and in direct contact with maternal blood, and underlying stromal cells, which are adjacent to fetal capillaries, and consist of fibroblasts (FIBs) and Hofbauer cells (i.e. HBCs, fetal tissue macrophages).^{1, 2} More than 100 years ago HBCs were first described in the placental villus by several investigators, and morphological studies by Hofbauer and others revealed these large (10–30 μm) pleiomorphic cells to be highly vacuolated with a granular cytoplasm.^{1, 2} HBCs appear on the 18th day of gestation and are found until term.³ Villous stromal compression in mid-gestation makes their identification difficult in the third trimester prompting the use of immunohistochemistry

with antibodies raised against macrophage proteins (*e.g.* CD68, CD163).⁴ 5 HBCs were demonstrated to be fetal in origin based on the use of Y chromosome-specific probes in pregnancies with a male fetus.⁵ 6

Phagocytosis of apoptotic bodies and cellular debris, as well antigen presentation in response to inflammation and infectious agents, are considered to be general functions of tissue macrophages.⁷ However, how these processes are modulated in HBCs during normal pregnancy, as well as potential disruption in pregnancy complications, remains unelucidated. Reports from several groups indicate that HBCs may play a key role in early placental development by regulating angiogenesis,⁸ vasculogenesis,⁹ and maturation of the placental mesenchyme.¹⁰ Changes in numbers of HBC's or alterations in their characteristics are noted in complications of pregnancy including villitis of unknown etiology (VUE), a destructive inflammatory lesion of the chorionic villi which is associated with intrauterine fetal growth restriction and significant perinatal morbidity and mortality.¹¹ 12 VUE is characterized by an influx of CD8⁺ maternal T lymphocytes and HBCs to the placental villus.⁵ 6 13 14 In contrast to VUE, histological chorioamnionitis (HCA) is most often caused by ascending genital tract microorganisms which stimulate the infiltration of neutrophils in maternal decidua and fetal membranes, with or without a neutrophilic response in the placenta and fetus.¹⁵ Levels of HBCs in the placental villus have been reported to increase¹⁶ and decrease¹⁷ in HCA. Based on immunohistochemical analysis, our group recently reported that there was a 2 to 3-fold focal increase in HBCs in the villus stroma of placentas from pregnancies with HCA compared to gestational age-matched controls.¹⁸

The employment of *in vitro* culture systems enables the analysis of HBC cell function and potential insight into the role HBCs play in placental pathophysiology in adverse pregnancy outcomes. Indeed, several studies have described biological responses of HBCs following their isolation from term placenta.¹⁹–²⁵ Early methodologies used digestion and homogenization to disrupt villous tissue, and then Percoll and Ficoll gradients to separate cell types based on their densities.²¹ 24 26 Strong adherence of HBCs to tissue culture plastic compared to other cell types was also utilized in purification strategies.⁸ 24 27 Later approaches removed contaminating cytotrophoblasts (CTs) through negative immunoselection techniques with antibodies to epidermal growth factor receptor and magnetic beads.²² 25 However, unlike methodologies used to isolate CTs which have remained largely unchanged for more than 20 years,²³ 28–³¹ there appears to be no consensus regarding a specific methodology which can be used to consistently isolate HBCs in high yield and purity, indicating that no standard technique is currently available. Thus, the goal of the current study was to develop a protocol for the isolation of HBCs in high yield and purity which could foster more mechanistic studies of HBC function.

MATERIALS AND METHODS

Collection of Placentas

Placentas (n=8) from uncomplicated term pregnancies were brought to the laboratory within 30 min following elective cesarean section without labor at New Haven Hospital. Infection was excluded on the basis of standard clinical criteria (absence of fever, uterine tenderness, maternal/fetal tachycardia, foul vaginal discharge) Tissues were then processed immediately for isolation of placental cell cultures. Each placenta was processed separately (*i.e.* tissues were not pooled). Approval for this study was granted by the Yale University School of Medicine Human Investigation Committee.

Isolation of cytotrophoblasts (CTs), Hofbauer cells (HBCs) and fibroblasts (FIBs)

Isolation of the three cell types was initiated through protocols previously employed to obtain CTs using Trypsin/DNase I digestion and discontinuous Percoll gradient fractionation, employing slight modifications of published methodologies.^{23, 28, 31, 32} Villous tissue was dissected free of membranes, minced, and rinsed with calcium- and magnesium-free phosphate-buffered saline (PBS) (see Table 1 for the sources of reagents used for cell isolation). Membrane-free villous tissue fragments were then subjected to sequential enzymatic digestions in a solution containing 0.25% trypsin, 0.2% DNase I, 25 mM HEPES, 2 mM CaCl₂, and 0.8 mM MgSO₄ in Hanks' Balanced Salt Solution (HBSS) at 37°C. Undigested tissue was removed by passage through gauze and a 100 µm sieve. The first digestion was carried out for 15 min in 150 ml of digestion solution, the supernatant was discarded, and the tissue was washed 3 times using PBS. The following digestions were carried out for 30 min in 150 ml and 200 ml of digestion solution, respectively. Cell pellets from the second and third digestions were resuspended using 1:1 mixture of DMEM/F12 with 10%FBS and 1% antibiotic-antimycotic (*i.e.* DF medium). Trypsin and DNase-treated tissue was saved for isolation of HBCs (see below). The resuspended cell pellets were separated on a discontinuous gradient of Percoll (50%/45%/35%/30%) by centrifugation using a Beckman TJ-25 centrifuge and a TS-5.1–500 swinging bucket rotor (Beckman Instruments, Fullerton, CA, USA) for 20 min at room temperature without brake at 1000 × g. Cells migrating to the 35%/45% Percoll interface were recovered by centrifugation at 300 × g for 10 min at room temperature, and were immunopurified by negative selection by simultaneous treatment with mouse anti-human CD9, mouse anti-human CD45 antibody, and then goat anti-mouse IgG conjugated DynaBeads (see Figure 1 for a description of the isolation protocol and Table 2 for antibodies used in cell isolations and flow cytometry). CD9 and CD45 antibodies were added at a ratio of 1 µg antibody per 10⁶ cells, and incubations were carried out for 15 min at 4°C on a BD Clay ADAMS® nutator (Franklin Lakes, NJ, USA). Cells were then incubated with goat anti-mouse IgG conjugated DynaBeads, at ratio of 50 µl beads per 10⁷ cells, for 30 min at 4°C with rotation. Cells were then pelleted and washed using DF medium to remove excess unbound antibodies. Dynabeads with attached contaminating cell types were removed by exposure to a magnet for 5 min, and were saved for culture of FIBs (see below). Cells from the supernatant were pelleted and purity of CTs was assessed by methodologies described below.

HBCs were isolated from trypsin-treated tissue fragments (see above and Figure 1) which were washed using PBS and further digested with collagenase A (1mg/ml)/DNase I (0.2 mg/ml) in RPMI-1640 containing 25 mM HEPES, 5% FBS, and 1% antibiotic-antimycotic (RPMI medium) for 1 h at 37°C. Cells were pelleted and resuspended in RPMI medium and were loaded onto a discontinuous Percoll gradient (40%/20%) and centrifuged for 20 min in a swinging bucket rotor without brake. Cells from the 40%/20% Percoll interface were pelleted and resuspended in RPMI medium. The cells were then loaded onto a second discontinuous Percoll gradient (35%/30%/25%/20%) and centrifuged for 30 min. Cells from 20%/25% to 30%/35% interfaces were combined and were immunopurified by negative selection using sequential treatment with anti-EGFR and then anti-CD10 antibodies conjugated to magnetic beads. The beads were prepared by incubating 1 µg of mouse anti-human EGFR25 or mouse anti-human CD10 antibody with 50 µl goat anti-mouse IgG antibody conjugated DynaBeads for 1 h at room temperature (Table 2). Beads were then exposed to a magnet and washed 3 times using PBS containing 0.1% BSA and 2 mM EDTA to remove unbound antibody, and were resuspended in this same solution and stored at 4°C. For HBC cell isolation, Percoll gradient purified cells were incubated on ice with anti-EGFR antibody-conjugated magnetic beads for 15 min followed by anti-CD10 conjugated magnetic beads for 15 min. Cells from the supernatant were counted using a hemocytometer, then plated in RPMI medium, and floating and weakly attached cells washed off after 1 h

and then DF medium or serum-free medium (DMEM/F12 with 50 µg/ml ascorbic acid and ITS+ Premix, an universal culture supplement which yields a final concentration of insulin of 6.25 mg/ml; transferrin, 6.25 mg/ml; selenous acid, 6.25 ng/ml; bovine serum albumin, 1.25 mg/ml; and linoleic acid, 5.35 µg/ml) was added. HBCs were maintained for the indicated period of time prior to analysis by flow cytometry and in phagocytosis assays. Cells were plated at a density of 1.2×10^6 cells per well of a 12-well plate for morphological analysis and phagocytosis assays, and at 7×10^6 cells per T-25 flask for flow cytometric analysis.

Cultures of FIBs were obtained from cells attached to magnetic beads containing CD9 and CD45 antibodies from CT isolations, and those attached to anti-CD10 beads from HBC preparations (Figure 1). The bead-cell mixtures were washed and cultured in DF medium and fresh medium was added every 2–3 days until confluency was reached after approximately 2 to 3 weeks. Following trypsinization of first passage cells, magnetic beads with attached cells, comprising approximately 10% of the cell population, were removed with a magnet. Passage three FIBs were used in flow cytometric and phagocytosis assays.

Flow cytometry

CTs were fixed on day 0 (*i.e.* prior to plating) by incubation with 4% paraformaldehyde/PBS for 10 min at room temperature and were stored in PBS at 4°C. For HBCs, cells were detached from the substratum using 5 mM EDTA and 4 mg/ml lidocaine-HCl in HBSS33[−]35 and fixed and stored as above after 1 h, 1 day and 2 days of culture. FIBs were analyzed following trypsinization of passage 3 cultures. To detect surface antigen, 2×10^5 paraformaldehyde fixed cells were washed once using staining buffer (0.5% BSA/PBS). For analysis of intracellular and surface antigen (*i.e.* total) expression, staining buffer containing 1% saponin was used to permeabilize cells. Blocking of Fcγ receptor was then carried out by incubating fixed cells with human IgG (100 µg/ml) in staining buffer for 15 min at room temperature. Appropriate primary antibodies and isotype-matched controls were then added (Table 2), and incubated for 45 min at 4°C. Cells were then washed twice with staining buffer. For conjugated antibodies, cells were then resuspended in 300 µl of PBS for analysis. For unconjugated antibodies, cells were resuspended in staining buffer and incubated with secondary antibodies conjugated to fluorescent probes for another 45 min at 4°C. Cells then were washed and resuspended in 300 µl of PBS for analysis. Ten thousand events were collected using FACSCalibur and CellQuest software (BD Biosciences). The results were analyzed using FlowJo software (Tree Star, Ashland, OR). The following placental cell specificities have been attributed to the chosen antibodies: CTs- EGFR,36 CD10,37 cytokeratin 7;38 HBCs- CD68, CD163-B;39 40 FIBs- CD10,41 vimentin,39 42 CD90;43 and leukocyte marker-CD45.40 44 Note, cells were not permeabilized to examine expression of EGFR, CD10, CD90, CD45, and CD163 (Figures 2 Figure 3); permeabilized cells were used to examine expression of cytokeratin 7; CD68, CD163, and vimentin. The percentage of positive cells was based on comparison with the isotype-matched control antibody, for which gating was set at 1%.

Representative flow cytometry results are shown from the following number of independent experiments from different placentas for each of the three cell types: HBCs (n=8); CTs (n=4); FIBs (n=4).

Phagocytosis assay

Ten µl containing 3.6×10^8 of 1 µm yellow-green fluorescent carboxylate microspheres (Invitrogen, Carlsbad, CA) were resuspended in 1 ml of DF medium or serum-free culture medium pre-warmed to 37°C. Cells then were then incubated with the suspension of microspheres for 1 h at 37°C. Cells were then washed at least four times with PBS (*i.e.* until

microscopy revealed that nearly all of the non phagocytosed microspheres were removed). Cells were fixed using 4% paraformaldehyde and stored in PBS at 4°C. Photographs were captured using a 40× objective with an Olympus IX71 fluorescent microscope. A representative experiment from 3 independently conducted ones from 3 different placentas is shown for each cell type.

RESULTS

Isolation of HBCs from human term placentas

To analyze cell purity, flow cytometric analysis with several cell-type-specific markers was carried out for HBCs maintained for 1 h in culture and compared to results obtained for CTs and FIBs. It is important to note that flow cytometry was carried out for HBC cultures detached after 1 h of culture, for uncultured CTs, and from third passage FIBs. As expected, HBCs, but not CTs or FIBs, expressed the leukocyte marker CD45 (Figure 2). This is indicated by the solid line histogram obtained in the presence of anti-CD45 antibody which is right-shifted in relation to signals obtained with an isotype-matched control antibody denoted by the dashed line. Of note, significant CD68 expression was observed for all three cell types, indicating that CD68 was not a specific marker for HBCs as determined by flow cytometry. This supports observations made by others showing expression of CD68 in non-myeloid cell types.⁴⁵ Conversely, the macrophage marker CD163 was not expressed by CTs and FIBs, but nearly 100% of HBCs expressed this protein. CD90, a protein expressed by FIBs, stem cells, and other non-macrophage cell types⁴³⁻⁴⁶, was strongly detected in FIBs, not in CTs, and very low levels of expression were noted in HBCs. As expected, the cytotrophoblast/epithelial cell marker cytokeratin 7 was only expressed by CTs, and the fibroblast/mesenchymal cell marker vimentin was only expressed in FIBs. The yield and purity of HBCs, using CD163 as a macrophage marker, were analyzed in 8 independent experiments and are summarized in Table 3. From 80 to 100 g of villous tissue we obtained a final yield of HBCs which ranged from 130 to 200×10⁶ cells per preparation with cell purity of 98 or 99%. These results indicate that these protocols can be used to consistently isolate HBCs from term placenta with excellent purity and high yield.

Culture of HBCs from human term placentas

Morphological characteristics of HBCs were evaluated after maintenance in DF medium, which contains 10% FBS, or in serum-free medium for up to 5 days (Figure 3). We noted that cells maintained in serum were approximately 10–20 μm in size, and manifested a pleiomorphic phenotype with vacuoles on day 1 (Figure 3A). The shape varied from round to partially elongate. These characteristics are similar to those described for HBCs in the placental villus.¹ On days 3 and 5 (Figure 3B to 3C) cells increasingly formed colonies, a characteristic previously noted for macrophages isolated from mouse placenta.⁴⁷ Under serum-free conditions (Figure 3D to 3F), the pleiomorphic appearance was also observed, but the vacuoles were more pronounced on days 3 and 5, and HBCs failed to form colonies. Cells maintained these described morphologies for at least one week in culture (not shown). Flow cytometry was used to assess the patterns of protein expression during the first 48 h of culture, difficulty in releasing cells from the substratum after >48 h of culture precluded flow cytometric analysis at longer culture periods. We observed that nearly 100% of permeabilized cells expressed CD163 at 1 h, 24 h and 48 h of culture (denoted by “Total” Panels in Figure 4), confirming that the macrophage phenotype is maintained in culture. It is of note that the percentage of non-permeabilized HBCs expressing CD163 increased with time in culture from 25–50% at 1 h, to 55 to 80% at 24 h and to 93–99% at 48 h (denoted by “Surface” Panels in Figure 4). This suggests that treatment of placental tissue with trypsin and or collagenase during isolation reduces surface expression of CD163 in HBCs, but cell surface levels CD163 are re-established during 1 to 2 days of culture. CD90, a marker of

FIBs, was expressed in 10–50% of HBCs after 1 h of culture, with no expression being noted at 24 and 48 h (Left Panels). Interestingly, expression of vimentin (Right Panels), the fibroblast/mesenchymal cell marker, markedly increased in HBC cultures from approximately 10–20% expression at 1 h to 99% at 48 h. Others have noted that HBCs and other tissue macrophages express vimentin.^{39, 42, 48}

Assessment of phagocytosis by HBCs, CTs, and FIBs

Levels of phagocytosis in cultures of HBCs, FIBs, and CTs were determined following a 1 h incubation with fluorescent microspheres in DF medium or serum-free medium. Phagocytosis, as indicated by the intracellular appearance of fluorescent particles, was observed in nearly all HBCs maintained in DF medium (Figure 5A to 5C) or serum-free medium (Figure 5D to 5F) for 1, 3, or 5 days of culture prior to assay. Highest levels of phagocytosis resulting in large bright intracellular signals was noted in HBCs maintained for 5 days in serum-free medium (Figure 5F). Conversely, neither FIBs (Figure 5G) nor CTs (Figure 5H) maintained in culture for 1 day prior to assay showed evidence of phagocytosis. Thus, morphological assessment, flow cytometric analysis, and phagocytosis assays support the conclusion that the described protocols isolated functional HBCs with high yield and purity.

DISCUSSION

HBCs were identified in the placental villus more than 100 years ago. Over the last several decades a wide range of techniques have been employed to isolate these fetal villous macrophage with the goal of examining their function in culture. Early methods for the isolation of HBCs from term placentas used digestion and homogenization of tissue, and then Percoll and Ficoll gradients to separate cell types base on density.^{21, 24, 26} Embedding of antibodies raised against HBC proteins in the membranes of erythrocytes by Rosetting was also used facilitate purification of HBCs.^{49, 50} Rosetting, as well as positive selection using anti-CD68 antibody²⁰, has a potential disadvantage in that HBCs may become activated during the isolation process, perhaps complicating isolation and subsequent *in vitro* studies. For this reason, researchers later employed negative immunoselection techniques using anti-epidermal growth factor receptor antibodies and magnetic beads to remove contaminating CTs.^{22, 25} To date, there appears to be no consensus regarding a specific methodology which could be consistently used to isolate HBCs in high yield and purity. Contributing factors precluding its development may include the loss of macrophage-specific markers during the digestion process which may complicate verification of purity, activation during isolation (see above), and long term culture may be compromised by outgrowth of fibroblasts. Despite the absence of uniform protocols for isolation and culture of HBCs, there have been several *in vitro* studies which have provided insight into potential HBC function. Early studies examined phagocytosis^{24, 26} and expression of the receptor for IgG-Fc and class I and class II MHC antigens.^{21, 24, 49} Absence of lymphocyte priming by HBCs suggested they serve an anti-inflammatory role in the villus.⁴⁹ Additional studies indicate that HBCs may regulate uptake of triglycerides and transfer of fatty acids to the fetus,¹⁹ as well as stimulating the proliferation²⁰ and differentiation⁸ of trophoblasts. Another study showed that hypoxic conditions decreased levels of prostaglandin E₂ in HBCs,²⁵ of specific relevance to preeclampsia, a condition associated with placental hypoxia.^{51, 52}

Modifications to the isolation protocols made in the current study have resulted in a standardized procedure which yielded 130 to 200 × 10⁶ HBCs per placenta in 8 independent experiments. Several notable features of our protocol include the use of 80 to 100 g of villous tissue, higher than what was previously been reported.^{19–25} Sequential digestion with trypsin, and then collagenase served two purposes: cells released by trypsin digestion

were used to obtain CTs, and the remaining tissue now had reduced levels of CTs which facilitated concomitant isolation of HBCs following collagenase treatment. Two sets of Percoll gradients were then used to better separate cells according to density. The negative immunoselection procedure employed a sequential treatment of cells with anti-EGFR antibodies and then anti-CD10 Abs. Since CTs still represented the major “contaminant” of cells released during collagenase digestion, we reasoned that an initial treatment with anti-EGFR Ab would remove the majority of CTs. Subsequent treatment with anti-CD10 antibody would then be expected to remove remaining CTs as well as FIBs based on the observation that this protein is expressed by both of these cell types.^{37, 41} Finally, these procedures were also used to isolate FIBs, following their detachment from magnetic bead/anti-EGFR and anti-CD10 antibody complexes and subsequent passage in culture. Thus CTs, FIBs, and HBCs, three major cell types of the placental villous, were isolated during these procedures.

Another novelty of this study is that purity of HBCs, as well as that of CTs and FIBs, was assessed by flow cytometry. Our results indicated, that following removal of unattached and weakly adherent cells 1 h after plating, HBCs were obtained with purities of 98 to 99% based on expression of the macrophage marker CD163. HBCs also expressed the pan-macrophage marker CD68, the pan-leukocyte marker CD45 as well, but did not express cytokeratin 7 (epithelial/CT marker), and weakly expressed both the mesenchymal protein marker vimentin as well as the fibroblast/stem cell marker CD90. Of note, CD68 was also expressed by CTs and FIBs, indicating that it was not a specific marker of macrophages as assessed by flow cytometry. Similar observations of CD68 expression in non-myeloid cell types were made by other groups using anti-CD68 antibodies different than the one employed in the current study.^{45, 53} Use of the “macrophage-specific” KP1 antibody revealed significant cross-reactivity with FIBs and endothelial cells by flow cytometry,⁴⁵ and with CTs, syncytiotrophoblast, and FIBs by immunohistochemistry.³⁹ This led authors to conclude that CD68 is not a selective marker of macrophages⁴⁵ and anti-CD163 antibody is the most appropriate for identifying HBCs.³⁹

Several noteworthy observations were made during culture of HBCs. Flow cytometry of HBCs permeabilized after 1 h of culture revealed that 98–99% of the cells expressed CD163. However, in non-permeabilized cells, in which only surface levels of proteins are detected, levels of CD163 ranged from 25 to 50% at 1 h of culture to 93 to 99% at 48 h of culture. This suggested that levels of CD163 on the surface of HBCs, reduced by trypsin and/or collagenase treatment during cell isolation, were enhanced during subsequent culture. This supports previous findings that enzymatic treatments used during cell isolation procedures may reduce proteins associated with the cell surface,⁵⁴ which may complicate determinations of cell purity. It is also of note that expression of vimentin, an intermediate filament protein classically found in fibroblasts and mesenchymal stromal cells,^{39, 42} increased in expression in HBCs from a value of 10–20% expression at 1 h to 99% at 48 h. Other investigators have observed that tissue macrophages express cell-associated vimentin, and secrete it as well.^{39, 42, 48} Since levels of CD90, a marker of FIBs, decreased with time in culture from 10–50% expression at 1 h to <1% at 24 and 48 h, this indicated that the time-dependent increases in vimentin expression in HBC cultures was not due to proliferation of a fibroblast contaminant. This suggests that HBCs may undergo transitions in mesenchymal phenotypes during the culture period. Flow cytometric measurements of cultured cells HBCs were made in cells maintained in medium supplemented with serum. Morphological assessment of HBCs under these conditions revealed a predominance of round, vacuolated cells at 1 h of culture. Following culture for 1 to 5 days, vacuoles were maintained but more elongated cells were noted, and prominent cell colonies formed. Colony formation during culture was previously noted for murine placental macrophages.⁴⁷ Elongation of cells was also noted in serum free-medium, however more prominent vacuoles were present compared

to those observed in the presence of serum, and HBCs failed to form colonies. This indicated that cell migration and colony formation required growth factors not present in serum-free medium.

High levels of phagocytosis by HBCs, as assessed by uptake of fluorescent carboxylate microspheres, were noted in the presence and absence of serum factors. Equivalent or elevated levels of phagocytosis were noted in Day 5 cultures compared to that observed on Day 1. Under the conditions studied, no evidence of phagocytosis was noted in cultures of CTs and FIBs, indicative of cell-specific phagocytosis by HBCs. These results indicate that viability and function of isolated HBCs are maintained in culture for at least 5 days in the presence and absence of serum.

In conclusion, HBCs were isolated from term placenta with high yield and purity using procedures in which CTs and FIBs were concomitantly obtained. Biochemical and functional assessment of cultured HBCs revealed that macrophage phenotype and function were maintained during extended culture in the presence and absence of serum. These results should facilitate examination of the molecular regulation of HBC function in future studies.

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Abbreviations

CTs	cytotrophoblasts
FCM	flow cytometry
FIBs	fibroblasts
HBC	Hofbauer cell
IP	immunopurification

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Figure 1.

Schematic representation of the protocol used to isolate CTs, FIBs, and HBCs.

CTs were generated following enzymatic digestion of placenta with trypsin, centrifugation on Percoll gradients, and negative immunoselection by simultaneous incubation with anti-CD45 and anti-CD9 antibodies. HBCs were isolated using collagenase digestion of trypsin-treated tissue, followed by centrifugation on Percoll gradients, and negative immunoselection by sequential incubation with EGFR and then CD10 antibodies. FIBs were obtained from the cells removed during negative immunoselection.

Figure 2.

Purity of CTs, FIBs, and HBCs as assessed by flow cytometry.

The three cell types were incubated with antibodies shown at the left of the figure and flow cytometric analysis of cellular fluorescence is shown by the solid line in each Panel. Right-shifting of the histogram relative to the signal obtained with isotype-matched control antibody, denoted by the dashed line, is indicative of specific expression. The number of positive events and the fluorescence intensity are indicated on the y- and x-axes, respectively. The results are representative of 4 independent experiments for CTs and FIBs and 8 independent experiments for HBCs.

Figure 3.

Morphology of HBCs in culture.

HBCs were maintained in the presence of culture medium with serum for 1 (Panel A), 3 (Panel B), or 5 (Panel C) days; or without serum for 1 (Panel D), 3 (Panel E) or 5 (Panel F) days. Cells were fixed in 4% paraformaldehyde prior to taking photomicrographs. Note that pleiomorphic, vacuolated, HBCs formed colonies when cultured in the presence of serum but not in its absence. A representative experiment of 3 identically conducted ones is shown. Bar, 10 µm.

Figure 4.

Analysis of protein expression in HBC cultures by flow cytometry.

Expression of CD163 (macrophage marker) CD90 (fibroblast marker), and vimentin (fibroblast/mesenchymal cell marker) was analyzed by flow cytometry in HBCs maintained in DF culture medium for 1, 24, and 48 h. Specific staining was indicated by the right shift of the solid line histogram obtained with antibodies to the indicated protein compared to signals obtained with isotype-matched control antibody indicated by the dashed line. Nearly 100% of permeabilized HBCs expressed CD163 at all time points tested (denoted “Total”), whereas the percentage of HBCs expressing CD163 only on their cell surface showed a time-dependent increase (denoted “Surface”). HBCs did not express CD90, and a time-dependent increase in vimentin was noted. A representative experiment of 3 identically conducted ones is shown.

Figure 5.

Assessment of phagocytosis in cultures of HBCs, FIBs, and CTs.

Cells were maintained for 1 h in the presence of DF medium containing yellow-green fluorescent carboxylate microspheres. Cells were then washed and fixed in paraformaldehyde. Phagocytosis is indicated by the appearance of bright intracellular signals. Photomicrographs indicate prominent phagocytosis in HBCs maintained in DF medium for 1 (Panel A), 3 (Panel B), and 5 (Panel C) days as well as HBCs maintained for 1 (Panel D), 3 (Panel E), and 5 days (Panel F) in serum-free medium. Conversely, phagocytosis was not detected in FIBs (Panel G) and CTs (Panel H). Bar, 10 μ m.

Table 1

Reagents used for isolation of HBCs, CTS, and FIBs.

Reagents	Source	Cat#
Dulbecco's Phosphate Buffered Saline (PBS, 1×)	Invitrogen, Carlsbad, CA, USA	14190-144
2.5% Trypsin (10×)	Invitrogen, Carlsbad, CA, USA	15090-046
Hanks' Balanced Salt Solution (HBSS, 10×)	Invitrogen, Carlsbad, CA, USA	14185-052
Dynabeads® Goat anti-Mouse IgG	Invitrogen, Carlsbad, CA, USA	110.33
DNase I (from bovine pancreas, grade II)	Roche Diagnostics, Indianapolis, IN, USA	10104159001
Collagenase A (from <i>Clostridium histolyticum</i>)	Roche Diagnostics, Indianapolis, IN, USA	11088793001
Percoll	GE Healthcare Biosciences, Piscataway, NJ, USA	17-0891-01
Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12)	Sigma, St Louis, MO, USA	D2906
RPMI-1640 Medium	Sigma, St Louis, MO, USA	R8755
Calcium Chloride dehydrate (CaCl ₂)	Sigma, St Louis, MO, USA	C7902
Magnesium sulfate (MgSO ₄)	Sigma, St Louis, MO, USA	M2643
Sodium bicarbonate (NaHCO ₃)	Sigma, St Louis, MO, USA	S5761
HEPES	Sigma, St Louis, MO, USA	H4034
Lidocaine-HCl	Sigma, St Louis, MO, USA	L5647
BenchMark™ Fetal Bovine Serum (heat inactivated)	Gemini Bio-products, West Sacramento, CA, USA	100–106
ITS+ Universal Culture Supplement Premix	BD Biosciences, San Diego, CA, USA	354352

Table 2

Antibodies used in cell isolation and flow cytometry

Antibody	Clone	Isotype	Application	Source
Unconjugated Primary Antibody				
CD9	209306	Mouse IgG2b	IP	R&D Systems, Minneapolis, MN, USA
CD10	MEM-78	Mouse IgG1	IP	Biolegend, San Diego, CA, USA
CD45	F10-89-4	Mouse IgG2a	IP	GeneTex, Irvine, CA, USA
Cytokeratin 7	OV-TL 12/30	Mouse IgG1	FCM	Dako, Carpinteria, CA, USA
EGF-R	528	Mouse IgG2a	IP	Santa Cruz biotechnology, inc., Santa Cruz, CA, USA
Vimentin	V9	Mouse IgG1	FCM	Dako, Carpinteria, CA, USA
Conjugated Primary Antibody				
CD45-FITC	HI30	Mouse IgG1	FCM	Biolegend, San Diego, CA, USA
CD68-FITC	298807	Mouse IgG2b	FCM	R&D Systems, Minneapolis, MN, USA
CD90-APC	Thy-1	Mouse IgG1	FCM	BD Biosciences, San Diego, CA, USA
CD163-APC	GHI/61	Mouse IgG1	FCM	Biolegend, San Diego, CA, USA
Unconjugated Isotype Control				
Mouse IgG1	11711		FCM	R&D Systems, Minneapolis, MN, USA
Conjugated Isotype Control				
Mouse IgG1-FITC	11711		FCM	R&D Systems, Minneapolis, MN, USA
Mouse IgG1-APC	MOPC-21		FCM	BD Biosciences, San Diego, CA, USA
Mouse IgG2b-FITC	MPC-11		FCM	Biolegend, San Diego, CA, USA
Conjugated Secondary Antibody				
Goat anti-Mouse IgG/IgM-FITC			FCM	BD Biosciences, San Diego, CA, USA

Table 3

Yield and purity of HBC cultures.

Experiment	Villous Tissue (g)	Yield ($\times 10^6$ cells)	% CD163-positive cells	
			Surface	Total
I	100	130	98	99
II	80	200	50	98
III	95	100	50	98
IV	80	180	97	99
V	85	140	71	98
VI	80	200	74	99
VII	80	190	40	98
VIII	80	160	84	99

Eight different placentas were used to isolate HBCs. Cells were cultured for 1 h and purity of non-permeabilized (surface expression examined) and permeabilized (total; surface+intracellular expression examined) cells was assessed by flow cytometry using anti-CD163 and isotype-matched control antibodies (see Table 2). Results are expressed as a percentage of CD-163-positive cells.