Culturing of Human Placental Macrophages

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Cells were isolated by successive dissociation of human placenta in dispase and collagenase solutions and separated by centrifugation in Ficoll-Verografin gradient. Cell suspension was enriched with macrophages by adhesion to plastic followed by washing with RPMI-1640. Isolated placental macrophages can be cultured for a long time. Cell culture homogeniety assessed by various methods was equal or exceeded 95%.

Key Words: *placenta; macrophages; culturing*

According to current views, mononuclear phagocytes are present in the placenta throughout the entire gestation period and constitute up to 40% of all nontrophoblast cells of chorionic villi [2,3]. This cell population plays an important role in the interaction between mother and fetus under normal and pathology, in particular, in miscarriage and abortion. Placental macrophages produce prostaglandins stimulating contractile activity of the uterus and platelet-activating factor modulating fetoplacental circulation, which may result in spontaneous abortion [1].

Cell culture technique is useful for the study of this problem, since this approach allows one to obtain viable cells and to study their properties and functions *in vitro*. To this end, macrophages should be isolated from the placenta consisting of various cell types, and the obtained cell population should possess all principal characteristics typical of these cells *in vivo*. Some procedures for preparing enriched cultures of placental macrophages were described previously [4,7,8,10,11]. However, they differ from each other and cannot always be well reproduced. Moreover, cells obtained by some authors cannot be definitely recognized as resident macrophages [6]. In light of this, we attempted to develop a standardized and reproducible procedure of isolation of highly homogenous long-term placental macrophage culture.

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MATERIALS AND METHODS

Cells were isolated from human placenta obtained during normal labors (38-41 weeks) at the D. O. Ott Institute of Obstetrics and Gynecology. Placenta fragment weighing 10-20 g free of membranes was washed with Tyrode solution containing 80 μg/ml gentamicin and 0.5 μg/ml amphotericin B. Tissues were cut to 2-3-mm^3 fragments and transferred to 100 ml RPMI-1640 medium (Sigma) containing 2 mg/ml dispase (Boehringer), 40 μg/ml gentamicin and 0.25 μg/ml amphotericin B and incubated for 30 min at 37°C with constant shaking. After sedimentation of tissue fragments supernatant was decanted and the fragments were washed three times with Hanks' balanced salt solution (HBSS, Flow). Then the fragments were incubated for 1.5 h in RPMI-1640 containing 2 mg/ml collagenase from hepatopancreas of the king crab *Paralithodes camtschatica* (Pacific Ocean Institute of Bioorganic Chemistry, Russian Academy of Sciences), 60 U/ml DNase (Boehringer), 20 mM HEPES, 3 ml fetal bovine serum (FBS, Flow), and antibiotics at constant shaking. After sedimentation of nondigested fragments, the supernatant was filtered through a nylon filter (100 μ pore diameter), centrifuged at 200*g* for 7 min, washed three times with HBSS, and allowed to stay at 4°C. Nondigested fragments were suspended in fresh portion of the incubation medium and incubated for 1.5 h and the procedure was repeated. Two portions of dissociated cells were pooled, resuspended in 40 ml

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RPMI-1640 medium containing 20 mM HEPES, and filtered through a nylon filter (40 μ pore diameter). Cell suspension was centrifuged at 400*g* for 40 min in Ficoll-Verografin gradient (d=1.077). The cells (upper of two rings at the interface) was cautiously gathered, washed three times with HBSS and resuspended in RPMI-1640 containing 15% FBS and antibiotics. Cell count and viability were evaluated in a hemocytometer in the Trypan Blue exclusion test.

The cells were seeded on coverslips (10^5 and 5×10^5 cells per 9×9 and 18×18 coverslips, respectively) and cultured in RPMI-1640 containing 15% FBS and antibiotics (37°C, 5% CO_2) for 2 h (type I cultures) or 18 h (type II cultures). Nonadherent cells were then removed by washing with incubation medium.

Adherent cells were counted in a hemocytometer after detachment from glass with 0.2% trypsin.

The cultures were assayed for nonspecific esterase after 1 and 7 days [5] (no less than 200 cells per glass).

Phagocytic activity was evaluated as described previously [9] with some modifications.

For immunocytochemical analysis the cells were fixed in absolute ethanol for 10 min, washed with phosphate-buffered saline (Flow) containing 0.2% bovine serum albumin (PBS-BSA), and incubated with mouse monoclonal antibodies against macrophage-specific CD68 antigen (PG-M1, Dakopatts) for 1 h at room temperature. Then the cells were washed three times with PBS-BSA and incubated for 20 min with fluorescein isothiocyanate-labeled anti-mouse IgG antibodies (Sorbent, Russia) at room temperature. Cultures were analyzed using an Orion-Axiophot luminescent photomicroscope.

RESULTS

Incubation in 0.2% dispase led to partial dissociation of the placenta samples and a massive release of blood cells from preserved microcapillaries into incubation medium. Dissociation of the chorionic villus cells was observed only after incubation in the presence of collagenase, this process was most intensive after the addition of fresh portion of the enzyme (this stage yielded to 70% isolated cells).

TABLE 1. Number of Cells in Type I and II Cultures on Days 1 and 7 of Culturing (% of Seeded Cells, *M±m*)

Duration of culturing, days	Type I cultures	Type II cultures
1	52±5	68±10
7	39±6	43±8

Centrifugation in Ficoll-Verografin density gradient eliminated the bulk of erythrocytes (bottom fraction) and tissue-forming cells, primarily trophoblasts (fraction at the interface below the macrophage fraction).

The total yield after enzymatic dissociation and gradient centrifugation was $2.6\pm0.4\times10^6$ cells per gram tissue. Cell viability was 95-97%.

As seen from Table 1, 75% adhesive cells were attached to the substrate during the first 2 h in culture. Attachment, when took more than 2 h, was unstable, and the cells were removed by subsequent washouts, so that the number of cells in cultures obtained by different methods became practically equal. Thus, washout 2 h after seeding promotes elimination of nonviable cells from cultures.

The obtained cell cultures were analyzed by methods which allowed precise identification of cells of the monocyte-macrophage lineage. The data are presented in Table 2 and Fig. 1.

All three tests confirmed that washout of cell cultures 2 h after seeding considerably elevated the content of macrophages even at the early stages of culturing. Further *in vitro* incubation enriched cultures with macrophages, presumably due to death of other adherent cell types that cannot survive long-term culturing. In type II cultures, the percentage of macrophages also rose during the 1st week, but it did not exceed 80%; 7 days after seeding we observed active proliferation of other cells and, therefore, a decrease in the percentage of macrophages in these cultures (data not shown).

Tests for nonspecific esterase and mediated phagocytosis underestimated the number of macrophages

TABLE 2. Content of Cells with Macrophage-Specific Characteristics in Type I and II Cultures (% of Total, *M±m*)

Tests	Type I cultures		Type II cultures	
	day 1	day 7	day 1	day 7
Test for nonspecific esterase	84±5	92±3	58±6	67±9
Fc-receptor-mediated phagocytosis	78±6	88±6	62±5	72±10
Expression of CD68	90±7	95±3	66±6	74±5

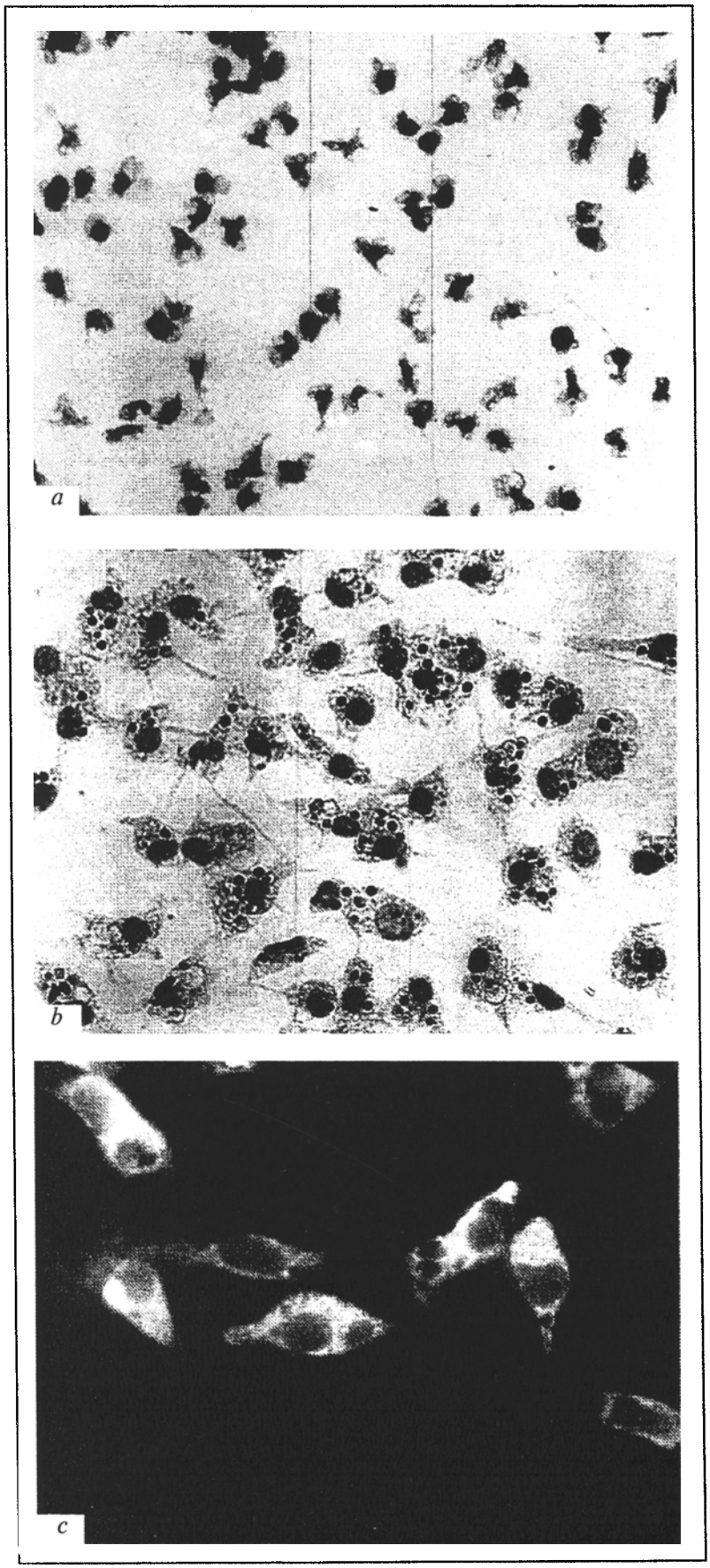

Fig. 1. Human Placental Macrophages, 7 days in culture (type I culture). *a*) staining for nonspecific esterase, ×220; *b*) Fc-receptor-mediated phagocytosis of sheep erythrocytes, ×450; *c*) immunofluorescent staining of CD68-positive cells, ×860.

in cultures in comparison to immunocytochemical test. This can be attributed to the fact that 10-15% cells that were morphologically identified as macrophages lacked nonspecific esterase and Fc-receptor-mediated phagocytosis [9]. Hence, it can be concluded that macrophages constitute no less than 95% cells in type I cultures.

Thus, very simple procedure, washout of cell cultures soon after seeding, eliminates the majority of proliferating cells, thus improving homogeneity of cell population. Unlike trypsin dispersion and enrichment of cell cultures, our approach preserves cell surface antigens allowing their further investigation.

Our experiments demonstrated that the differences in cell adhesiveness, which are most pronounced at the initial stages of the cell-substrate interaction, allow one to effectively separate different cell types and to obtain highly enriched placental macrophage cultures for investigation of their morphology and function.

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