Research Journal of Biological Sciences 1 (1-4): 63-68, 2006

Preliminary Identification of Cultured Human Trophoblasts

[1]Chunmei Wang, [1]Xiaofeng Huang, [1]Zhen Li, [2]Dezhong Xu, [2]Ke Men, [2]Anhui Wang,
[1]Jiaji Yang, [1]Hua Yu, [1]Dan Chen, [1]Xiaojun Huang and [1]Shuang Wang
[1]Electron Microscopic Center, Fourth Military Medical University, Xi'an China
[2]Department of Epidemiology, Faculty of Preventive Medicine,
Fourth Military Medical University, Xi'an 710033

Abstract: To identify the characteristics of the cultured cells isolated from human villous. The purified and subcultured cells were derived from human placentae villous. The cellular morphology was observed under inverted phase contrast microscope. The nucleus and cytoplasm were demonstrated by Haematoxylin and Eosin (HE) staining. The superficial structure was observed under Scanning Electron Microscope (SEM) and the intracellular ultrastructures were revealed by transmission electron microscopy (TEM). The Immunofluorescent Histochemistry (IH) combined with Laser Scanning Confocal Microscopy (LSCM) was used to examine the expression of cytokeratin 18 (CK18), vimentin (Vim), β-human Chorionic Gonadotropin (β-hCG) and human Placental Lactogen (hPL). The cultured cells appeared as patchy spreading and irregular polygonal with big oviform nucleus and transparent cytoplasm. SEM observation showed that abundant microvilli existed on the cell surfaces. The typical desmosomes between cells were observed with TEM. The numerous microvilli were also found on the surface of the cells. The abundant mitochondria, glycogen, developed Golgi complexes, a few of endoplasmic reticulum and many lipid droplets appeared in cytoplasm. Cytokeratin 18 expressed in all cells and vimentin was expressed in part of the cells, hCG and hPL immunoreactive positive substances were distributed in cytoplasm.

Key words: Trophoblast, microscopy/electron, scanning, transmission, Laser Scanning Confocal Microscopy (LSCM)

INTRODUCTION

Human trophoblastic cells originate from embryonic trophectoderm, which play a major role in embedding of embryo and have close relations with many cyesis diseases in the occurrence, development, proliferation and functional disorder. It is impossible to investigate these diseases *in vivo,* so human villous trophoblasts should be cultured *in vitro*. At present, there are many methods of separation and culture of trophoblastic cells. Whereas, every method still has some flaws, such as other cells contamination in the course of primary culture, characteristics changes in long-term culture and so on, so it is necessary to identify the source and kind of culture cells. At present, there is no any unified criterion about identification of trophoblastic cells; only some relatively credible markers have been put forward in correlative international conferences and simultaneously, the using standards of them were established[1].

The purified and subcultured villous trophoblastic cells, obtained from the hosts of a 6 to 10 weeks' pregnancy, were isolated by the trypsinization[2]. The cellular morphology was observed by inverted phase contrast microscope, the nucleus and cytoplasm were observed by Haematoxylin and Eosin (HE) staining. The superficial structures were observed by Scanning Electron Microscope (SEM) and the intracellular ultrastructures were revealed by Transmission Electron Microscope (TEM). The Immunofluorescent Histochemistry (IH) combined with Laser Scanning Confocal Microscopy (LSCM) was used to examine the function of hormonal excretion. Accordingly, the cells origin and differentiation were identified, which offered favorable cytological basis for further experimental investigation.

MATERIALS AND METHODS

Materials: The purified and subcultured villous tissue from the hosts of a 6 to 10 weeks' pregnancy was isolated by trypsinization. The culture cells were seeded in a concentration of 4×10^5 mL^{-1} and grew in DMEM supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 U mL^{-1} and streptomycin 100 mg mL^{-1}). The medium was changed every 2 days. Cultures were

Corresponding Author: Chunmei Wang, Electron Microscopic Center, Fourth Military Medical University, Xi'an China

observed daily with an inverted phase contrast microscope. The 50th generation of the cultured cells were employed in the present study. Human skin fibroblast (9th generation) was taken as the control cells.

High-sugar DMEM (Gibco), Hepes (Promega Co). Super fetal bovine serum (Hangzhou purpleflower holly leaf Co). FITC Monoclonal Anti-cytokeratin Peptide 18 (Clone CY-90), Cy3 Monoclonal Anti-Vimentim (mouse IgG1 isotype), Monoclonal Anti-human chorionic gonadotropin (β-Subunit Specific) and Rabbit anti-human human placental lactogen (hPL), FITC goat anti-mouse IgG, anti-rabbit IgG biotin conjugate and Texas red avidin conjugate (SIGMA).

CO_2 culture cabinet (Heraeus Co, Germany), inverted phase contrast microscope BX-50 (Olympus, Japan), scanning electron microscope S-520 (Japan), ultramicrotome LKB-Nova (Nova, Sweden), JEM-2000EX transmission electron microscope (JEOL,Japan), Bio-Rad MRC-1024 laser scanning confocal microscope (Bio-Rad,America).

HE staining: When the cells grew as a monolayer on 12×12 mm^2 carry sheet glass in culture bload lamina, the cells were washed with 0.01 mol L^{-1} PBS, 5 min×3 times, fixed with 40 g L^{-1} Formaldehyde solution for 30 min, washed with 0.01 mol L^{-1} PBS, 5 min×3 times, stained with haematine for 5 min, rinsed with tap-water for 1 min, treated with diluted hydrochloric acid and alcohol for a few seconds, tap-water for 1 min, light ammonia water for 3~5 min, tap-water for 1 min, 700 mL L^{-1},800 mL L^{-1},900 mL L^{-1} alcohol 1 time, 950 mL L^{-1} alcohol 2 times, 1 000 mL L^{-1} alcohol 3 times, 1 min per times, xylene clearing, 1 min×3 times and finally observed by light microscope.

Scanning electron microscopy: When the cells grew as a monolayer on 12×12 mm^2 carry sheet glass in culture bload lamina, washed with 0.01 mol L^{-1} PBS, 5 min×3 times, fixed with 30 mg L^{-1} glutaral f or 30 min, washed with 0.01 mol L^{-1} PBS, 5 min×3 times and treated with 500 mL L^{-1}, 700 mL L^{-1}, 900 mL L^{-1}, 1 000 mL L^{-1} acetonitrile in sequence, 10min×1 time. After the second 1 000 mL L^{-1} acetonitrile treatment, air dried for 2 h, plating gold 10 min and were observed with SEM.

Transmission electron microscopy: The monolayer cells in log growth phase were prepared into cell suspension, then were shaped into 1 mm^3 cell mass through centrifugation at 1000 rpm, 10 min, then fixed with 30 mg/L glutaral for 2 h, washed with 0.01 mol L^{-1} PBS, 5 min×3 times, dehydrated through 500 mL L^{-1}, 700 mL L^{-1}, 900 mL L^{-1}, 1 000 mL L^{-1} gradient acet saturated 0.5 h in 1: 1 acet and epoxy resin; epikote, embedded with Epon-812, aggregated for 24 h at 65°C, stained with uranyl acetate and lead citrate and observed with TEM.

Laser scanning confocal microscopy: When the cells grew as monolayer on 12×12 mm^2 carry sheet glass in culture bload lamina, the cells were washed with 0.01 mol L^{-1} PBS, 5 min×3 times, fixed with 950 mL L^{-1} alcohol for 30 min, washed with 0.01 mol L^{-1} PBS, 5 min×3 times. 1) The cells were blocked with goat serum, 37°C, 30 min; treated with FITC Monoclonal Anti-cytokeratin Peptide 18 and Cy3 Monoclonal Anti-Vimentim (1:1)50 μL overnight at 4°C. Blank (only PBS, no first antibody) and positive controls were set up; rewarmed to37°C for 60 min, washed with 0.01 mol L^{-1} PBS, 5 min×3 times. After sealed with 500 mL L^{-1} glycerin, the cells were observed with LSCM. 2) The cells were blocked with goat serum, 37°C, 30 min; treated with Monoclonal Anti-human chorionic gonadotropin (β-Subunit Specific) and Rabbit anti-human human placental lactogen (hPL) (1:1) 50 μL overnight at 4°C. Blank (only PBS, no first antibody), specific (only first Ab or second Ab) and negative controls were set up. Then rewarmed to at 37°C for 60 min; washed with 0.01 mol L^{-1} PBS, 5 min×3 times, treated with FITC goat anti-mouse IgG and anti-rabbit IgG biotin conjugate (1:1)50 μL, 40 min at 37°C; washed with 0.01 mol L^{-1} PBS, 5 min×3 times, treated with Texas red avidin conjugate 50 μL, 40 min at 37°C; washed with 0.01 mol L^{-1} PBS, 5 min×3 times. After sealed with 500 mL L^{-1} glycerin, the cells observed with LSCM.

RESULTS

Light microscopy: As shown by inverted phase contrast microscope, the cells appeared irregular polygonal in shape and grew in a monolayer flaky way (Fig. 1a). They had abundant cytoplasm with one or more large oviform nuclei and mixed into multinuclear giant cell in local area. HE staining showed that the cells appeared polygonal in shape with blue-violet nucleus, pink cytoplasm and sometimes multinucleate giant cells (Fig. 2a). But fibroblast had long-shuttle apparence, arranged very closely and grew in weaving way (Fig. 1b and 2b).

Ultrastructural characteristics: As shown by SEM, the cells grew in a spread monolayer way with abundant microvilli on the surface (Fig. 3a), but fibroblast displayed long-shuttle apparence (Fig. 3b). The desmosomes were found between the cells under TEM, so the cells connected with each other and became patchy. There

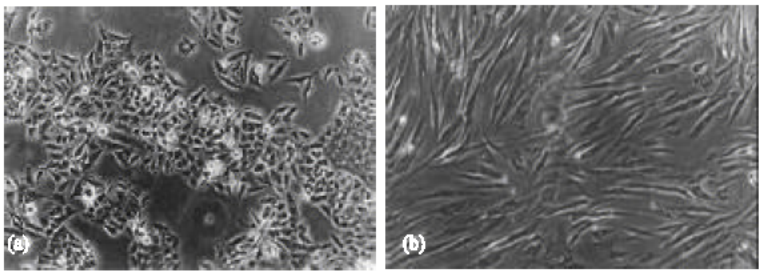

Fig. 1: The characteristics of the culture cells (a) and the control cells (b, fibroblast) under inverted contrast microscope. a, cells are irregular polygonal and arranged in patch *in vitro*. b, fibroblasts display long-shuttle apparence, arrange very closely and grow in weaving way. (original magnification: a and b,×100)

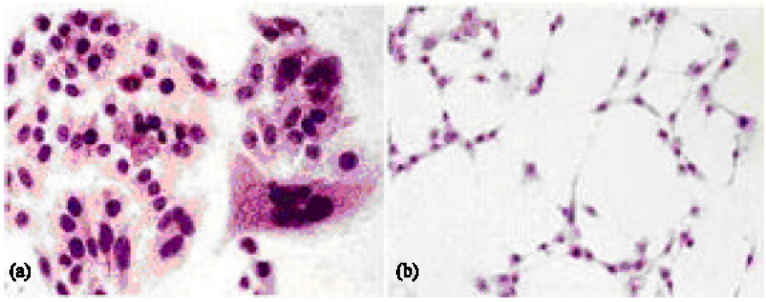

Fig. 2: HE staining of the culture cells (a) and the control cell s(b, fibroblast). a, Cells are polygonal, the nucleus are localized centrally in cells. Karyokinesis and polynuclear cell can be seen. b, Fibroblasts are in long-shuttle apparence and grow in weaving way. (original magnification: a and b,×100)

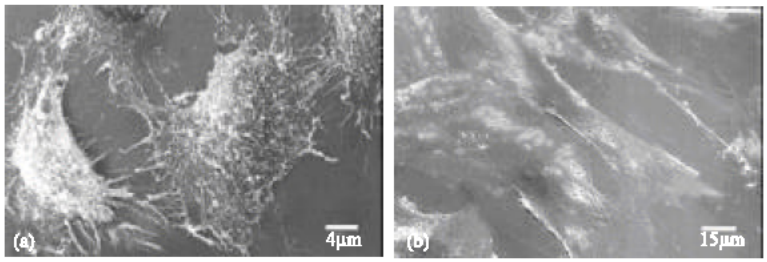

Fig. 3: The characteristics of the culture cells (a) and the control cells (b, fibroblast) observed by SEM. a, Cells are irregular polygonal and the numerous microvilli are found on cell surface. b, Fibroblasts are long-shuttle, there are abundant microvilli on its surface. (original magnification: a,×1500; b,×400)

were a great number of lipid droplets, mitochondria, developed Golgi complex, glycogen and dissociative ribosome in cytoplasm (Fig. 4a-c). No desmosomes were found between fibroblast cells (Fig. 4d), so they couldn't joint (Fig. 4e), but smooth endoplasmic reticulum in cytoplasm were proliferated and expended, there were large quantity of protein-like excretion substance, which divided cytoplasm into some "island" structure (Fig. 4f).

Expression of CK18, Vim , |B-hCG and hPL: As shown immunofluorescent histochemically, CK18 and Vim were expressed in cytoplasm. CK18 was arranged in a loose reticular way and scattered all over the cytoplasm, while Vim was expressed only in the limited number of sites, often in the peripheral parts of the cells (Fig. 5a). But fibroblast cells only expressed Vim. Other control groups were stained negatively (Fig. 5b). Both β-hCG and hPL were expressed in the cells, the former was in the cytoplasm (Fig. 6a) and the latter was in the areas close to the nucleus (Fig. 6b). But these were negative in fibroblast cells.

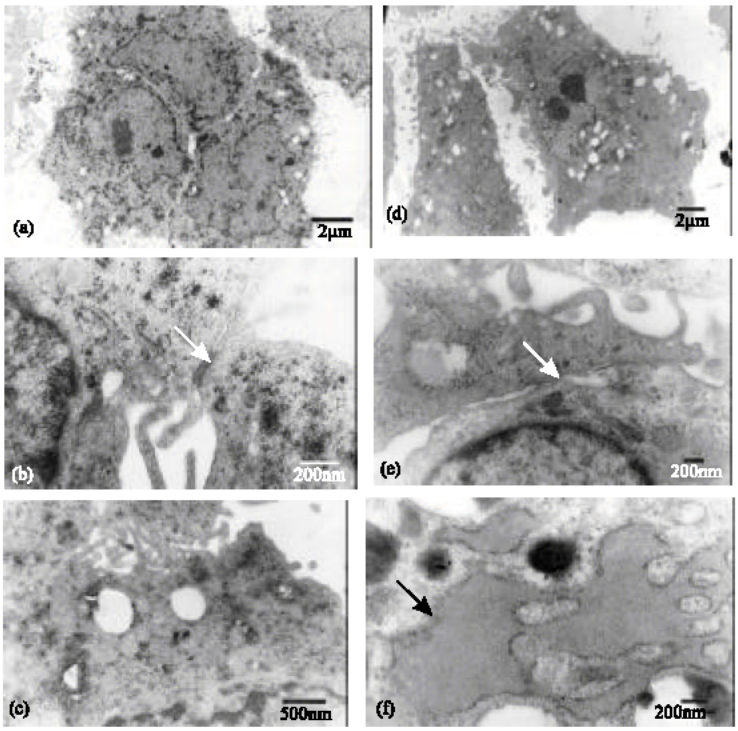

Fig. 4: The culture cells (a) and the control cells (b, fibroblast) observed by TEM. Cells are contacted tightly. a,×3000; b, The desmosome can be seen between cells; c, There are abundant microvilli on cell surface and some lipid droplets in cytoplasms; d, apparent interspace between fibroblasts; e, No desmosome can be seen between cells; f, Many protein secretions in SER can be seen in cytoplasms. (original magnification: a,×3000; b,×30000; c,×15000; d,×3000; e,×20000; f,×25000)

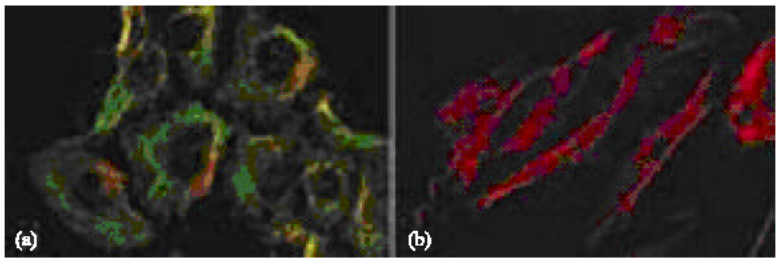

Fig. 5: Immunofluorescent histochemistry of the culture cells (a) and the control cells (b, fibroblast).a, Co-localization (yellow) of CK18(green) and Vim (red) in cytoplasm; b, Vim (red) in cytoplasm. (original magnification : a,×800; b,×400)

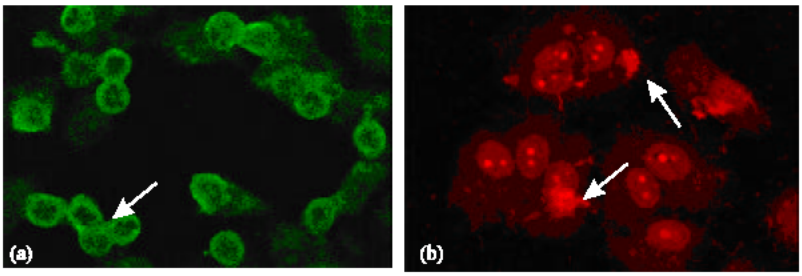

Fig. 6: Immunofluorescent histochemistry staining of the culture cells (a) and the control cells (b, fibroblasts) . a,The expressions of |B-hCG-immunoreactive positive substances are detected in cytoplasms. b,The expression of hPL is found in cytoplasm close to the nuclear areas. (original magnification : a,×600; b,×800)

DISCUSSION

Trophoblastic cells originate from epithelia, so they have the characteristics of epithelial cells. As shown in this study, the culture cells appeared irregular short or long polygonal in shape, clung the wall and grew in a monolayer flaky way. They have abundant microvillous on surface and the desmosomes between the cells and quite whole organelles. The TEM observation accords with the morphology characteristics. Placenta trophoblasts include two different modalities parts: Cytotrophoblast (CT) and Syncytiotrophoblast (ST). CT are simple, haven't entire organelles; but ST, that translate from the former, have quiet entire organelles and more importantly, can synthesize the hormones. Some studies reported that ST formation was mainly due to cell division and multiplication of CT, or CT fusion through cell migration, drawing close and being diamorph. In this study, some big and multinuclear giant cells were observed under light microscope in higher magnification. So we supposed that ST may be formed through the fusion of single-nuclear CT[3-6].

Up to date, there has not been any accordant criterion for the identification of trophablastic cells, only some identified markers, such as Cytokeratin (CK), Vimentim (Vim), β-human chorionic gonadotropin (β-hCG) and human Placental Lactogen (hPL) and so on were put forwarded. Cellular skeleton has intimate relation with origin, differentiation, modality and action of cell of mammal. Cytokeratin and vimentin belong to the intermediate fibroin, which was one of Cellular skeleton. Generally, epithelial cells express only CK, but no Vim. In this cellular skeleton staining experiment, it was observed that CK18 and Vim were coexpressed in cytoplasms. So it is supposed that the culture cells occurred the character conversion toward interstitial cells, because of long culture process *in vitro*. Furthermore, some studies indicated that Vim were not the specific marker, which can express in epithelia, so the positive expression of Vim in culture cells cannot deny its source of epithelia[7]; the other possibility is that the cells root in interstitial trophoblast, because of the fact that Vim is positively expressed in interstitial trophoblast had been found. Recently, some researchs reported that trophoblastic cell could express Vim[8].

Human trophoblastic cells can secrete many hormones; the representative ones are Human Gonadotropic Hormone (hCG) and human Placental Lactogen (hPL). Abundant organelles such as endoplasmic reticulum, Golgi apparatus related with hormonal secretion had been found in the cytoplasm of the culture cells by TEM. The expression of β-hCG-immunoreactive positive substances are detected in cytoplasm of most cells in the experiment. The expression of hPL is found in cytoplasm of some cells close to the nuclear areas. Previous research showed that CT had the ability of producing only α-hCG and could produce β-hCG after changinginto ST. Furthermore, ST also has the ability of producing hPL[9]. Some researchers reported that single-nuclear CT still excreted hCG and hPL[10]. Our study showed that β-hCG and hPL had different expression patterns; the former expressed in whole cytoplasm; but the latter expressed in limited areas close to the nucleus, which maybe related with some organelles, especially Golgi apparatus, which would need further studies.

our results suggest that the culture cells have the characteristics of epithelial cells and function of secretory cell through the identification of the source, differentiation, general and special moldality and function of the cells, so we presume that the cells cultured in vitro can be identified as human trophoblastic cells.

CONCLUSION

The cellular morphological features and cytoskeleton correspond with those of epithelial cells. And they can secrete the hormones of trophoblast. It may be confirmed that the cultured cells *in vitro* maybe derived from interstitial trophoblast.

ACKNOWLEDGEMENT

Supported by grants from the National Natural Science Foundation of China (No. 30230320).

REFERENCE

1. King, A., L. Thomas and P. Bischof, 2000. Cell culture models of trophoblast II: trophoblast cell lines-a workshop report. Placenta, 2000, 14: S113-119.
2. Wang, X.P., D.Z. Xu, Y.G. Li, Y.P. Yan, J.X. Zhang and J. Huang, 2002. *In vitro* culture of human trophoblasts [in Chinese]. J. Fourth Mil. Med. Univ., 23: 2023-6.
3. Kong, L.H., Z.H. Liu, S.H.L. Chen, 2000. Culture of human trophoblastic cells. Di-yi Junyi Daxue Xuebao (J First Mil Med Univ), 20: 10-11.
4. Kliman, H.J., J.E. Nestker and E. Sermasi *et al*., 1986. Purificateon, characterization and *in vitro* differentiation of cytotrophoblasts from human term placentae. Endocrinol., pp: 118:1567.

5. Lu, X.D., C.H.F. Xu and R.Q. Guo *et al*., 2001. The expression of Human Chorionic Gonadotropin (HCG) and Epiderma Growth Factor Receptor (EGRF) in human trophoblast during Early Pregnancy. Zhenjiang Yixueyuan Xuebao (J. Zhenjiang Med. Coll), 11: 289-290.
6. Ushizawa, K., T. Takahashi, K. Kaneyama, M. Hosoe, K. Hashizume, 2006. Cloning of the bovine antiapoptotic regulator, BCL2-related protein A1 and its expression in trophoblastic binucleate cells of bovine placenta. Biol Reprod., 74: 344-51.
7. Knerr, I., S.W. Schubrt, C. Wich, K. Amann, T. Aigner T. Vogler, R. Jung J. Dotsch, W. Rascher, A.J.G. Potgens, G. Gaus and H.G. Frank *et al*., 2001. Characterization of trophoblast cell isolations by a modified flow cytometry assay. Placenta, 22: 251-255.
8. De-Souza, P.C. and S.G. Katz, 2001. Coexpression of cytokeratin and vimentin in mice trophoblastic giant cells. Tissue Cell, 33: 40-45.
9. Tu, A.Y., M.C. Cheung, X. Zhu, R.H. Knopp, J.J. Albers, 2004. Low-density lipoprotein inhibits secretion of phospholipid transfer protein in human trophoblastic BeWo cells. Exp. Biol. Med. (Maywood), 229: 1046-52.
10. Pavlov, N., E. Hatzi, Y. Bassaglia, J.L. Frendo, D.E. Brion and J. Badet, 2003. Angiogenin distribution in human term placenta and expression by cultured trophoblastic cells. Angiogenesis, 6: 317-30.