Abstract

Human amniotic epithelial cells were isolated from a piece of fresh amnion. Using immunocytochemical methods, we investigated the expression of neuronal phenotypes (microtubule-associated protein-2, glial fibrillary acidic protein and nestin) in human amniotic epithelial cells. The conditioned medium of human amniotic epithelial cells promoted the growth and proliferation of rat glial cells cultured in vitro, and this effect was dose-dependent. Human amniotic epithelial cells were further transplanted into the corpus striatum of healthy adult rats and the grafted cells could integrate with the host and migrate 1–2 mm along the nerve fibers in corpus callosum. Our experimental findings indicate that human amniotic epithelial cells may be a new kind of seed cells for use in neurograft.

Key Words: human amniotic epithelial cells; cell transplantation; microtubule-associated protein-2; glial fibrillary acidic protein; nestin; neurotrophin; central nervous system injury; neural regeneration

RESULTS

Morphology of the isolated and cultured HAECs and the expression of neuronal specific markers

The HAECs became adherent within 2 days after the isolation and 1 × 10^5 HAECs could be harvested from an amniotic membrane at a size of 15 cm × 15 cm. Under light microscopy, the adherent HAECs were growing in clusters, in round or polygonal shapes, and contained large nuclei and characteristic vesicular fat droplets in the cytoplasm (Figure 1). The cells proliferated slowly and were passaged every 2–3 weeks.

Figure 1 Morphology of the human amniotic epithelial cells in primary culture (hematoxylin-eosin staining, light microscopy, × 200).

Adherent cells were round or polygonal, with large nuclei and characteristic vesicular fat droplets in cytoplasm (arrow).

INTRODUCTION

Human amniotic epithelial cells (HAECs) have the potential to differentiate into nervous system cells\(^1\), and can synthesize and release brain-derived neurotrophic factor, neurotrophin-3 and nerve growth factor\(^9\). Therefore, HAECs are potential seed cells for cell transplantation. Importantly HAECs do not express HLA-associated antigen, which avoids the immune rejection present in cell transplantation\(^9\).

A number of existing studies demonstrate that HAEC transplantation may be an effective treatment for ischemic neurological impairment, Parkinson’s disease and spinal cord injury\(^4-9\), with no limitations such as scarcity of seed cell sources, difficulty in sample harvesting, or legal and ethical issues. However, a systematic study of these cells has not been undertaken and their biological characteristics still remain unclear. This study sought to establish stable HAEC culture conditions, elucidate the biological characteristics, and observe the migration of HAECs after transplantation, in a broader attempt to provide novel seed cells for stem cell transplantation in the treatment of neurological diseases.
Immunohistochemical and cytochemical staining demonstrated that microtubule-associated protein-2 (MAP-2), glial fibrillary acidic protein (GFAP) and nestin were expressed in both the amniotic membrane and the cultured HAECs. Nestin expression was markedly reduced in the cultured HAECs (Figure 2).

**Morphology of the cultured rat glial cells**

Under light microscopy, the adherent rat glial cells were irregular spindle-shaped cells, with small cell bodies and long protrusions. Cells were vigorously proliferating and were passaged every 4–5 days (Figure 3).

**Effect of HAECs conditioned medium on the morphology of rat glial cells**

Hematoxylin-eosin staining showed that rat glial cells could not survive in serum-free RPMI1640 and the majority of cells died. A small number of residual cells could adhere but showed thin shape and poor refraction. Rat glial cells grew well in the HAEC conditioned medium, with extended bodies and long processes (Figure 4).

**Effect of HAEC conditioned medium on proliferation of rat glial cells in vitro**

The proliferation of rat glial cells was determined with trypan blue staining and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Results found that HAEC conditioned medium promoted the
proliferation of rat glial cells (Figures 5, 6).

MTT assay also showed that the nutrition effects of the HAEC conditioned medium were concentration-dependent (Figure 6).

Survival and migration of HAECs transplanted in rats
The nuclei of Hoechst33342-labeled HAECs emitted blue fluorescence under a fluorescent microscope. A large number of Hoechst33342-labeled HAECs were visible under fluorescence microscopy at 1 month after transplantation into the corpus striatum of rats, integrated well with the host and showed no obvious dysplasia. Some HAECs could migrate 1–2 mm along the nerve fibers in corpus callosum (Figure 7).

**DISCUSSION**

In embryonic development, both HAECs and nerve cells are derived from the ectoderm cell mass of the morula. HAECs may contain some nerve cells or stem cells that can differentiate into nerve cells, suggesting that HAECs may exhibit nerve cell-associated biological characteristics. In this study, both amniotic membrane tissue and the cultured HAECs expressed the nerve tissue markers GFAP, MAP-2 and nestin, as detected by immunohistochemical/cytochemical staining. In addition, the nestin-positive cells were present in HAECs before and after culture, but the number was significantly reduced after culture. This is evidence that the cultured HAECs and amniotic tissue are likely to contain neural stem cells that can differentiate and the number of neural stem cells decreases after culture.

In order to ensure cell viability during culture, the time from the isolation from the placenta to the culture of cells was controlled within 2 hours. Results of the present study showed that HAECs adhered within 2 days and showed a clustered growth pattern. Under *in vitro* culture conditions, HAECs proliferated slowly and were passaged every 2–3 weeks. Nevertheless, there are a large number of cells on the amniotic membrane, up to...
2 × 10⁶[8]. Our experiments obtained 1 × 10⁵ cells from 15 cm × 15 cm amniotic membrane, which is sufficient for cell transplantation.

The mechanism underlying cell transplantation in treatment of nerve injury depends on two aspects, one is that seed cells can provide the lost tissue cells to restore functions, and the other is that seed cells provide nutritional factors to promote axonal regeneration and prevent the apoptosis of remaining nerve cells[9]. The HAECs and their secreted amniotic fluid play an important role in nutritional support during early nervous tissue development[9], this strongly suggests that HAECs can provide nutrition for nerve cells. To confirm this speculation, we cultured rat glial cells with HAEC conditioned medium. Results of the present study showed that HAEC conditioned medium has a significant nutritional role in maintaining the integral morphology of rat glial cells and promotes rat glial cells to proliferate and extend processes in serum-free culture conditions. Low-concentration (one-third) HAEC conditioned medium had equal effects to culture medium containing 10% fetal bovine serum on the proliferation of rat glial cells. This proliferation increased with the HAEC conditioned medium concentrations, indicating a concentration-dependent feature.

To serve as seed cells for cell transplantation, the cells need to survive after transplantation and cannot induce any rejection[10]. In this experiment, 1 month after the Hoechst33342-labeled HAECs were transplanted into the rat striatum, a large number of fluorescein-labeled cells were found in the brain tissue and were well integrated with the host. Interestingly, many cells migrated 1–2 mm along the nerve fibers in the corpus callosum. No previous study has reported the migration of HAECs and further studies are required to conclusively determine whether HAECs have features of migrating cells, such as those displayed by neural stem cells[11].

In summary, the present experiment was the first demonstration that both amniotic membrane and the cultured HAECs express neuron-specific markers, their secretion solution has neurotrophic effects, and the transplanted cells can survive long term in nerve tissue. This is evidence that HAECs are a good seed cells for cell transplantation.

MATERIALS AND METHODS

Design
A randomized controlled animal experiment.

Time and setting
Experiments were performed from March 2004 to May 2005 at the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China.

Materials
Animals
Twenty healthy, pathogen free, adult, female Sprague-Dawley rats, weighing 250–300 g, were utilized for cell transplantation (license No. SCXK (Hu) 2004-0006). The experimental disposal of animals complied with the Regulations for the Administration of Affairs Concerning Experimental Animals, issued by the Ministry of Science and Technology of China[12].

Amnion samples
Human placenta was harvested after cesarean section from the First Maternal and Child Health Hospital of Shanghai, China, in accordance with the Guidance Suggestions for the Pregnant Placenta after Live Birth, formulated by the Ministry of Public Health of China[13], and all puerperants and their families gave written consent.

Methods
HAEC isolation, culture and morphology
The duration from the isolation of amniotic membrane to cell separation and culture was controlled within 2 hours. Human amniotic membrane at a size of 15 cm × 15 cm was obtained after cesarean surgery and washed with phosphate buffered saline (PBS) following the removal of residual choriocarcinoma. Samples were digested with 0.125% trypsin (Sigma, St. Louis, MO, USA) at 37°C for 15 minutes, repeated four times, then centrifuged at 1 000 r/min for 6 minutes. Cells were collected, washed three times with PBS and inoculated with 10% fetal bovine serum (Hyclone, Logan, Utah, USA) + RPMI1640 culture medium (Gibco, Carlsbad, CA, USA) at 37°C in a humidified 5% CO₂ incubator. 3 days later, adhered HAECs were collected for morphologic study.

GFAP, MAP-2 and nestin expression in amniotic membrane and HAECs detected by immunohistochemistry/immunocytochemistry staining
Fresh amniotic membrane and HAECs adhered for 3 days were fixed with 4% paraformaldehyde for 20–30 minutes and underwent immunocytochemical staining for GFAP, MAP-2 and nestin expression.

Preparation of HAECs conditioned medium
After HAECs were adherent to the 6-well culture plate for 3–5 days, the culture medium was removed, and cells were washed three times with PBS. Next, 5 mL of serum-free RPMI1640 medium was added to each well and cells were cultured at 37°C in a 5% CO₂ incubator for 24 hours. The culture medium was then centrifuged in tubes at 7 500 r/min for 20 minutes at 4°C. The supernatant (that is, the HAECs conditioned medium) was removed and stored at 4°C.

Isolation and culture of rat glial cells
Fetal mice were used on postnatal day 2. The frontal cortex Cells were collected and seeded into the petri dishes containing 10% fetal bovine serum at a density of 1 × 10⁵ cells per well. The dishes were incubated at 37°C in a humidified 5% CO₂ incubator.

Morphology of rat glial cells under different culture conditions detected by hematoxylin-eosin staining
Three generations of cultured rat glial cells were digested, centrifuged and seeded onto the 6-well plate, with serum-free RPMI1640 or HAEC conditioned medium. Three days later, cells were collected, fixed and stained...
The number of living rat glial cells under different culture conditions detected by trypan blue staining
Three generation of cultured rat glial cells were seeded in 24-well plates at 2 x 10^5 cells per well and incubated at 37°C and 5% CO₂ for 7 days. Cells were treated with serum-free RPMI1640 or HAEC conditioned medium. Cells in three wells were collected everyday and digested, then the number of living cells was counted with trypan blue staining and the mean values were calculated. The growth curve was plotted to observe the effect of HAEC conditioned medium.

Proliferation of rat glial cells under different culture conditions detected by MTT assay
Third generation cultured rat glial cells were digested, centrifuged and seeded onto a 96-well plates at a density of 2 000 cells per well. Cells were divided into three groups according to the culture conditions: serum-free culture group (RPMI1640); fetal bovine serum culture group (10% fetal bovine serum + RPMI1640); and HAEC conditioned medium group. Furthermore the HAEC conditioned medium was assigned into three concentrations: one-third HAEC conditioned medium (plus serum-free RPMI1640); two-thirds HAEC conditioned medium (plus serum-free RPMI1640); and complete HAEC conditioned medium. After 5 days of culture the reaction in each well was terminated with 20 μL of 5 mg/mL MTT (Gibco) at 37°C for 4 hours. The supernatant was discarded, 150 μL DMSO was added to each well, and the culture plate was oscillated for 1 minute. The absorbance at 490 nm was read with an automatic microplate reader (Shanghai Jianglai Bio-Technology Co., Ltd., Shanghai, China).

The survival and migration of the HAECs transplanted detected by fluorescence microscopy
The HAECs adhered for 5 days were stained with Hoechst33342 (Sigma). The cell density was adjusted to 2 x 10^5 cells/mL. Cell suspension (5 μL) was injected into the left striatum of rats via the following coordinates: 0.5 mm posterior to the bregma, 2 mm lateral to the center and 5 mm deep. One month later, rat's brain tissue around the needle tract was obtained and cut into 30 μm-thick frozen sections. The survival and migration of the cells were observed under a fluorescent microscope.

Statistical analysis
Data were statistically analyzed with SPSS 10.0 statistical software (SPSS, Chicago, IL, USA). Both cell counts and absorbance values were expressed as mean ± SD. Comparisons between multiple groups were done using one-way analysis of variance and a level of P < 0.05 was considered a statistically significant difference.

Author contributions: Guozhen Hui and Lihe Guo had full access to the study concept and design. Zhiyuan Wu, Tianjin Liu and Qin Huang provided, integrated, and analyzed experimental data. Zhiyuan Wu and Yi Lu drafted and validated the manuscript. Guozhen Hui was responsible for the fund.

Conflicts of interest: None declared.

Funding: The pilot was sponsored by the National Natural Science Foundation of China, No. 30271325 and the Natural Science Foundation of Jiangsu Province, No. BK2001170; the National Basic Research Program of China (973 Program), No. 2005CB522604.

Ethics approval: The animal experiments have been approved by the Animal Ethical Committee of Soochow University, China, and the amniotic membrane was obtained under the approval of the Animal Ethical Committee of the First Maternal and Child Health Hospital of Shanghai, China.

Acknowledgments: We would like to express our thanks to Zhihua Jiang, from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China for the guidelines in morphological observations.

REFERENCES


(Edited by Wang X, Sa YL/Yang Y/Wang L)