Human amniotic epithelial cells express specific markers of nerve cells and migrate along the nerve fibers in the corpus callosum ☆

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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

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[2]. 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[3].

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[4-5], 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.

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 1×10^6 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.
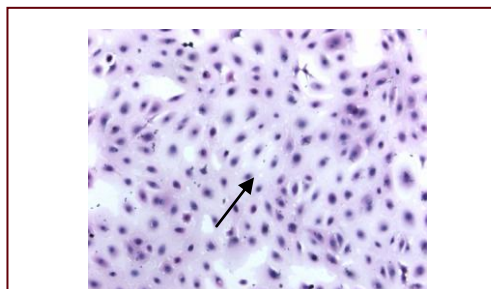

Figure 1   Morphology of the human amniotic epithelial cells in primary culture (hematoxylin-eosin staining, light microscopy, × 200).

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

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).
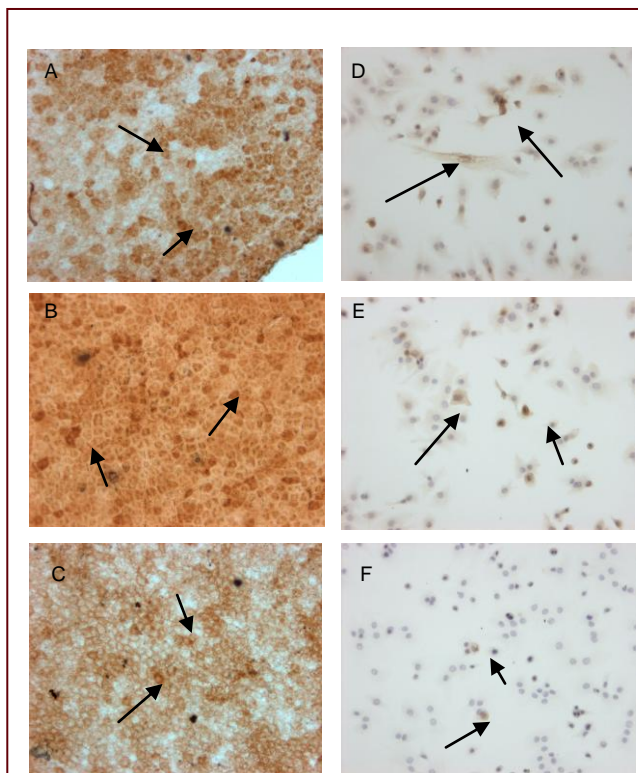

Figure 2　Expression of glial fibrillary acidic protein (A, D), microtubule-associated protein-2 (B, E), and nestin (C, F) in amnion tissue and the cultured human amniotic epithelial cells (light microscopy, × 200).

(A−C) Amniotic membrane (immunohistochemistry).

(D−F) Primary cultures of human amniotic epithelial cells (immunocytochemistry).

Glial fibrillary acidic protein (arrows), microtubule-associated protein-2 (arrows), and nestin (arrows) were expressed in both the amnion membrane and the cultured human amniotic epithelial cells, and the number of nestin-positive cells was reduced in the cultured cells.

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).
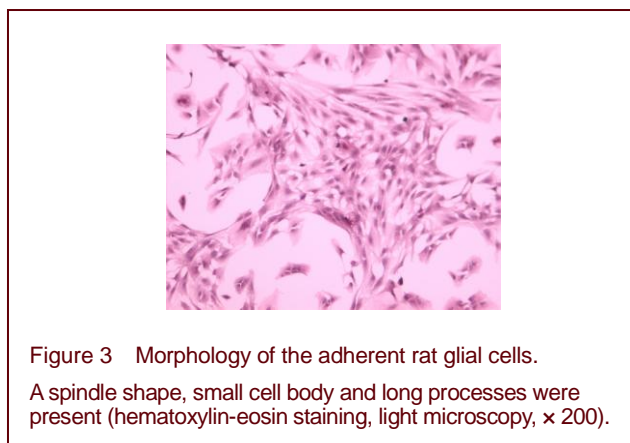

Figure 3　Morphology of the adherent rat glial cells.

A spindle shape, small cell body and long processes were present (hematoxylin-eosin staining, light microscopy, × 200).
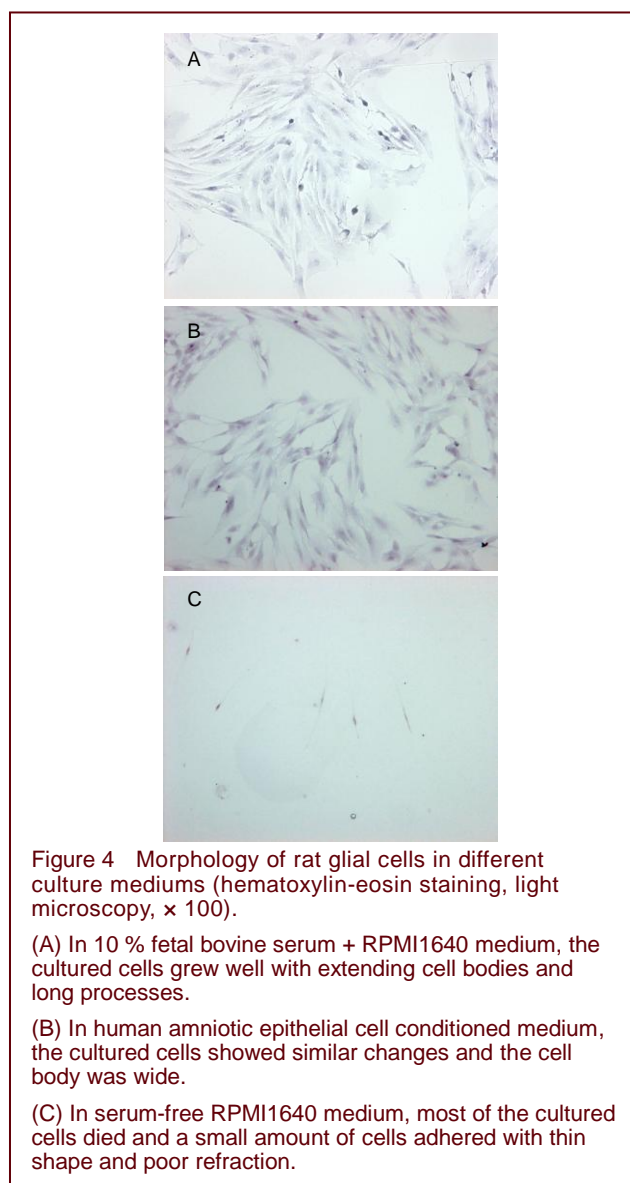

Figure 4　Morphology of rat glial cells in different culture mediums (hematoxylin-eosin staining, light microscopy, × 100).

(A) In 10 % fetal bovine serum + RPMI1640 medium, the cultured cells grew well with extending cell bodies and long processes.

(B) In human amniotic epithelial cell conditioned medium, the cultured cells showed similar changes and the cell body was wide.

(C) In serum-free RPMI1640 medium, most of the cultured cells died and a small amount of cells adhered with thin shape and poor refraction.

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

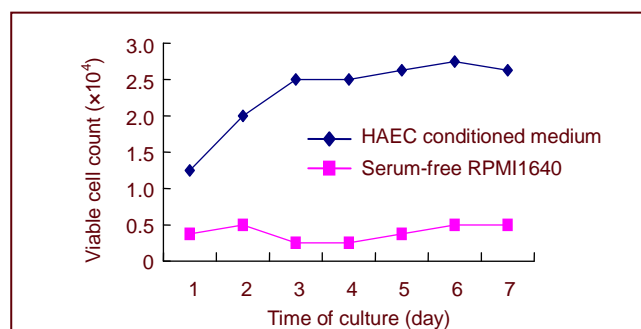proliferation of rat glial cells (Figures 5, 6).

Figure 5   Growth curve of rat glial cells cultured in different culture conditions.

Data are expressed as the mean values of three samples collected at each time point in a group. In human amniotic epithelial cell conditioned medium, the growth of cultured cells increased exponentially at 2−3 days, while cells could not survive in serum-free RPMI1640 medium.
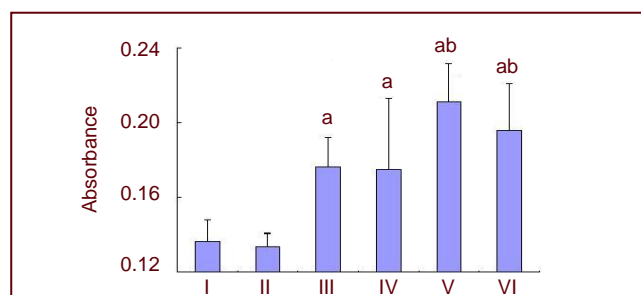

Figure 6   Effect of conditioned medium on the proliferation of rat glial cells by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay.

A total of 16 cell samples were cultured in each culture medium for 5 days. Data are expressed as mean ± SD. $^aP < 0.01$, *vs.* serum-free culture medium; $^bP < 0.01$, *vs.* fetal bovine serum culture group (one-way analysis of variance). I: Control group; II: serum-free culture group; III: fetal bovine serum culture group; IV: one-third HAEC conditioned medium group; V: two-thirds HAEC conditioned medium group; VI: complete HAEC conditioned medium group. HAEC: human amniotic epithelial cell.

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[6]. 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[7].
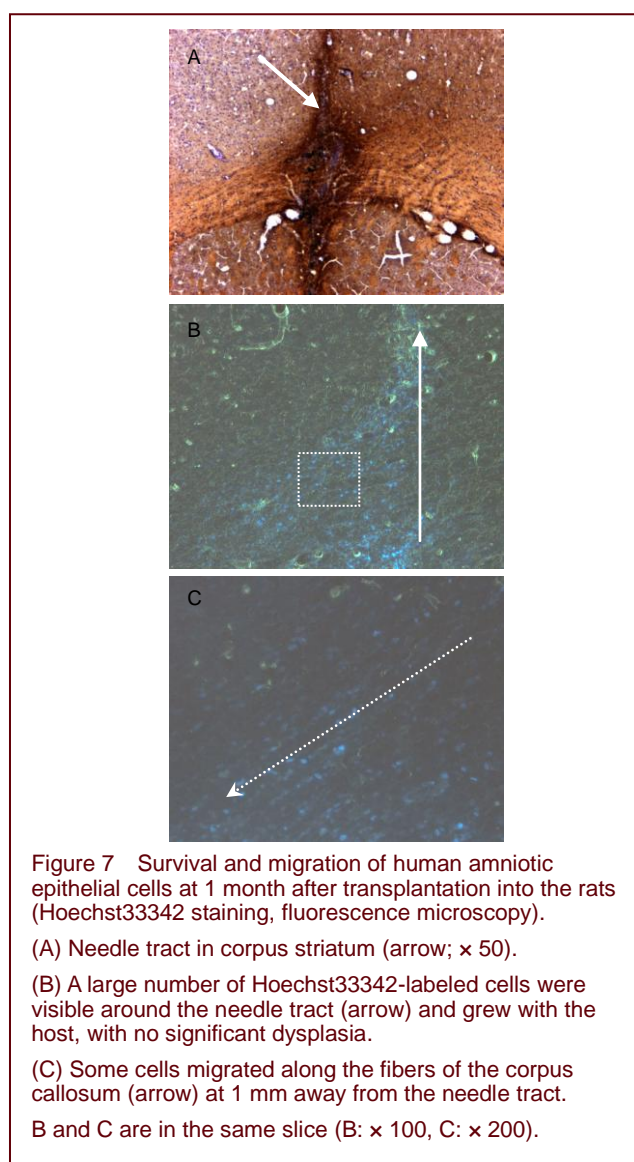

Figure 7   Survival and migration of human amniotic epithelial cells at 1 month after transplantation into the rats (Hoechst33342 staining, fluorescence microscopy).

(A) Needle tract in corpus striatum (arrow; × 50).

(B) A large number of Hoechst33342-labeled cells were visible around the needle tract (arrow) and grew with the host, with no significant dysplasia.

(C) Some cells migrated along the fibers of the corpus callosum (arrow) at 1 mm away from the needle tract.

B and C are in the same slice (B: × 100, C: × 200).

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

2×10^{8}[8]. Our experiments obtained 1×10^{6} 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[5]. 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 fluorescence-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% CO_2 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% CO_2 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 1×10^{5} cells per well. The dishes were incubated at 37°C in a humidified 5% CO_2 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

for morphologic study.

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 2×10^4 cells per well and incubated at 37°C and 5% CO_2 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 2×10^7 cells/mL. Cell suspension (5 μL) was injected into the left striatum of rats[14] *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.

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