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Urothelial differentiation of human amniotic fluid stem cells by urothelium specific conditioned medium

Seyung S. Chung, Ph.D.¹, Henry Kang², and Hyung-Gyoo Kang, Ph.D.²

¹Division of Cancer Research and Training, College of Medicine, Charles Drew University of Medicine and Science, 1731 East 120th street, Los Angeles, California, U.S.A. 90059

²Department of Pathology, Saban Institute for Research, Children's Hospital in Los Angeles, Los Angeles, California 90027 U.S.A

Abstract

Human amniotic fluid stem cells (HAFSCs) have a high proliferative capacity and a good differentiation potential, and may thus be suitable for regenerative medicine. To date, urothelial differentiation mechanisms of HAFSCs are poorly understood. We have investigated the urothelial differentiation potential of HAFSCs so that they can be therapeutically applied to cure defective diseases of bladder. To induce the stem cell differentiation, HAFSCs were cultured in a bladder cancer-derived conditioned medium. After 2 weeks of culture, HAFSCs began to express the urothelial lineage-specific markers (UPII, CK8 and FGF10). Meanwhile, pluripotency markers (Oct-4, Sox-2 and Nanog) were downregulated at both RNA and protein levels in the differentiated HAFSCs. Immunocytochemistry data revealed that differentiated HAFSCs expressed urothelial markers of UPII and CK8. We have screened the receptor tyrosine kinase arrays with the differentiated HAFSCs. The screening showed that MuSK, Tie-1 and EphA4 receptor tyrosine kinases were upregulated, whereas EphA7 and FGF R1 kinases were downregulated in HAFSCs. The data suggest that HAFSCs can be an important urothelium cell source, which can be use for urinary tract engineering.

Keywords

bladder cancer; differentiation; Immunohistochemistry; receptor tyrosine kinase; RT-PCR; stem cell

1. Introduction

The current technique for the reconstruction of the urinary tract is utilizing the autologous segments of the gastrointestinal tract. This technique, however, may cause various complications, such as urinary calculi, intestinal adhesions, chronic infections and secondary malignancies (Atala, 2011). Cell-based tissue engineering can provide an alternative way for bladder reconstruction. Atala et al. (2006) reported a clinical trial of tissue-engineered urinary bladders with urothelium and smooth muscle cells expanded from the biopsy specimen. However, this approach has a limitation; when patients have cancer, infection or organ loss, the organ-specific autologous cells cannot be used for tissue engineering. Therefore, more attention has been brought to HAFSCs as a source for the regenerative medicine.

Correspondence to: Seyung S. Chung, Ph.D. Division of Cancer Research, College of Medicine, Charles Drew University, 1731 East 120th street, Los Angeles, California 90059, U.S.A., Phone: 1-323-563-4999; Fax: 1-323-563-4889, seyungchung@cdrewu.edu.

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HAFSCs are multi-potent stem cells with a mesenchymal origin extracted from amniotic fluid (De Coppi et al., 2007). The use of HAFSCs is less controversial since human embryos need not be destroyed for stem cell extraction. HAFSCs can differentiate into various tissue types, such as skin (Siegel et al., 2009), cartilage (Siegel et al., 2008), kidney (Perin et al., 2007), nerve (Antonucci et al., 2009), neuron (Prusa et al., 2004) and heart (Schmidt et al., 2008). With good multi-potency, HAFSCs could be an alternative endless source for bladder tissue engineering.

We hypothesized that microenvironment changes induced by a bladder-specific culture medium was sufficient to induce differentiation of HAFSCs into urothelial cells. To this end, we used a conditioned medium derived from the human bladder cancer line, LD605. HAFSCs were cultured in this conditioned medium and examined for the bladder lineage specific gene expression. Our data suggest that HAFSCs can be useful cell source for urothelial cells in urinary tract tissue engineering.

2. MATERIALS and METHODS

2.1 Cell lines and culture

HAFSCs were isolated and maintained as previously described (Canazi et al., 2009). Samples of human amniotic fluid from male fetuses (12–18 week of gestation) were provided by Genzyme Genetics Corporation (Mongovria, CA, USA) after karyotyping analysis. The stem cell population was separated from the general human amniotic cellular milieu using standard Magnetic Sorting (MACS) techniques (Miltenyi Biotech, Auburn, CA, USA) against the cell surface marker, c-kit. Cancer cell line LD605 was used to derive the conditioned medium. LD605 is a patient-derived immortalized bladder cancer line. Multiple apoptosis-associated genes were methylated in LD605. Vascular endothelial growth factor (VEGF) receptor and EphB4 receptor tyrosine kinase were highly expressed in LD605 (Xia et al., 2006). LD605 was grown in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal calf serum (Life Technologies, Rockville, MD, USA).

2.2 Conditioned medium culture of HAFSCs

HAFSCs ($1,500\text{--}2,000\text{ cells/cm}^2$) were cultured with LD605 bladder cancer cells-derived conditioned medium for 14 days. The conditioned medium cultures of HAFSCs were triplicated for each experiment. HAFSCs culture experiments were repeated at least 3 times. Conditioned medium was collected from cultured human bladder cancer line LD605 with 60–70% confluence. The collected media were centrifuged at 3,000 rpm for 5 min to remove cells, filtered (0.22 μM) and diluted with one-fourth of the volume of DMEM with 10% FBS before use.

2.3 Urothelial Gene Expression Determination

To monitor the differentiation effect of conditioned media, urothelial lineage-specific gene transcription was detected by RT-PCR after 14 days induction. The urothelial genes explored were uroplakin II, CK8 and FGF10. Total RNAs were isolated from cells Rneasy kit (Qiagen, Valencia, CA, USA), and cDNA synthesized using OneStep RT-PCR kit (Qiagen, Valencia, CA, USA). The sequences of primers are listed in Table 1. Each RT-PCR experiments were repeated at least 3 times.

2.4 Western blot analysis

Whole cell lysates were prepared from both HAFSCs cultured in DMEM medium and HAFSCs cultured in conditioned medium. The 2 lysates were subjected to immunoblotting, loaded side-by-side on the gel to compare the urothelial lineage-specific gene expressions. Cells were lysed in RIPA lysis buffer (Thomas Scientific Co, Swedesboro, NJ, USA).

Protein (50 µg) was resolved using a 10% SDS-polyacrylamide gel. The antibodies used for the western blots were uroplakin II, cytokeratin 8, Oct-4, Sox-2, Nanog, FGF10 and β-actin. Antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Proteins were detected using the Super Signal femto reagent (Thomas Scientific Co, Swedesboro, NJ, USA). Each western blot was repeated at least 3 times.

2.5 Urothelial lineage specific proteins by immunofluorescence

The cells were washed 3 times with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. The cells were incubated with the following primary antibodies at 4°C overnight: cytokeratin-8 (1:100), uroplakin 2 (1:100). An appropriate secondary antibody was conjugated to fluorescein isothiocyanate (FITC) for Uroplakin 2 and Cytokeratin 8. Finally, the cells were mounted in DAPI Nucleic Acid Stain (SIGMA, St Louis, MO, USA) for staining nuclei. Cells were viewed under a fluorescent microscope (Leica, Germany).

2.6 Activated tyrosine kinase arrays

HAFSCs were treated with conditioned medium from LD605 for 24 h. Cells were washed with PBS containing 100 µmol/L Na₃VO₄, solubilized in lysis buffer, and 300 µg of total protein were incubated with Human Phospho-RTK Arrays (R&D Systems, Catalog number ARY001B, Minneapolis, MN, USA). Arrays were incubated with whole-cell lysates overnight at 4°C with shaking and washed with the supplied washing buffer. Arrays were then incubated with anti-phosphotyrosine-HRP antibodies for 2 h at room temperature on a rocking platform shaker before incubation with a chemiluminescent reagent and film exposure. The arrays were scanned with the Bio-Rad Molecular Imager Gel Doc XR system (Bio-Rad, catalog number 170–8195, Hercules, CA, USA). After the array images were obtained, free software Image J was utilized to quantify the intensity of tyrosin kinase arrays.

3. Results

3.1 HAFSCs culture in conditioned medium expressurothelial-lineage specific genes

The morphology of LD605, HAFSCs and differentiated HAFSCs were observed (Fig. 1A). After 14 days of culture in conditioned medium, 75–80% HAFSCs showed elongated and polygonal shapes, which are typical urothelial phenotypes. In agreement with the morphology, urothelial lineage-specific genes (UPII, CK 8) and the urothelial proliferation marker, FGF10, were expressed from the differentiated HAFSCs (Fig. 1B). Consistently, urothelial lineage markers were also expressed at protein levels from the HAFSCs (Fig. 1C).

3.2 Immunocytochemistry show UPII and CK8 expressions from HAFSCs cultured in conditioned medium

Immunocytochemistry for UPII and CK8 was used to confirm urothelial differentiation at the cellular level. The differentiated HAFSCs expressed both UPII and CK8 markers (Fig. 2A and B). The results suggest that a directed-differentiation is accomplished in a conditioned-medium culture.

3.3 Pluripotency genes of Oct-4, Sox-2 and nanog are downregulated in HFASCs cultured in conditioned medium

We hypothesized that, if the urothelial differentiation was induced, the pluripotency markers would be downregulated. RT-PCR and western blots were used. Oct-4, Sox-2 and nanog were expressed in the undifferentiated HAFSCs; however, HAFSCs cultured in the conditioned medium did not express Oct-4, Sox-2 and Nanog at both RNA and protein

levels (Fig. 2C and D). The data suggest that urothelial marker genes began to be expressed at the expense of stemness gene expression.

3.4 Multiple receptor tyrosine kinases are activated during urothelial differentiation of HAFSCs

We determined which receptor tyrosine kinases were activated during HAFSCs differentiation induction by using receptor kinase arrays. MuSK (Muscle-specific kinase) receptor, Tie-1 (Tyrosine kinase with immunoglobulin-like and EGF-like domains 1) receptor and EphA4 (Ephrin type-A4) receptor tyrosine kinases were concurrently upregulated in the urothelial-lineage differentiated HAFSCs (Figure 3 A and B). EphA7 (Ephrin type-A7) and FGFR1 (Fibroblast growth factor receptor 1) kinases were downregulated with urothelial differentiation (Figure 3A and B). Quantitative analyses of receptor tyrosin kinase arrays using software Image J showed that In undifferentiated control HAFSCs, the average intensity levels of EphA7 and FGFR1 were 135 and 155, respectively. However in HAFSCs cultured in conditioned medium, the average intensities of EphA7 and FGFR1 are 110 and 134, respectively (Figure 3C). In control HAFSCs, the MuSK, Tie-1 and EphA4 average intensity units are 121, 128 and 110, whereas in HAFSCs cultured in conditioned medium, the average intensity units increased to 138, 155, 129, respectively (Figure 3D).

4. Discussion

Three urothelial specific markers were chosen to monitor the HAFSCs differentiation. They are cytokeratin 8, a cell marker expressed in all cell layers of urothelium (Erman et al., 2006), uroplakin II, which represents molecular marker for urothelial differentiation (Wu et al., 1990), and FGF10, the urothelial cell proliferation marker (Bagai et al., 2002).

We selected bladder cancer cell-derived conditioned medium expecting a rapid induction and strong paracrine effects during the differentiation. Cancer cell-derived conditioned medium has been used as a source for growth factors required for cellular changes of stem cells. For instance, media conditioned on the A549 human lung adenocarcinoma cell line are particularly effective in driving type II pneumocyte differentiation from both murine and human embryonic stem cells (Rippon et al., 2005). MT1-MMP and MMP-11 have both been reported to be induced in human fibroblasts treated with conditioned medium from MDA-MB-231, a highly invasive mammary tumor cell line (Polette et al., 1997). The comparative studies on the bladder cancer cell line and normal bladder urothelium for urothelial induction is underway.

We found 2 kinases, EphA7 and FGF R1, were downregulated during urothelial differentiation. Eph and Eph-related receptors have been implicated in mediating developmental events, particularly in the nervous system. FGF R1 family member binds both acidic and basic fibroblast growth factors, and is involved in limb induction. However, to date the mechanisms by which EphA7 and FGF R1 activation leads to urothelial specific lineage differentiation are not well understood. We also uncovered that 3 receptor tyrosine kinases were activated concurrently with urothelial differentiation. MuSK is a receptor tyrosine kinase required for the formation of neuromuscular junction (Glass et al., 1996), and is activated by the proteoglycan, agrin. Tie-1 receptor tyrosine kinase is essential for vascular endothelial cell integrity (Rodewald et al., 1996). Tie-1 upregulates the cell adhesion molecules through p38-dependent mechanism. EphA4 is a receptor tyrosine kinase involved in developmental events especially in the nervous system (Flanagan and Vanderhaeghen, 1998). So far, how upregulation of these kinases contributes to induction of urothelial differentiation is not known. It remains to be seen how the up and down regulated kinases elicit the urothelial differentiation from HAFSCs.

The potential induction mechanisms for stem cells may be that cells secrete cytokines and growth factors to their adjacent tissues, which stimulate the cells by paracrine signaling. In this regard, Tian et al. (2010) have detected several growth factors, epidermal growth factor, platelet-derived growth factor-BB, transforming growth factor- β 1, and vascular endothelial growth factor, from the urothelium derived conditioned medium. The embryonic urogenital sinus mesenchyme can induce stem cells to form prostate through a mechanism that requires stromal TGF-beta signaling (Li et al., 2009). Finally, Rider et al. (2008) reported that FGF-2 was an important growth factor for the proliferation and differentiation of adipose derived mesenchymal stem cells via the wnt pathway. However, to date it is not understood which receptor tyrosine kinases are activated during the urothelial differentiation of HAFSCs.

This is the first report that shows that multiple receptor tyrosine kinases are involved in the human stem cell differentiation into urothelium. Further studies are geared toward cross-talking between the growth factors in the conditioned-medium and activated receptor tyrosine kinase in the differentiated stem cells. In conclusion, urothelial differentiation of HAFSCs was achieved by utilizing culture medium conditioned on bladder cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HAFSCs	human amniotic fluid stem cells
FGF10	Fibroblast growth factor 10
CK	cytokeratin
UP	Uroplakin
MuSK	muscle specific kinase
Tie-1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1

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Figure 1.

Characterization of urothelial differentiation of HAFSCs.

A: Morphologies of LD605, HAFSCs and HAFSCs cultured in the conditioned medium were shown. Scale bar 100 μ m. **B:** RT-PCR data of urothelial-specific marker genes. UPII, CK8 and FGF10 gene expressions were examined from the LD605, HAFSCs and differentiated HAFSCs. **C:** Western blot analysis of urothelial-specific marker protein expression. UPII, CK8 and FGF10 protein expression levels were monitored by PAGE gel electrophoresis.

Figure 2.

Immunocytochemistry analysis of HAFSCs and pluripotency marker gene expression in stem cells. A: The panels show the expression of urothelial-specific marker UPII. B: The panels show the expression of CK8. Scale bar: 50 μ m. C: Characterization of pluripotency markers of HAFSCs by RT-PCR. Oct-4, Sox-2 and nanog expressions were monitored with the undifferentiated HAFSCs and HAFSCs cultured in a conditioned medium. D: Western blot results on the PAGE gel for the Oct-4, Sox-2 and Nanog.

Figure 3. Receptor tyrosine kinase (RTK) array screening. A: The array is done with the undifferentiated HAFSCs. B: EphA7 and FGF R1 kinases are down-regulated whereas MuSK, Tie-1 and EphA4 receptor kinases are activated in the urothelial-differentiated HAFSCs. C: The average of relative intensity of EphA7 and FGF R1 kinases were presented in a graphical presentation. The kinase intensity was decreased in HAFSCs cultured in conditioned medium. D: The average kinase intensity of MuSK, Tie-1 and EphA4 were presented. The kinase intensity was increased in HAFSCs cultured in conditioned medium.

Table 1

RT-PCR primer sequences for the assay.

Genes	Sequences
UPII	forward primer 5'-AACAAACCCACACTTACCACTG-3' reverse primer 5'-GGAGCTTGCTGGAATACACCTC-3'
CK8	forward primer 5'-GCCTGGTTCGGCCCGCCTGCCTCC-3' reverse primer 5'-GGCTGAGGGCTAGGGCTGAGCCTC-3'
FGF10	forward primer 5'-ATGTGGAAATGGATACTGACACATTG-3' reverse primer 5'-TACATTTGCCTCCCATTATGCTGCC-3'
Oct-4	forward primer 5'-TCCCTTCGCAAGCCCTCAT-3' reverse primer 5'-TGACGGTGCAGGGCTCCGGGGAGG-3'
Sox-2	Forward primer 5'-CCCCCGGCGGCAATAGCA-3' Reverse primer 5'-TCGGCGCCGGGAGATACAT-3'
Nanog	Forward primer 5'-ATCCAGCTGTCCCAAA-3' Reverse primer 5'-ATTTCATTGCTGGTTCTGG-3'
GAPDH	forward primer 5'-CCCAATGTATCCGTTGTGGA-3' reverse primer 5'-GCCTGCTTACCACCTTCTT-3'