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Original Research

Human amniotic fluid derived mesenchymal stem cells cause an anti-cancer effect on breast cancer cell line in vitro

M. Ghafarzadeh[1*], A. Eatemadi[2], Z. Fakhravar[3]

[1] Assalian Hospital, Center for Obstetrics and Gynecology, Lorestan University of Medical sciences, khoramabad, Iran
[2] Department of Medical Biotechnology, School of advance Science in Medicine, Tehran University of Medical sciences, Tehran, Iran
[3] Razi Drug applied research center, Lorestan University of Medical sciences, khoramabad, Iran

Abstract: Human amniotic fluid stem cells (hAFSCs) have the ability to self-renew, and multipotent differentiation into three germ layer cells. We obtained 5 ml amniotic fluid from ten 16-20 week pregnant women undergoing amniocentesis. hAFSCs were isolated from all samples, co-cultured with T47D breast cancer cell line and characterized using flow cytometry and RT-PCR. After 3, 4 and 5 days, T47D and HSFCs viability were evaluated with MTT assay. After 5 days of co-culture T47D cells viability were decreased. Our findings showed that hAFSCs can release soluble factors in cell culture, causing an efficient anticancer effect.

Key words: Stem cell, T47D, co-culture, anticancer therapy.

Introduction

One of the lethal gynecology malignancy is breast cancer Breast cancer threatens the mental and physical health of females, so as their quality of life. Since most of breast cancer patients have gotten to an advanced stage when they are diagnosed with this disease, metastasis occurs and abdominal implantation occur, leading to a rapid disease reducing the quality of life. Over the past 30 years, radiotherapy, chemotherapy, and gene therapy have been utilized by gynecological oncologist for the treatment of this disease using cytoreductive surgery. However, in most advance stage, metastasis persisted and thereby resulting to death (1).

Stem cell therapy has experience growth as an area of concentration in regenerative medicine (2), cancer therapy, and other applications, due to their properties. Mesenchymal stem cells (MSCs) were initially isolated from bone marrow as adherent elements, they are non-hematopoietic precursors, can self-renew, highly proliferating, and possess multi-lineage differentiation capacity (3,4). Ethical issues and possibility of initiating teratoma may eventually prohibit their usefulness clinically.

In order to avoid these problems, scientists are looking into other sources as pluripotent cells substitute, such as amniotic fluid. Isolation of novel type of stem cell from amniotic fluid has been said to be possible. Amniotic fluid from fetus is a safe and reliable screening tool for congenital and genetic diseases in the fetus, they are also more pluripotent as compared to bone marrow derived stem cells. Just like Human embryonic stem cells (ESCs), Human amniotic fluid stem cells (hAFSCs) possess several characteristics including expression of embryonic markers, ability to give rise to multiple lineage, ability to maintain their telomeric length, and the ability to remain stabilized in an undifferentiated state for long period. They can as well be induced with different growth factors, molecules and medium, when needed to differentiate into several cell types (5). Unlike ESCs, hAFSCs cells don't form teratoma in vivo, this low tumorigenicity risk would be a great asset for their therapeutic use in future.

In this study, we co-cultured hAFSCs isolated from amniotic fluid with T47D breast cancer cell line, our result shows that this cells have natural tumor tropism, and can release soluble factors in cell culture, causing an efficient anticancer effect.

Materials and Methods

Sampling

Ten patients undergoing amniocentesis, for routine karyotype screening serves as the donor of 5ml each of human amniotic fluid, and evaluation was performed in Assalian Hospital, Center for Obstetrics and Gynecology, (Lorestan, Iran). The Amniocentesis was performed according to sonographer guides using a 22G Needle under a gynecologist supervision.

Cell culture and Cell Lines

Human amniotic fluid serves a source of hAFSCs (5). Undifferentiated stem cells were kept in Chang's medium (alpha-minimal essential medium 400ml, L-glutamine 5ml, Penicillin/Streptomycin 5ml, Chang Supplements 12.5ml and fetal bovine serum 100ml; Irvine Scientific, Irvine, CA) and incubated at 37°C in a 95% humidified chamber with 5% carbon dioxide. Breast cancer cell line T47D were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% heat inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml; Life Technologies, Rockville, MD). This cancer cell line was incubated at 37°C in a 95% humidified chamber with a 5% carbon dioxide.

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*** Corresponding author:** Masoomeh Ghafarzadeh, Center for Obstetrics and Gynecology, Lorestan University of Medical sciences, khoramabad, Iran. Email: m.ghafarzadeh95@gmail.com

Flow cytometric analysis

After 3rd-5th passage of hAFSCs, cells were separated with trypsin, washed twice in FACS wash (PBS+ 2% FBS), and then centrifuged at 1500 rpm for 3 minutes. Supernatant was discarded, and resulting cell pellet was resuspended and filtered out using 40 µm cell strainer. Then, the cells were incubated with a 1/30 dilution of fluorescein(FITC)-conjugated antibody. Then, cells were diluted in 100µl of FACS wash containing 1/1000 concentration of PI (propidium iodide) in order to remove dead cells, and analyzed using flow cytometer with a BD FACS Calibur Flow cytometer (Ref: 342976), at total of 10000 events for each of the cell line. We checked CD90 (BD pharmigen, cat# 560977), CD 44 (BD pharmigen, cat# 555596), mesenchymal stem cell markers, and CD31 (PECAM-1, cat# FAB3567c) and CD 45(abcam, cat# ab65952) as hematopoietic markers.

Pluripotency Characterization

Before cryopreservation cell line pluripotency statuses were evaluated at 3rd-5th passage. Cells were checked using reverse transcription polymerase chain reaction (RT-PCR) for gene expression, using NANOG and Oct4 pluripotent markers, which were visualized by 2% gel electrophoresis. Furthermore, cells were prepared for FACS analysis to assess mesenchymal stem cells markers like as CD90 and CD44, and also hematopoietic markers like CD31 and CD45.

Total RNA extraction, cDNA synthesis and RT-PCR

RNX-Plus kit (CinnaGen, cat# RN7713C) was used to extract total RNA from 5-6×10^5 undifferentiated hAFSCs from 3rd-5th passages, according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total RNA using Termo Scientific Revert Aid First Strand cDNA Synthesis Kit (Fermentas, cat# K1621) with oligo-dT primer in a 20 µl reaction mixture performed in a Thermal Cycler (PeQLab). Primers used for amplification are listed in Table 1. All samples were verified for b-actin (housekeeping gene), as an internal control to check the progress of the RT reaction, other pluripotent genes were verified with its specific primers. RT-PCR using the Hyper Script RT master mix (GenAll, cat# 601-710) was carried out with 2µl of the single stranded cDNA sample for detection of NANOG and Oct4 (POU5F1) gene expression. The experiment proceeded with an initial denaturation at 94°C for 4 min, amplification at 94°C for 30 sec, followed by annealing temperature at 56°C for 30 sec, and 72°C for 30 sec, and extension at 72°C for 5 min, for a total of 35 cycles. PCR products amplified were separated on a 2% agarose gel using electrophoresis, bands were visualized by dye, and photographed with a gel doc.

Cryopreservation

The temperature for warming and thawing HAFSCs was optimized using cryopreservation technique. We initially calculated the number of cells using trypan blue, 1× 10^6 cells per cryotubes. We then centrifugate cells at 400g for 3 min, the supernatant was discarded, and the cell lumps were resuspended into four different combinations of cryoprotectants: a solution of 10% (v/v) DMSO and 90% FBS, a solution of 5% (v/v) DMSO and 95% FBS, a solution of 4% (v/v) DMSO and 96% FBS or one with 2% DMSO and 98% FBS.

Aliquots of 1×10^6 cells were transferred in all groups, and homogenized into 1 mL cryotubes containing each cryoprotectant solution. Just after the addition of the cells, the cryovials were refrigerated at -20°C for 45 minutes (optional), and then to -70°C for 24h. All samples were then transferred to a liquid nitrogen tank for further analysis. After one week, cryopreserved cells were thawed by rapidly dipping the cryovials in a water bath set at 37°C. Then the cells were transferred to a 15mL Falcon tube, and 10mL medium was gradually added. The supernatant was discarded after centrifugation at 400g for 3 min. The pellet obtained was resuspended in the medium, and the numbers of cells were calculated using trypan blue.

Co-culture of T47D Breast cancer cell and isolated hAFSCs

Undifferentiated hAFSCs and T47D Breast cancer cell line were co-cultured indirectly (without contact, but exchanges of soluble factors) using 0.4 µm pore-sized membrane tissue culture inserts (Becton Dickinson labware, Catalog number 353495, translucent polyethylene terephthalate membrane, Franklin Lakes, NJ) for five days. Firstly, cell suspensions containing 40,000 T47D in 24-well tissue culture plates was prepared, after 24h a trans-well (0.4 µm pore-sized) was inserted, and seed 25,000 hAFSCs in trans-well followed. The cells were co-cultured in DMEM containing 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin, and incubated at 37°C in a 95% humidified chamber with 5% carbon dioxide. Human skin fibroblast cells were used as negative control.

MTT

Three, four and five days after co-culturing hAFSCs with SKOV3, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich, USA) 5 mg/ml was added to each well (10 µl per well) and the plates were incubated for 4 h at 37˚C, followed by lysis reagent overnight. Supernatants were removed and 100 µl of 99% dimethyl sulfoxide (DMSO) Junsei

Table1. Primers used for RT-PCR.

	Gene name	Primer forward (F) and revers (R)	PCR size
1	Oct4 (POU5F1)	F 5' CCATGCATTCAAACTGAGGT 3' R 5' CCTTTGTGTTCCCAATTCCT 3'	146bp
2	NANOG	F 5' AGTCCCAAAGGCAAACAACC 3' R 5' TGCTGGAGGCTGAGGTATTT 3'	161bp
3	Beta actin	F 5 GGCACCCAGCACAATGAAGA 3' R 5 CGACTGCTGTCACCTTCACC 3'	342bp

Chemical Co., Tokyo, Japan) were added to each well to dissolve the resultant total dye was quantified by absorbance at 600 nm for 1 second. A standard curve of known cell numbers was prepared and analyzed in parallel (6).

Statistical analysis

All data were analyzed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Data from the in vitro and in vivo experiments are presented as the means ± SD and the means ± SEM, respectively. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison tests. A P-value >0.05 was considered to indicate a statistically significant difference. All tests were done in triplicated manner.

Results

Cell isolation

We successfully isolated hAFSCs, after one week of first culture in medium, all samples were visualize using inverted microscope, and results showed the excessive cell size growth and numbers. The Multilayer growths of cells appeared after 3 weeks of first culture. In this condition, the confluency of cells was 90% (Figure 1).

Total RNA extraction, cDNA synthesis and RT-PCR

All samples between 3rd -5th passages that were analyzed by RT-PCR showed mRNA expression of stem cell markers (pluripotency markers) such as Oct-4 (POU5F1), and NANOG, which were detectable relative to the housekeeping gene b-actin in 2% gel electrophoresis (Figure 2).

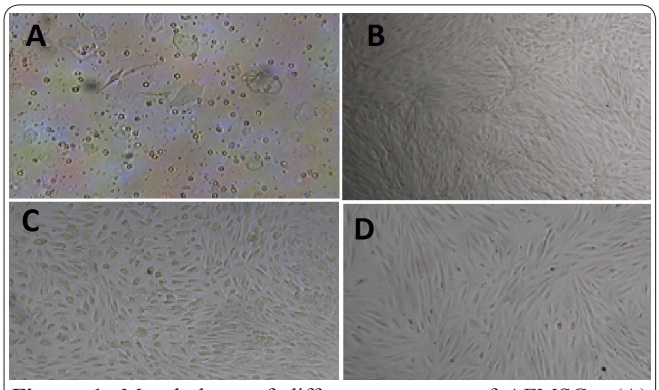

Figure 1. Morphology of different passages of AFMSCs. (A) Primary passage of DMEM-L. The non-adherent cells. (B) The third passage. (C) Cells in primary passage those were isolated from supernatant. D) Passage 1 the epithelioid population rapidly disappeared from culture and AFSCs showed a fibroblast-like morphology.

Figure 2. Gene expression profile of amnion-derived stem cells in 2% gel electrophoresis. Human skin fibroblast cells were used as negative control. β-action served as the internal control. Oct4 and Nanog expressed positively in AFMSCs.

Flow Cytometry

Monoclonal antibodies were used for the detection of surface antigens expression by flow cytometry. This technique showed that hAFSCs were successfully isolated based on cell surface marker profiles. All cultures were positive for CD90 (99%) and CD 44 (87%) confirming positive for MSCs markers. However, expressions of CD45 and CD31 were negative confirming that they did not express hematopoietic markers (Figure 3).

Cryopreservation of HAFSCs

After one week cryopreservation of 10^6 viable cells for each cryovial, in two different media using 2.5, 5, 10 and 20% DMSO, cryopreserved cells were thawed by rapidly immersing the vials in a water bath set at 37°C. In the next step, the cell viability was calculated, stained with trypan blue after thawing. The results showed that, the media containing DMSO 5% and 4% had more cell viability than those containing DMSO 2% and 10%. All p-value were significant (figure 4).

Co-cultivation induces early ovarian cell differentiation

To determine whether hACSC have cytotoxcicity effect on T47D breast cancer cell line, we performed

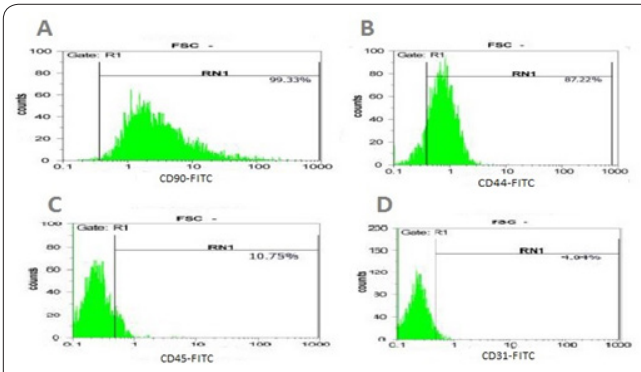

Figure 3. Flow cytometric analysis of surface-markers expression of Human amniotic fluid stem cells (HAFSCs). The cells were labeled with FITC-conjugated antibodies. Human amniotic fluid stem cells were positive for (A) CD90 and (B) CD44 and negative for (C) CD45 and (D) CD31.

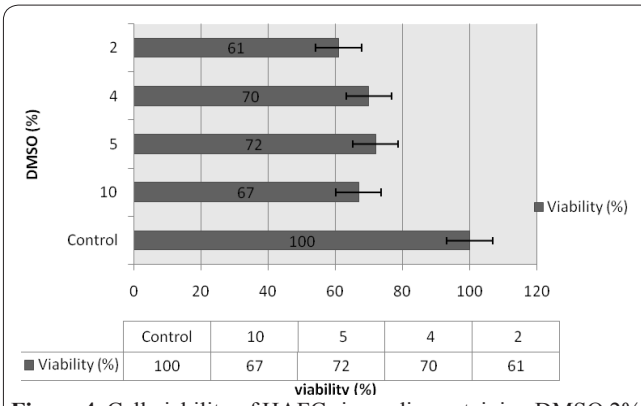

Figure 4. Cell viability of HAFCs in media containing DMSO 2%, 4%, 5% and 10%. As shown in this figure, the media containing DMSO5% and 4% had more cell viability than those containing DMSO 2 and 10%. All p-value were significant and p-value for media containing DMSO 2% vs. control, media containing DMSO 4% vs. control, media containing DMSO 5% vs. control and media containing DMSO 10% vs. control were .025,.009, .004 and .013, respectively.

the hAFSC-cancer cell co-culture *in vitro*. After three, four and five days of co-culture with ovary cancer cell T47D, MTT assay were performed (figure 5, 6 and 7). According to these results and in comparison with Human skin fibroblast cells (as negative control), hAFSC showed a significant anti-cancer effect on T47D Breast cells, indicating that T47D cells are highly sensitive to the cytotoxicity of hAFSC. This cytotoxicitic effect was time dependent, which shown in Fig. 5, 6 and 7 for 3, 4 and 5 days (respectively) after co-culture of hAFSC-cancer cell.

Discussion

In this study, we isolated hAFSCs and co-cultured it with T47D Breast cancer cell lines. These cells were easily isolated from patients undergoing amniocentesis for routine karyotype screening. Amniocentesis is a moderately gentle modality applied for prenatal and karyotype screening. These methods propose an easy procedure to access fetal stem cells without harming fetus itself.

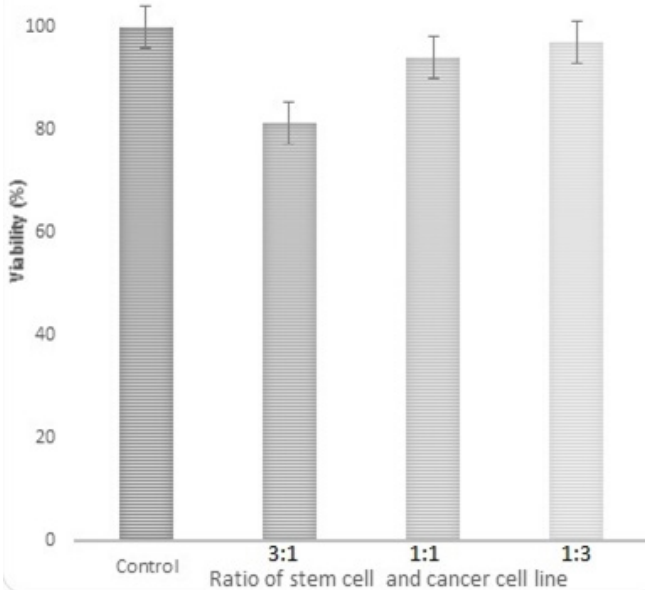

Figure 5. MTT assay results of therapeutic effects of hAFSC on ovarian cancer cell line after 3 day co-culture. All p-value was significant. P-value for ratio of stem cell and cancer cell line 3:1, 1:1 and 1:3 were 0.03, 0.05 and 0.09, respectively.

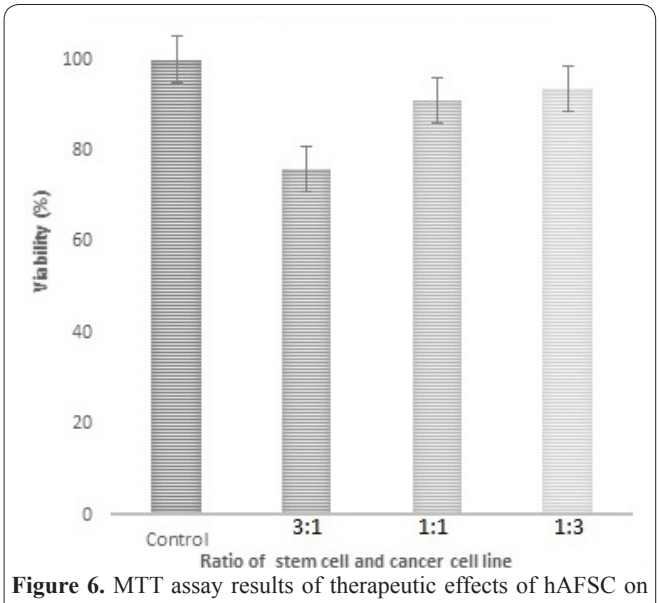

Figure 6. MTT assay results of therapeutic effects of hAFSC on ovarian cancer cell line after 4 day co-culture. All p-value was significant. P-value for ratio of stem cell and cancer cell line 3:1, 1:1 and 1:3 were, 0.014, 0.019 and 0.023, respectively.

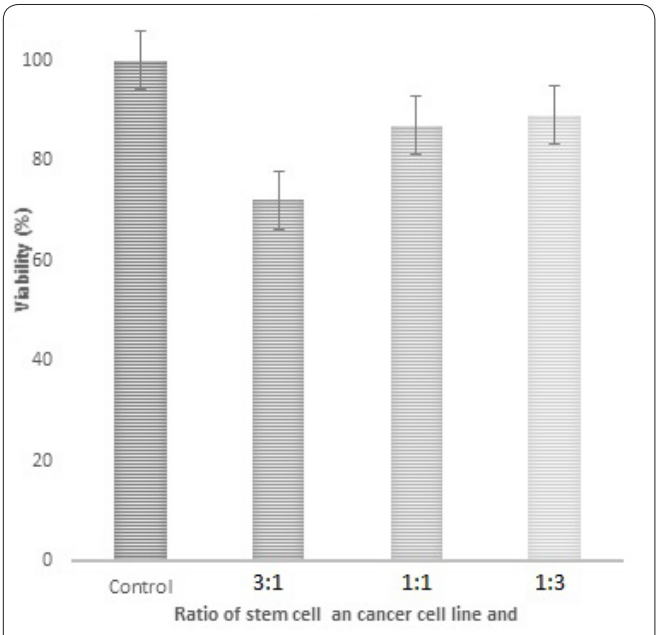

Figure 7. MTT assay results of therapeutic effects of hAFSC on ovarian cancer cell line after 5 day co-culture. All p-value was significant. P-value for ratio of stem cell and cancer cell line 3:1, 1:1 and 1:3 were 0.002, 0.005 and 0.010, respectively.

The multipotency of hAFSCs was analyzed using RT-PCR for (Oct-4 and NANOG), and flow cytometry. Our study is similar to that of Guan et al., where neonatal rat cardiomyocytes were co-cultured in monolayer with human hAFSCs to determine their in vitro differentiation capacities (7), a redistribution of connexin43 and N-cadherin proteins leading to gap junction formation occurred (7).

We focused on two positive markers of hAFSCs (CD44and CD90), and also two negative markers (CD 31, CD 45). Based on the results of RT-PCR, Oct-4 (POU5F1), and NANOG was detected relative to the housekeeping gene, b-actin. Furthermore, flow cytometry shows that over 99% of hAFSCs for CD90 and 87% for CD 44 was positive, and expression of CD45 and CD 31 in all hAFSCs was negative. Nanog and Oct4 are two transcription factors necessary to sustain the pluripotency and self-renewal of embryonic stem (ES) cells (8,9). HAFSCs are also identified to express cells surface markers of embryonic stem cells, such as Oct4, Nanog, mesenchymal markers (vimentin, alpha smooth muscle actin α-SMA, N-cadherin) as well as endothelial markers (CD144, von Willebrandt factor). Thus, for this reason, hAFSCs are known for being an intermediate stage between embryonic and adult stem cells. This feature proposes they will be excellent candidates for clinical applications. To access optimum condition for freezing and thawing of hAFSCs, cells were freezed in four different combinations of cryoprotectants: a solution of 10% (v/v) DMSO and 90% FBS, a solution of 5% (v/v) DMSO and 95% FBS, a solution of 4% (v/v) DMSO and 96% FBS or one with 2% DMSO and 98% FBS. After thawing and calculating cell viability, the results

were shown that, the media containing DMSO 5% and 4% had more cell viability than those containing DMSO 2% and 10%

hAFSCs is potential precursors to a wide range of differentiated cell linages. hAFS cells can therefore yield differentiated cells corresponding to each of the three embryonic germ layers. Furthermore, it is remarkable that HAFSCs were positive for Oct-4 and NANOG genes, which is a characteristic of the embryonic and adult stem cells, thus proposing their potential multipotency. The surface marker expression of hAFSCs, and their potentials for expression of the transcription factor Oct4 proposes that they are intermediate stage between pluripotent ESCs cells and adult stem cells.

In conclusion, our results showed the elasticity of hAFSCs, and their favorable potential as a multipotent cell source for regenerative stem cell therapy, being capable of giving rise to multiple lineages, and can release soluble factors in cell culture, causing an efficient anticancer effect. Thus, we can use hAFSCs for complete anticancer therapy on T47D Breast cancer cell line at cell culture condition and in vivo, in the future.

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