Isolation and Differentiation of Human Mesenchymal Stem Cells Obtained from Second Trimester Amniotic Fluid; Experiments at Chang Gung Memorial Hospital

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Background: The aims of this study were to evaluate the efficacy of current techniques for isolating mesenchymal stem cells (MSCs) from amniotic fluid obtained by second-trimester amniocentesis as well as to determine their differentiation potential.

Methods: We collected 50 samples of amniotic fluid by second-trimester amniocentesis. To obtain MSCs from amniotic fluid, the fluid was cultured using the two-stage culture protocol described in previous literature. Reverse transcription-polymerase chain reaction (RT-PCR) of a stem cell-specific transcription factor, octamer-binding protein 4 (Oct-4), was used to identify the characteristics of the MSCs cultured from amniotic fluid. Osteogeneic differentiation of these MSCs was confirmed by the presence of osteocalcin (a mineral-binding protein uniquely synthesized by osteoblasts) using RT-PCR and Von Kossa staining. Adipogenic differentiation of these MSCs was displayed by RT-PCR of adipocyte lipid-binding protein (a lipid-binding protein specifically in adipocytes) and Oil Red O staining.

Results: Amniotic fluid-derived MSCs were successfully isolated and cultured from six samples. These cells could express the pluripotent stem cell-specific transcription factor Oct-4 as confirmed by RT-PCR. Under specific culture conditions, amniotic fluid-derived MSCs could be successfully induced to differentiate into adipocytes and osteocytes, based on product analysis by RT-PCR and specific staining.

Conclusion: Based on our experiment, we estimate the efficacy of isolating mesenchymal stem cells from second-trimester amniotic fluid obtained by amniocentesis to be about 12%. Human MSCs from second-trimester amniotic fluid had the ability to differentiate *in vitro* into adipocytes and osteocytes under specific culture conditions. The multilineage differentiation potential of these amniotic fluid-derived MSCs may be applicable to cell transplantation and regenerative medicine.

(Chang Gung Med J 2007;30:402-7)

Key words: mesenchymal stem cells, amniotic fluid, differentiation

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Received: Jul. 7, 2006; Accepted: Feb. 5, 2007

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Mesenchymal stem cells (MSCs) are capable of differentiating into different mesenchymal lineages. Recent reports have described the identification of pluripotent or multipotent stem cells from umbilical cord,[1] cord blood,[2-5] human placenta[6-9] and amniotic fluid.[10-13] Although such observations suggest that amniotic fluid might be a useful source of stem cells, the data on the efficacy of culturing mesenchymal stem cells from amniotic fluid has been scarce. The differentiation potential of mesenchymal stem cells from amniotic fluid had been previously demonstrated by cell morphology and staining. However, analyzing the end product by reverse transcription-polymerase chain reaction had not been done. These questions inspired further investigation in our laboratory.

METHODS

Collecting samples of amniotic fluid

Fifty amniotic fluid samples (20 ml of amniotic fluid each) were obtained for fetal karyotyping by amniocentesis performed between 16 and 20 weeks of gestation. Specimens were obtained with informed consent signed by the patients. Cytogenetic analyses revealed normal karyotypes in all cases.

Cell culture of MSC from amniotic fluid and in vitro differentiation culture conditions

The cells in amniotic fluid were prepared following the procedures previously described by Tsai MS, with some modifications.[13] Amniotic fluid cells were plated on three 35 mm diameter cell culture dishes using Chang medium (Irvine Scientific, Santa Ana, California) as the culture medium. The attached cells were used for Giemsa-stained chromosome banding under standard protocol. The non-attached cells in the supernatant medium (about 4.5 ml -5 ml) were collected and grown in HyQ MEM Alpha Modification (α-Minimum Essential Medium, HyClone, Logan, UT) containing 20% fetal bovine serum (FBS; Hyclone, Logan, UT) and 4 ng/ml basic fibroblast growth factor (bFGF; Sigma) in a 25 cm^2 flask and incubated at 37°C with 5% humidified CO_2. When the cells achieved 80%-90% confluency, they were dissociated by trypsin and plated in two 25 cm^2 flasks. At cell passage 3-4, these cells were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) for octamer-binding protein 4 (Oct-4). Further differentiation protocols were carried out for these Oct-4 positive cells. For osteogenic differentiation, the cultured cells were shifted to osteogenic induction medium (α-MEM supplemented with 10% FBS, 0.1 μmol/l dexamethason, 10 mmol/l β-glycerol phosphate, 50 μmol/l ascorbate). For adipogenic differentiation, the cultured cells were shifted to adipogenic medium (α-MEM supplemented with 10% FBS, 1 μmol/l dexamethasone, 5 μg/ml insulin, 0.5 mmol/l isobutylmethylxanthine and 60 μmol/l indomethacin) for 1 to 3 weeks.

Oct-4 expression and differentiation assay by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cultured cells by using RNeasy Minikit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. In order to display Oct-4 expression, we used primers of Oct-4 (product size 247 bp) forward, 5'-CGTGAAGCTGGAGAAGGAGAAGCTG -3' and reverse, 5'-CAAGGGCCGCAGCTTACACATGTTC -3" and β-actin (product size 396 bp) forward, 5'-TGGCACCACACCTTCTACAATGAGC -3' and reverse, 5'-GCACAGCTTCTCCTTAATGTCACGC -3' as an internal control. For osteogenic differentiation, the cells were assessed by RT-PCR using specific primers as follows: human osteocalcin (product size 279 bp) forward, 5'-ACACTCCTCGCCCTATTG-3' and reverse, 5'-GATGTGGTCAGCCAACTC-3'. For adipogenic differentiation, the primers used were adipocyte lipid-binding protein (product size 274 bp) forward, 5'-TACCTGGAAACTTGTCTCCAGTGAA -3' and reverse, 5'-CCATTTCTGCACATGTACCAGGACA -3'. The RT-PCR procedure was performed using the OneStep RT-PCR kit, beginning at 50°C for 30 min and 95°C for 15 min for reverse transcription, then followed by 35 cycles, with each cycle consisting of denaturion at 94°C for 1 min, annealing at 57°C for 1 min, elongation at 72°C for 1 min, and the final extension at 72°C for 10 min. The amplified DNA fragments were visualized through 2% agarose gel electrophoreses and photographed under UV light.

Differentiation assay by staining

To demonstrate osteogenic differentiation, the mineralization of calcium accumulation in the cells was made visible by von Kossa staining. To view

adipogenic differentiation, intracellular lipid droplets were revealed by Oil Red O staining.

RESULTS

Cell culture of MSC from amniotic fluid

After plating the non-adhering amniotic fluid cells from primary amniocyte culture, colonies of cells began to appear in the 25 cm^2 flask in all samples 3-5 days later. After 1-2 weeks of culture, the cells became 80%-90% confluent. They were then dissociated by trypsin and plated in two 25 cm^2 flasks for further cell culture. These cells had a fibroblast-like appearance under microscopy.

Detection of Oct-4 expression

At passage 3-4, these cells were collected to analyze Oct-4 expression. Oct-4 positive cells were detected in 6 of the 50 samples. Figure 1 demonstrated the positive and negative results of those samples examined for Oct-4 by RT-PCR.

Assay of osteogenic differentiation

Osteogenic differentiation was observed after induction with osteogenic medium for 2 to 3 weeks. Osteogenic differentiation was demonstrated by RT-PCR identification for human osteocalcin (Figure 2). Von Kossa staining of the cells showed calcium mineralization aggregates within the cells (Figure 3).

Assay of adipogenic differentiation

Adipogenic differentiation was observed after induction with adipogenic medium for 1 to 2 weeks. Adipogenic differentiation was demonstrated by RT-PCR analysis for adipocyte lipid- binding protein (Figure 4). Oil Red O staining of the cells showed multiple lipid-droplets within the cells (Figure 5).

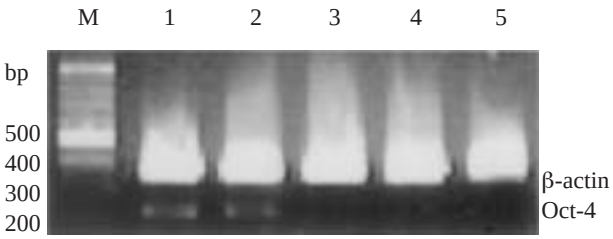

Fig. 1 RT-PCR analyses showed that Oct-4 mRNA expression was detectable in our cultured AFMSCs, and the PCR products of β-actin served as an internal Control. (column 1 and 2, samples positive for Oct-4; columns 3, 4, and 5, samples negative for Oct-4).

DISCUSSION

Amniotic fluid cells are traditionally used for prenatal diagnosis of a wide range of chromosomal anomalies.[14] Recently, amniotic fluid cells were discovered to be a potential source of mensenchymal stem cells.

The cells in the amniotic fluid are heterogenous, including cells shed from embryonic and extra-

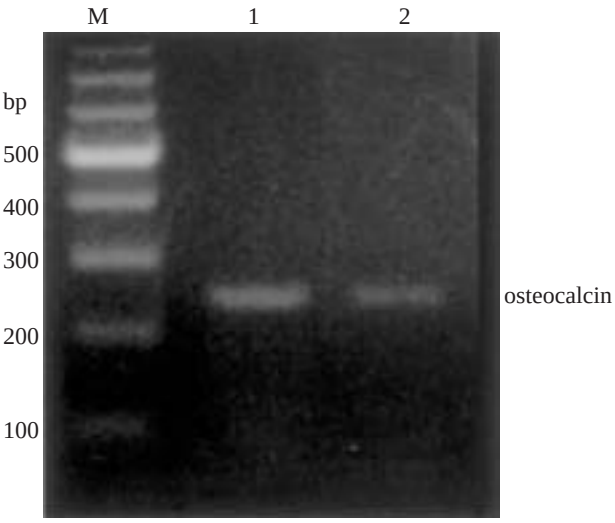

Fig. 2 After culture of the AFMSCs with osteogenic induction medium, RT-PCR for these differentiated cells tested positive for human osteocalcin.

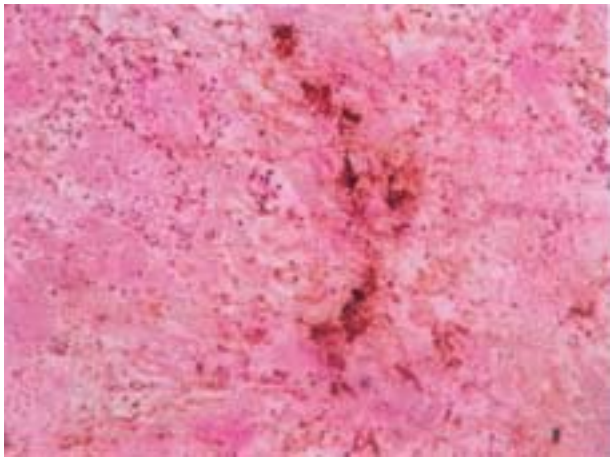

Fig. 3 Von Kossa staining of these differentiated cells showed aggregates of calcium mineralization within the cells.

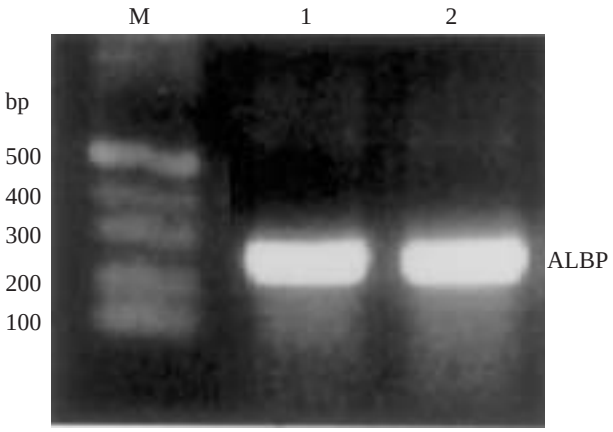

Fig. 4 After culture of the AFMSCs with adipogenic induction medium, RT-PCR for these differentiated cells tested positive for adipocyte lipid-binding protein (ALBP).

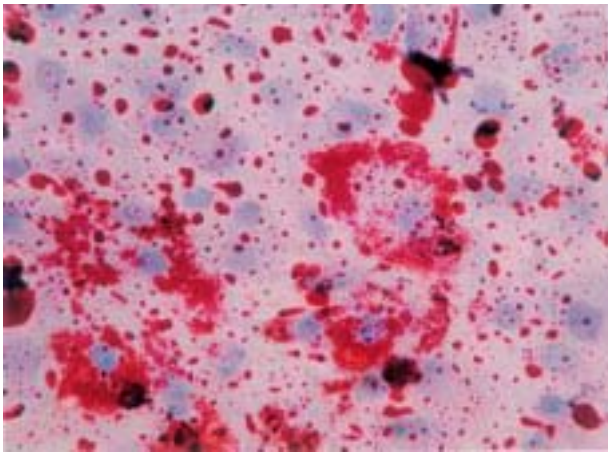

Fig. 5 Oil Red O staining of these differentiated cells showed multiple lipid vacuoles within the cells.

embryonic tissues, such as cells from amnion, placenta, fetal skin, as well as the respiratory, gastrointestinal and urinary tracts.[15] Ectodermal, mesodermal and endodermal cells can all be found in amniotic fluid. Placental amnion may be derived from the epiblast as early as eight days after fertilization. Thus, amnion epithelial cells are thought to retain the pluripotent properties of early epiblast cells, and these found in amniotic fluid may serve as a source for mensenchymal stem cells.

Research on amniotic epithelial cells claims they possess stem cell potential, capable of differentiating into a wide variety of different tissues, including heart, liver and brain.[16] Amniotic epithelial cells have also been demonstrated to express neuronal stem cell factors,[17] and can survive and function in the brain of a rat model of Parkinson's disease.[18] The amniotic epithelial cells isolated from human term placenta revealed the potential to differentiate into all three germ layers in vitro, including endoderm (liver, pancreas), mesoderm (cardiomyocyte), and ectoderm (neural cells).[19] As amniotic epithelial cells are one type of amniotic fluid cells, previous studies suggested that the amnion was likely contributing to the presence of MSCs in amniotic fluids.[6]

Amniotic fluid was first mentioned as a novel source of mensenchymal stem cells for therapeutic transplantation in 2003.[10] Furthermore, Oct-4-expressing cells were identified in human amniotic fluid.[11,12] Subsequently, human mensenchymal stem cells isolated from second-trimester amniotic fluid by two-stage culture protocol were shown by RT-PCR and immunocytochemical staining to harbor positive Oct-4-expressing cells.[13] In't Anker PS had reported that MSCs were cultured from two out of ten term amniotic fluid samples from patients who had term cesarean deliveries and ten out of ten second-trimester amniotic fluid samples taken from patients who underwent termination of pregnancy.[6] Another study reported that five of eleven samples from second trimester amniocentesis revealed Oct-4 mRNA expression.[12] In this study, we have demonstrated that Oct-4-positive cells were detectable in 12% (6/50) of samples cultured from amniocentesis specimens at the second trimester. Oct-4 positive cells appear to be more prevalent in native amniotic fluid specimens obtained from second trimester pregnancies. The relatively lower yield of cells presenting Oct-4 in this study was partly due to the fact that we collected the non-adhering amniotic fluid cells suspended in the culture medium, after a majority of the fluid cells had settled and would be cultured for fetal karyotyping. Secondly, a different culture protocol was used.

Regarding the differential potential of MSCs from amniotic fluid, our results are consistent with those of previous reports, one of which claimed MSCs could be isolated from 2 ml of second-trimester amniotic fluid collected transcervically from termination cases. These were successfully induced to differentiate into adipocytes and osteocytes.[10] Human mensenchymal stem cells isolated from second-trimester amniotic fluid by two-stage

culture protocol have also showed their capacity to successfully differentiate into morphologically osteocyte, adipocyte and neuronal cells *in vitro*.[13] In this study, we have further demonstrated that these morphologically osteocyte and adipocyte cells can express specific genes for human osteocalcin and adipocyte lipid-binding protein.

MSCs from other sources have been applied to tissue engineering and cell-based therapies. Adult human bone marrow is the most common source of MSCs for clinical use, but MSCs are rare, constituting approximately 0.001%-0.01% of adult bone marrow.[20] While embryonic or fetal tissue is a rich source of stem cells, there are ethical restrictions on access and the chance of bacterial and fungal contamination is higher. As a result of our research and that of others, future efforts may find a way where these mesenchymal stem cells from amniotic fluid may be applied to the field of cell transplantation and regenerative medicine.

Acknowledgements

This work was supported by a research grant (CMRPG 340201) to Hsiu-Huei Peng from Chang Gung Memorial Hospital, Lin-ko Medical Center, Tao-Yuan, Taiwan.

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自懷孕中期的羊水當中分離人類間葉幹細胞的效能—長庚醫院之經驗

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背 景： 本文主要的目的是要了解自懷孕中期的羊水細胞中分離出人類間葉幹細胞之效能。

方 法： 我們搜集了懷孕中期做羊膜穿刺的個案共50 位，共取得50 個羊水檢體各20 西西。經由二階段的細胞培養方式之後以 RT-PCR 的方式偵測 Oct-4 (幹細胞特定轉錄因子)表現細胞。這些羊水中的間葉幹細胞經成骨分化後，以 Von Kossa 染色看鈣鹽聚集並用 RT-PCR 的方式偵測 osteocalcin 的形成 (專一於成骨細胞)。此外，這些羊水中的間葉幹細胞經脂肪分化後，以 Oil Red O 染色看脂肪小滴並用 RT-PCR 的方式偵測 adipocyte lipid-binding protein 的形成 (專一於脂肪細胞)。

結 果： 在 50 個羊水檢體當中有 6 個檢體測得 Oct-4 表現細胞。這些羊水中的間葉幹細胞可以成功分化爲成骨細胞 (以 Von Kossa 染色看到鈣鹽聚集及 RT-PCR 的方式偵測到 osteocalcin 表現)。此外，這些羊水中的間葉幹細胞也可以成功分化爲脂肪細胞 (以 Oil Red O 染色看到脂肪小滴及 RT-PCR 的方式偵測到 adipocyte lipid-binding protein 表現)。

結 論： 在我們的經驗當中，自懷孕中期的羊水細胞中分離出人類間葉幹細胞之效能爲12%。這些羊水中的間葉幹細胞可以成功分化爲成骨細胞及脂肪細胞。這些來自懷孕中期的羊水細胞中分離出的人類間葉幹細胞可望應用於細胞移植及再生醫學。

(長庚醫誌 2007;30:402-7)

關鍵詞： 間葉幹細胞，羊水，分化

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