Laboratory Research

Role of 5-azacytidine in differentiation of human mesenchymal stem cell into cardiomyocytes in vitro

Fang-Ge Deng¹², Yu-Lin Li¹, Xiu-Ying Zhang¹

1. Key Laboratory of Pathobiology, Jilin University, Changchun 130021, China
2. National Key Laboratory of Respiratory Diseases, Guangzhou Institute of Respiratory Diseases, First Affiliated Hospital of Guangzhou Medical College, Guangzhou 510120, China

Objective 5-azacytidine could induce the differentiation of stem cells into cardiomyocytes (CMs). The aim of this study was to screen the optimal condition for 5-azacytidine inducing differentiation of human mesenchymal stem cells (hMSCs) into CMs, and the effect of 5-azacytidine on adherence, cell vigor and chromosome karyotype of hMSCs. Methods hMSCs were isolated from human bone marrow and cultured in vitro. The phenotypes of hMSCs were identified by flow cytometric analyses. MTT test was used to investigate the effect of different concentrations of 5-azacytidine on proliferation of hMSCs. Four weeks after 5-azacytidine induction, semi-quantitative RT-PCR, transmission electron microscopy (TEM), single-cell action potentials, detection of cardio-enzyme AST and LDH, cell adherence, cell viability and chromosome karyotype test were performed. Results The typical morphological features of hMSCs were fibroblast-like in shape. hMSCs expressed CD44 and CD105 and did not express CD34, CD45 and CD31. The optimal concentration of 5-azacytidine was 10 µ mol/L. The shape of hMSCs treated with 5-Azacytidine changed from fusiform to polygon or astrocyte gradually, and passed cells were evenly arranged as polarity structure. Induced-hMSCs connected with neighbouring cells, forming myotube-like structures 4 weeks later. It was confirmed that induced hMSCs shaped myotube-like structure and had some of micro-histologic structures of CMs by TEM. RT-PCR showed that induced hMSCs expressed cardiac specific product BNP and early cardio-myogenesis specific transcription factor NKX2.5 mRNA. Besides, induced-MSCs led to the weak action potential and secreted cardio-enzyme AST and LDH. There was no significant difference in cell adherence and viability before and after induction. Both hMSCs and induced-hNSCs kept stable normal diploid nucleus. Conclusion The optimal condition for inducing effect of 5-azacytidine is 10 µ mol/L and 24-hour incubation; and under this condition, the adherence, vigor and chromosome karyotype of hMSCs would not be affected (J Geriatr Cardiol 2009; 6:182-188).

Key words Human mesenchymal stem cells; 5-azacytidine; differentiation; cardiomyocytes

Introduction

As adult cardiomyocytes (CMs) have limited regeneration ability to compensate myocardial infarction-induced loss of CMs, the necrotic CMs are progressively replaced by fibroblasts to form scar tissues.¹ Cellular cardiomyoplasty is an ideal strategy to repair necrotic CMs and to improve cardiac function, but critical donor shortage remains a great limit to this.

Mesenchymal stem cells (MSCs) are derived from mesenchymal tissue. They had functional capacity both to self-renew and to generate a number of differentiated progeny.² They have been investigated as promising candidates for use in new cell-based therapeutic strategies, such as mesenchymal-derived tissue repair,³ including cardiac infarction and hind limb ischemia.⁴

5-azacytidine, as a kind of rare anticancer medicine, was used to cure acute non-lymphocytic leukemia and myelodysplastic syndrome. It is reported that 5-azacytidine could induce the differentiation of stem cells into CMs.⁵⁶ In this study, we isolated hMSCs from human bone marrow in vitro and used 5-azacytidine to treat the cells. The purpose was to screen the optimal condition for 5-azacytidine to induce differentiation of hMSCs into CMs, and to investigate whether, under this condition, induced-hMSCs have ultrastructural characteristics, cardio-gene expression, action potential, as well as the secretion of cardio-enzyme function of MCs, at the same time, exert no effect on cell adherence, viability, and chromosome karyotype of hMSCs.

Methods

Isolation, culture and phenotypes of hMSCs

hMSCs were isolated and cultured as previously described.⁷ 2.5ml heparinized human bone marrow of healthy volunteers after informed consent was obtained aseptically. Bone mononuclear cells (MNCs) were prepared by density gradient centrifugation (below 1.073 g/ml, Pharmacia, USA).
The cells were washed, and plated in a 24-well plate at 2x10^3 - 3x10^3 cells / ml in Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA), and incubated in humidified 5% CO₂,95% air at 37°C. After 48-72 hours, the suspending cells were removed through the first medium change, and the adherent cells, namely hMSCs, were continuously incubated. The medium was replaced every 3-4 days. When individual colonies shaped, the monoclonoy was collected and isolated for further culture and proliferation. After reaching 80% confluen, the cells were digested with 0.25% trypsin-0.02% ethylenediamine tetraacetic acid (EDTA, Pharmacia, USA) and followed by routine processes.

Flow cytometric analysis

The phenotypes of hMSCs were identified by flow cytometric analyses. Suspending MSCs (0.5 x 10^6 to 1 x 10^6) at passages 5 to 8 were incubated with FITC-conjugated anti-CD31, anti-CD34, anti-CD44, anti-CD45 and anti-CD105 monoclonal antibodies, or control isotype antibodies for 30min at 4°C. After wash with FACS buffer, the cells were fixed with 10µl polyformaldehyde, and went through FACS (Becton Dickinson, USA) analysis, and the data were analyzed by using the CELL QUEST software program (Becton Dickinson, USA).

Effect of different concentrations of 5-azacytidine on cell proliferation of hMSCs by MTT test

The stably passaged hMSCs were prepared into cell suspension and plated in 96-well tissue culture plates at 5 x 10^3 cells / ml with 200µl DMEM-LG supplemented with 10% FBS. After 24-hour culture, the cells were incubated with 7 different concentrations of 5-azacytidine in conditional medium for 24 hours. Then 5-azacytidine was removed and normal complete medium was added for further incubation. The morphology of the cells were observed under inverted microscope every day, and the medium was replaced every 3-4 days. The MTT test was performed as routinely described.

5-Azacytidine induced medium treated with hMSCs

It was determined the optimal inducing concentration of 5-azacytidine as 10µmol/L by MTT test. After reaching 50%-60% confluence, the stably passaged hMSCs were incubated with 10µmol/L 5-azacytidine for 24 hours. The medium was replaced every 3-4 days. After 4 weeks, the cells were harvested and kept till use.

Determination of cell adherence

hMSCs with/without 5-azacytidine treatment were seeded onto 6-well tissue culture plates at 1x10^4 cells / ml. Viable and adherent cells were counted by trypan blue exclusion at the point of the 2^nd, 5^th, 8^th, 11^th, 14^th, 24^th hour and cell adherence were counted and depicted by using the following formula: cell adherence=score of viable and adherent cells / total cellular score.

Determination of cell viability

hMSCs with/without 5-azacytidine treatment were seeded onto 24-well tissue culture plate at 1x10^4 cells/ml. Viable and adherent cells in every 3 wells were counted by trypan blue exclusion for one week. Viable cellular score (%)= viable total cellular score / dead & viable total cellular score ×100%.

Semi-quantitative RT-PCR

Total RNA of the cells was extracted by Trizol and the purity and content were detected by Biophotometer 8.5 (Eppendorf, GERMANY) for semi-quantitative RT-PCR. The primer for human NPPB (Product size: 233bp) was 5’-TTTTCTGGAAGCTCGTCGCC-3’ and 5’-GGTGTAAGGACCATTTGCT-3’. The primer for human NKX2.5 (Product size: 46bp) was 5’-CCAAGAACCTAGAGCCGAA-3’ and 5’-ATAGCCGGGTTAGCGTTAT-3’. The primer for GAPDH (Product size: 700bp) was 5’-GGTGTAGCTGGTCGAGATTGCT-3’ and 5’-AAGATGAGGATTGTCTGAAGTC-3’. PCR conditions were as follows: 94°C for 5 minutes, then 30 amplification cycles (30 seconds denaturation at 94°C, 30 seconds annealing at 58°C , 30 seconds extension at 72°C), and a 10 - minute terminal extension at 72°C. The PCR products were electrophoresed on 2% agarose gel.

Histology & transmission electron microscope (TEM)

The induced-hMSCs were harvested following 0.25% trypsin-0.02% EDTA digestion, and washed with 0.1ml/L PBS. In each case, 1 x 10^4 - 1 x 10^5 cells were acquired and centrifuged to form pykno-micell. The samples were fixed by 4% glutaraldehyde and postfixed by 1% osmic acid, dehydrated with graded ethanol. They were then embedded in Epon812 (epoxy resin). Each ultrathin section was double stained with uranyl acetate and lead citrate for TEM (JEM-1200EX, JAP) analysis.

Single-cell action potentials

With diameter range of 0.8 - 1.5 µm, and resistance range of 1 - 3M Ω, microelectrodes tip was polished liquid irrigation electrode. Action potential of the cells were induced by application of current-clamp method, with given time 1-5 ms, once per second, ranging from 0.5-1 nA inward current pulse. At the same time, the action potential of the control cells (normal cultured neonatal rat cardiomyocytes and hMSCs) were recorded identically.

Detection of AST & LDH

Induced hMSCs and control cells (Beating CMs as positive control and hMSCs as negative control) were
washed with PBS 2 or 3 times in order to remove dead cells and serum. Then the three kinds of cells were cultured with DMEM-LG (without serum) for 24 hours. The DMEM-LG (without serum) were centrifuged to remove dead cells and cell debris and the supernatant was kept at -20°C for following test. H₂O₂-L-DMEM (without serum, and final concentration of H₂O₂ was 300µmol/L ), with the same capacity in medium with original L-DMEM basal medium, was added into the cell culture flask. After 6 hours, H₂O₂-L-DMEM was collected and centrifuged for remaining supernatant for next testing. Induced hMSCs, hMSCs and CMs were washed with PBS and incubated with L-DMEM basal medium (without serum). After 24 hours, supernatant was collected. Then H₂O₂-L-DMEM medium harvested at the above three time points, 24-hour before or 6-hour and 24-hour after coincubation, were used to AST and LDH detection by standard holo-automatic biochemistry analyzer (Hitachi, Jap).

**Determination of chromosome karyotype**

The chromosome karyotype test was performed as routinely described. When reaching 70%-80% confluence, hMSCs with/without 5-azacytidine in increased logarithmic phase were treated with 1% phytohemagglutinin 250 µl, and incubated in CO₂ homoisothermy incubator at 37°C for 36 hours. Colchicine 100 µl was added for 2-4 hour incubation before the cells were digested with 0.25% trypsin-0.02% EDTA and centrifugated as a micelle. Nice ml prewarmed KCL was added to dissolve the micelle at 37°C for 30 min. The mixture was centrifuged again and 0.5 ml deposition was dispersed into cell suspension. The cells were fixed with up to 5ml of fresh fixing solution (carbinol : acetic acid = 3 : 1) at room temperature for 30 min. Fixed the cells twice and dropped the cells onto pre-cooled slides from high distance (about 1 foot), the specimens were dried and stained by Giemsa for 15 min, and observed under immersion objective.

**Results**

**Isolation and culture of hMSCs**

MSCs were isolated from human bone marrow by density gradient fractionation and attached to plastic flasks 2-4 days later. The adherent cells were single or by several colonies and showed uniform spindle shape. After fully exchanging medium, the suspending cells and various kinds blood cells were gradually removed, and the remaining adherent cells were hMSCs. The individual colonies were cultured and proliferated in tissue dishes. After reaching 80% confluence, cells were trypsinized and passed every 5-6 days for 14 passages without morphologic alteration. The typical passaged hMSCs displayed fibroblast-like morphological features (Fig. 1).

**Characterization of MSCs**

The FACS analyses indicated that MSCs were negative for CD31, CD34 and CD45, markers for vascular endothelial cells, early hematopoietic progenitor cells and more mature hematopoietic cells, respectively, and were positive for CD44 and CD105, a matrix receptor marker.

**Effect of different concentrations of 5-azacytidine on proliferation of hMSCs by MTT test**

Every concentration of 5-azacytidine affected the proliferation of hMSCs to different extent. ≧ 10µmol/L 5-azacytidine had slightly effect on hMSCs and 20µmol/ L 5-azacytidine influenced hMSCs, while over 20µmol/L 5-azacytidine had obvious cytotoxicity and refrained the

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**Fig. 1** Morphology of original and passaged hMSCs. (A) Original hMSCs display uniform spindle shape (×100); (B) Passaged hMSCs display fibroblast-like morphological features (×100)
proliferation of hMSCs significantly (Fig. 2). Thus, the optimum concentration of 5-azacytidine for inducing differentiation of hMSCs into CMs was taken as 10μmol/L.

**Fig. 2** Effect of different concentrations of 5-azacytidine on proliferation of hMSCs.

**Morphological features of induced hMSCs**

The morphological changes of induced hMSCs were observed by inverted microscopy or laser scanning confocal microscopy (LSCM) after treatment with 5-azacytidine. hMSCs generally stretched and grew slowly. They began to proliferate until reaching a certain cell density, but their capacity of proliferation was obviously decreased. Induced hMSCs displayed little change 1 week later, except parts of induced-hMSCs became long and magnanimous. After 2-3 weeks, the passaged induced hMSCs arranged as polarity structure and parts of them changed their shape from spindle to polygon-like or star-like morphological features, which was as same as CMs. Induced hMSCs stretched out pseudopodium to connection and linked with each other and intercrossed to shape myotubule-like structure after 5-azacytidine treatment (Fig. 3).

**Cell viability of hMSCs and induced-hMSCs**

Determination of trypan blue exclusion showed that cell viability of hMSCs reached 99%, and that of induced-hMSCs was 95%.

**Adherence of hMSCs and induced-hMSCs**

The adherence of hMSCs at the point of 2nd, 5th, 8th, 11th, 14th, and 24th hour was 30%, 61%, 80%, 89%, 92% and 96% respectively. Whereas the adherence of induced-hMSCs was 28%, 53%, 72%, 78%, 84% and 88%. There was no significant difference between the two groups of cells (Fig. 4).

**Semi-quantitative RT-PCR of induced-hMSCs**

Both induced-hMSCs and hMSCs expressed GAPDH mRNA, which indicated that in each sample of the two

**Fig. 3** Morphology of 5-azacytidine induced hMSCs. (A)Induced-hMSCs display little change 1 week later except parts of induced-hMSCs become long and magnanimous (×250); (B,C)23 weeks later, parts of passaged induced-hMSCs display polygon-like or star-like morphological features (×250); (D)Passaged induced-hMSCs arrange as polarity structure (×100); (E,F)Induced hMSCs connect with adjoining cells forming myotube-like structures after 5-azacytidine treatment for 4 weeks (×100).
groups, cDNA was good and RNA was not degraded. Semi-quantitative RT-PCR showed that human BNPP or human NKK2.5 was expressed in induced-hMSCs while not in control hMSCs (Fig. 5).

Histology & transmission electron microscope (TEM) of induced-hMSCs

TEM analysis discovered that the micromorphology of induced-hMSCs was irregular, including spherical, oval or irregular shape. There was abundance of microvilli on the cells’ surface. It is thus evident that there were many sedimentary hepatin granules and thin myofilament-like structure in the intracytoplasm, among those thin myofilamen were sporadic punctiform black sedimentary electron dense speckles. However, the thin myofilament-like structure and sedimentary electron dense speckles were not shown in hMSCs (Fig. 6).

Single-cell action potentials

The results showed that neonatal rat cardiomyocytes had a clear action potential, hMSCs did not lead to the action potential, and 5-azacytidine induced hMSCs could lead to the weak action potential (Fig. 7).

Detection of AST & LDH

Detection of AST at the three time points, 24 hours before damage, 6 hours after damage and 24 hours after damage, showed that the level of AST in induced hMSCs and un-induced hMSCs were at the same lower level before H₂O₂ damage. The level of AST in induced hMSCs was increase obviously after the cells were damaged by H₂O₂, and the tendency of increase was as the same as that in CMs. However, the level of AST in un-induced hMSCs was not increased. The result of detection of LDH was similar to AST (Fig. 8).

Chromatosome karyotype of hMSCs & induced-hMSCs

According to detection and contrast analyses of chromatosome karyotype of different passaged hMSCs and induced-hMSCs, both hMSCs and induced-hMSCs kept stable normal diploid nucleus (46XX or 46XY).

Discussion

The bone marrow contains at least two different populations of stem cells, each of which is capable of both self-renewal and differentiation down multiple lineages. One is

![Fig. 4 Adherence of hMSCs & induced-hMSCs.](image)

![Fig. 5 NKK2.5 and BNPP mRNA in induced hMSCs detected by RT-PCR.](image)

![Fig. 6 Ultrastructural characteristics of induced-hMSCs.](image)
hematopoietic stem cells, which can differentiate into all blood cell types, another is MSCs. Cells referred as MSCs share a minimum of three primary characteristics: they grow as adherent cells in tissue culture plates; have a finite lifespan of approximately 30 to 50 cell doublings; and they can differentiate, under appropriate specific in vitro conditions, into osteoblasts, chondroblasts, and adipocytes.

In 1995, Wakitani et al. found that mesenchymal stem cells appeared to have the capacity to be induced to differentiate in vitro into myogenic and adipocytic phenotypes. In their experiment, rat bone marrow-derived mesenchymal stem cells were exposed to 5-azacytidine beginning 24h after seeding twice-passaged cells into culture dishes. After an exposure of 24h, long multinucleated myotubes were observed in some of the dishes 7-11 days later. But, the characteristics of CMs derived from MSCs were not identified in their report. Until 1999, Makino et al. had isolated firstly a cardiomyogenic cell line (CMG) from murine bone marrow stromal cells. They confirmed that MSCs could differentiate into CMs by detection of electron microscopy, action potentials, and protein genes. At following years, many research teams also reported that MSCs had potential in differentiation to CMs. But the main research about MSCs in these studies was derived from animal, and rare from human. Antonitisis et al. reported human adult bone marrow mesenchymal stem cells had cardiomyogenic potential in vitro when treated with 5-azacytidine.

At present, the regulation mechanism of MSCs differentiation into CMs is still uncertain. But, 5-azacytidine is only the effective chemical in inducing up to now in cardiomyogenic differentiation of hMSCs. It is considered generally, 5-azacytidine decided the direction of MSCs differentiation into CMs in the level of gene for it was an enzyme inhibitor of methyltransferases and incorporated in DNA during the S phase. 5-azacytidine was a poor inducer of muscle cells and a poor inhibitor of DNA methylation, and which played an important role for DNA modification in differentiation. Konieczny et al. reported that 5-azacytidine cytidine converted 10T1/2 cells by hypomethylation of “determination” regulatory loci which established lineages of stem cells with a potential to differentiate into muscle, cartilage, or fat cells.

However, 5-azacytidine is an effective anticancer drug. It has certain cytotoxic effect on hMSCs when it was only one effective chemical inducer. So the key is how to determine the most optimal and effective inducing concentration meanwhile lowest cytotoxicity to hMSCs. The aim of this study was to investigate whether the optimal inducing concentration of 5-azacytidine selected by MTT detection affect the property of hMSCs’ adherence and viability, and transform the chromosome karyotype of hMSCs after 5-azacytidine treatment.

According to MTT detection, 10 μmol/L 5-azacytidine conditional medium and 24-hour incubation was most optimal inducing condition. The morphology of induced-hMSCs gradually transformed to that of CMs with the inducing time passing by. Critically, the passed induced-hMSCs arranged as polarity structure and began to connecting with adjoining cells to form myotube-like structures, and there were many myofilaments in the cells after treating with 5-azacytidine over 4 weeks. Besides, RT-PCR examination indicated that induced-hMSCs expressed early cardiomyogenesis transcription factor BNP and Nkx2.5 mRNA, which identified the differentiation of hMSCs into CMs. To our interest, the 5-aza induced hMSCs could lead to the weak action potential, indicating that the induced hMSCs had certain excitability. In order to detect whether cardi-like induced-hMSCs have the cardio-enzyme secretory function, we detected AST and LDH in CMs, hMSCs and induced-hMSCs, and the positive results further illustrated that hMSCs had successfully differentiated into CMs after 5-azacytidine induction. The cell adherence and viability of induced-hMSCs were lower than that of hMSCs, but with no significant difference. The detection of chromosome karyotype showed that different passed hMSCs with 5-azacytidine treatment still kept stable normal diploid nucleus.

Taken together, the optimal condition for inducing effect of 5-azacytidine is 10μmol/L and 24-hour incubation, and under this condition, the adherence, viability and chromosome karyotype of hMSCs would not be affected.

References

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