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Characterization of smooth muscle differentiation of purified human skeletal muscle-derived cells

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Abstract

The purpose of this study is to characterize the smooth muscle differentiation of purified human muscle-derived cells (hMDCs). The isolation and purification of hMDCs were conducted by modified preplate technique and Dynal CD34 cell selection. Smooth muscle cell differentiation was induced by the use of smooth muscle induction medium (SMIM) and low-serum medium. The gene expressions at the mRNA and protein levels of undifferentiated and differentiated hMDCs were tested by RT-PCR, Western blot and immunofluorescence studies. Western blot and immunofluorescence studies demonstrated the purified hMDCs cultured in SMIM for 4 weeks and expressed significant amount of smooth muscle myosin heavy chain (MHC) and α-smooth muscle actin (ASMA). The cells cultured in low-serum medium for 4 weeks also expressed ASMA, while the control group did not. RT-PCR analysis showed increased gene expression of smooth muscle markers, such as ASMA, Calponin, SM22, Caldesmon, Smoothelin and MHC when purified hMDCs were exposed to SMIM for 2 and 4 weeks when compared to the controls. In conclusion, we confirmed the smooth muscle differentiation capability of purified hMDCs. The gene expression of smooth muscle differentiation of purified hMDCs was characterized. These cells may be potential biomaterials for human tissue regeneration.

Keywords: gene expression • smooth muscle • stem cells • bladder reconstitution

Introduction

Tissue engineering is promising in bladder reconstruction [1]. Several different biomaterials have been used as scaffolds for tissue repair. Neobladders obtained by culturing urothelial cells on collagen matrix reproduced normal bladder mucosa, whereas platelet-fibrin gels, which specifically inhibited neoplastic urothelial cell growth, could be used as scaffolds in surgical bladder reconstitution [2]. Nevertheless, poor contractility and compliance of the new bladder are still major problems in the present bladder reconstitution methods and stress urinary incontinence management. Adult skeletal muscle possesses remarkable regenerative capacity that has conventionally been attributed to the satellite cells. These precursor cells are thought to contain distinct populations with varying myogenic potential. Recently, the identification of multi-potent stem cells capable of new myofibre formation has expanded the general view on the muscle regenerative process [3, 4]. These precursor cells are normally quiescent but proliferate in response to muscle injury, producing myoblasts that repair the muscle. The porcine muscle-derived cells (MDCs) possess long-term expansion capacity and a multi-lineage differentiation capacity [5]. MDCs isolated from rat skeletal muscle injected into the bladder wall and urethra has been shown to enhance urethral closure and bladder function [6]. The rat MDCs has found successfully forming myotubes in the smooth muscle layers of the lower urinary tract that appeared to enhance urethral closure and detrusor contractility [7]. MDCs isolated from rat skeletal muscle are reported to be capable of forming myotubes and incorporating into acellular scaffold (small intestinal submucosa) making contractile biomaterials with better compliance for the treatment of urinary incontinence and bladder reconstitution [8, 9].

Previous studies differentiated human myosphere-derived progenitor cells or skeletal muscle stem cells in culture to produce cardiac, smooth muscle and endothelial cells; the differentiated cells are transplanted into ischaemic hearts in NOD/scid mice,

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promoting angiogenesis with substantial cardiovascular regeneration and exhibiting cardiac-like responses [10, 11]. An *in vitro* developmental model found that bone morphogenetic protein (BMP)2 rather than BMP4 agarose beads enhanced smooth muscle differentiation of mouse embryonic stem cells, and increased gut-like structures showing spontaneous contractions and expressing intensive α-smooth muscle actin (ASMA) immunoreactivity. This increase was confirmed by up-regulation of SM22 mRNA shown by real-time PCR [12]. Injection of skeletal MDCs into the penis was also found to facilitate recovery of injured penile innervation and improve erectile function [13]. Due to the capability of the smooth muscle differentiation of these progenitor cells, these stem cell-based therapy may have the potential to be administered into the urinary external sphincter, penis or bladder detrusor layer with or without scaffolds to be functional biomaterials for the management of urinary incontinence, erectile dysfunction and bladder reconstitution in human.

Our laboratory has previously shown that the isolation and characterization of human MDCs (hMDCs) from skeletal muscle was feasible [14]. The purified hMDCs have been proven to be capable of differentiating toward the myogenic, osteogenic, adipogenic and chondrogenic lineages [15]. This study describes the feasibility of smooth muscle differentiation of purified hMDCs to evaluate the potential of these cells in the application of human tissue repair.

Materials and methods

Stem cell purification with Dynal cell selection system

This study was granted approval in Taipei-Veterans General Hospital with the IRB number: VGHIRB No: 92-11-07A. Human MDCs were isolated from skeletal muscles by modified preplate technique as described previously [14, 16]. After isolation, the hMDCs were cultured in control medium (F12 medium supplemented with 15% foetal calf serum and 1% penicillin/streptomycin). Purification of these isolated cells were performed by CD34 selection; cells were subjected to immunomagnetic separation using Dynal CD34 progenitor cell selection system (Dynal Biotech, Oslo, Norway), following the protocol provided by the manufacturer. Briefly, cells were incubated with CD34 microbeads (microbeads conjugated to monoclonal mouse anti-human CD34 antibody) for 30 min. at room temperature and then placed in a magnet for 2 min. The supernatant was discarded and the bead-bound cells were gently washed 4 times with buffer 1 (phosphate buffer solution [PBS] w/0.1% BSA, 2 mM EDTA, pH 7.4, without Ca^{2+} and Mg^{2+}). Finally, the cells were resuspended in control medium and cultured in T-60 cell culture dishes.

Smooth muscle induction of $CD34^+$ purified hMDCs

Expanded populations of previously purified $CD34^+$ hMDCs were seeded at high densities [$(1.0–1.5) \times 10^4$ cells/cm^2] directly on glass coverslips and 6-well cell culture plate. The effect of a low-serum medium and a smooth muscle induction medium (SMIM) on the induction of a differentiated smooth muscle phenotype was evaluated. The purified hMDCs were cultured at 37°C either in control medium (F12 medium [Gibco, Paisley, UK] with 15% foetal bovine serum [FBS] and 1% penicillin/streptomycin) or in low-serum medium (F12 medium [Gibco, Paisley, UK] with 1% FBS and 1% penicillin/streptomycin). SMIM consists of F12 medium (Gibco, Paisley, UK) with 1% FBS and 1% penicillin/ streptomycin plus 100 units/ml heparin (VWR International, West Chester, PA, USA). Passage 2 cells were harvested, plated in control medium at $(1.0–1.5) \times 10^4$ cells per cm^2 in regular culture dishes for 72 hrs, and then incubated in SMIM, low-serum medium or control medium for an additional 2 or 4 weeks. The medium was replaced every 3–4 days.

Smooth muscle differentiation was assessed by gene expressions using immunofluorescence, Western blot and RT-PCR studies.

Immunofluorescence (IF) studies

Glass coverslips were placed at the bottom of the plate before co-culturing. When the induction of differentiation reached 60–70% confluence, the culture medium was cleared and the wells were washed twice with PBS. The glass coverslips were placed into a 6-well cell culture plate, where parafilm had been placed first at the bottom for easy manipulation. Fixation was performed by adding 100 μl 3.7% paraformaldehyde (Sigma, St. Louis, MO, USA) and washing with PBS 3 times. For permeabilization, 100 μl 1% Triton/PBS was added, which was followed by 0.02% Tween 20/PBS. 100 μl PBS containing primary antibody (1:100) was then added, and the plate was placed in a 37°C water bath for 45 min. The plate was washed by 0.02% Tween 20/PBS for 5 min., 3 times; then, 100 μl PBS containing 1:50 FITC-conjugated IgG was added to each well, and the plate was placed in the 37°C water bath for 45 min. The antibodies of smooth muscle myosin heavy chain (MHC) and ASMA are both from Millipore Billerica, MA, USA. When the reaction time ended, the plate was washed by 0.02% Tween 20/PBS for 5 min., 3 times and then washed by PBS for 5 min. The coverslips were dried at 37°C for 45 min. Before analysing under the microscope, 10–15 μl Dabco mounting media (PBS, 50–60% glycerol, 2.5% 1, 4 diazobicyclo (2, 2, 2)-octane) was added and coverslips were sealed by the transparent nail polish. The coverslips were observed under the fluorescent microscope; the wavelength for FITC excitation was set at 488 nm and emission was set at 515 nm. Images were captured by Leica TCS SP2 CLSM analysis.

Western blotting

$CD34^+$ cells, which were differentiated toward the smooth muscle cell lineage after a period of 4 weeks in SMIM medium, were washed in PBS, and lysed in lysis buffer (20 mM Hepes, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF and 1 μg/ml each of aprotinin, pepstatin and leupeptin, pH 7.5). Equal amounts of proteins from each lysate were resolved by denaturing PAGE (SDS-PAGE) and analysed using standard immunoblotting protocols. Lysates were examined for the expression of MHC and ASMA (Millipore Billerica, MA, USA). Expression of β-actin was used as an internal control for the Western blot procedure. Non-induced $CD34^+$ cells were also analysed as a negative control.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

The levels of expression of various differentiation markers were assessed by RT-PCR; primers designed are listed in Table 1. Briefly, total RNA was

Table 1 Primer pairs used in the RT-PCR analysis

Lineage	Target gene	Primers	Product size (bp)
Smooth muscle	SM 22	ATGGCCAACAAGGGTCC CTTCAAAGAGGTCAACAG	349
	Smoothelin	ATGGCGGACGAGGCCTTAG CCTCAATCTCCTGAGCCC	358
	Caldesmon	AGATTGAAAGGCGAAGAGCA TTCAAGCCAGCAGTTTCCTT	397
	MHC	GGACGACCTGGTTGTTGATT GTAGCTGCTTGATGGCTTCC	656
	Calponin	ATGTCCTCTGCTCACTTCA TTTCCGCTCCTGCTTCTCT	453
	ASMA	ACCCACAATGTCCCCATCTA TGATCCACATCTGCTGGAAG	595
	β-actin	GTAGATGGGCACAGTGTGGG GTAGATGGGCACAGTGTGGG	500

extracted using commercial kits (Protech Technology, Taipei, Taiwan) and stored in –80°C degree freezer before use. One microgram of total RNA was reverse transcribed by MMLV reverse transcription kit (Epicenter, Madison, WI, USA) in 20 microlitre volume and the PCR was performed by taking one microlitre of 1st-strand cDNA to amplify specific genes in 25 microlitre. All primer sequences were determined using established GenBank sequences. Amplification of β-actin was used as a control for total RNA amount. The PCR products were visualized on 2% agarose gel by ethidium bromide. Non-induced $CD34^+$ cells were examined as a negative control.

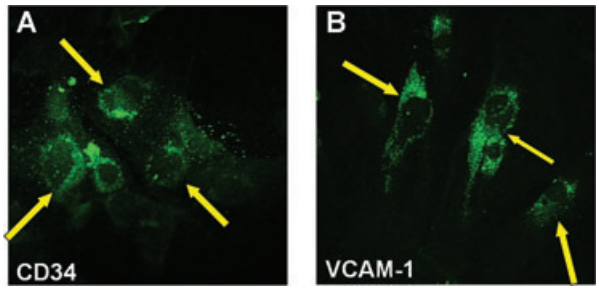

Fig. 1 Immunofluorescence study (200× magnification) shows the presence of stem cell markers, CD34 (**A**, arrows) and VCAM-1 (**B**, arrows) in the purified $CD34^+$ hMDCs.

Results

Purified hMDCs using Dynal cell selection system

After isolation of hMDCs using modified preplate technique, the cells were then purified by the use of Dynal CD34 cell immunoselective system. Immunofluorescence study showed the presence of stem cell markers CD34 (Fig. 1A) and VCAM-1 (Fig. 1B) in the purified CD34+ hMDCs.

Gene expression at the protein level determined by immunofluorescence studies

By the use of SMIM or low-serum medium, smooth muscle cell differentiation of purified $CD34^+$ hMDCs was induced. Immunofluorescence studies using smooth muscle-specific marker such as ASMA showed the expression of ASMA in purified hMDCs after being cultured in low-serum medium for 4 weeks (Fig. 2B and C), which is found absent in the control medium for 4 weeks (Fig. 2A). However, smooth muscle differentiation seems to be poor and the cells revealed exiguous by the use of low-serum medium.

There is an increased expression of MHC (Fig. 3, top panel) and ASMA (Fig. 3, bottom panel) as shown by immunofluorescence studies when purified hMDCs are cultured in SMIM when compared to the control medium group (Fig. 3, left panel) for 4 weeks.

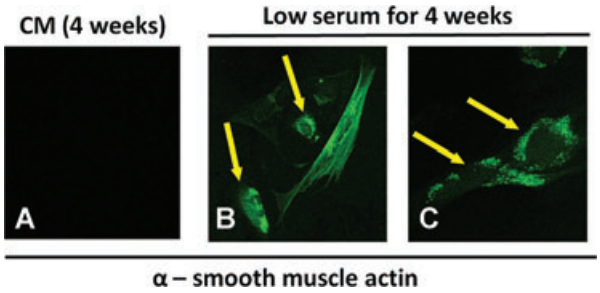

Fig. 2 Immunofluorescence study showing the presence of smooth muscle-specific marker, α-smooth muscle actin (ASMA) in purified hMDCs cultured in low-serum medium for 4 weeks [**B** (100× magnification) and **C** (200× magnification), arrows], which is not found in the control medium group for 4 weeks (**A**, 100× magnification). The cells revealed exiguous using this low-serum medium.

These results indicate that the purified hMDCs are differentiated into smooth muscle cells in SMIM culture.

Gene expression at the protein level determined by Western blot studies

Western blot studies confirmed the immunofluorescence results with increase in the expression of these smooth muscle cell-specific

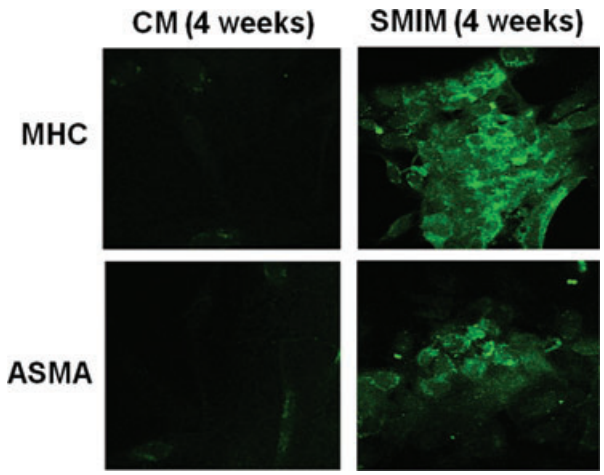

Fig. 3 There is an increased expression of smooth muscle-specific markers, smooth muscle myosin heavy chain (MHC) (top panel) and ASMA (bottom panel), when purified hMDCs are cultured in SMIM when compared to the control medium (left panel) for 4 weeks as revealed by immunofluorescence study (200× magnification).

genes at the protein level. The results from the Western blots are shown in Figure 4A and B. There is an increased expression of MHC and ASMA in the cells cultured in the SMIM medium when compared to the controls (Fig. 4B).

Gene expression at the mRNA level determined by RT-PCR analysis

There is an increased expression of smooth muscle-specific markers such as ASMA, Calponin, SM22, Caldesmon, Smoothelin, and MHC when purified hMDCs are exposed to SMIM for 2 and 4 weeks when compared to the control medium for 4 weeks as determined by RT-PCR (Fig. 5A). Increased gene expressions of smooth muscle-specific markers when purified hMDCs are exposed to SMIM for 2 and 4 weeks were determined by semi-quantitative RT-PCR using β-actin as a control gene (Fig. 5B).

Discussion

In this study, we used the modified preplating method to isolate human skeletal MDCs; the growth doubling time of this cell group is about 24 hrs [14]. The immunohistochemical and flow cytometry studies suggest that these cells mimic the muscle-derived myoendothelial cells reported by Zheng *et al.* [17] which also showed positive expressions of CD34 and CD56. In the muscle-derived myoendothelial cells report, the authors did not show the smooth muscle differentiation. In the present study, we further purified the cells from hMDCs by Dynal CD34 cell selection system. The growth-doubling time of this cell group is about 35 hrs, which suggests that this purified hMDCs group is more compatible with slow growing property of stem cells. The $CD34^+$ purified hMDCs demonstrate that the stem cell marker CD34 and myoblast marker CD56 show an increased expression when compared with the cells before Dynal cell selection [18]. Suga *et al.* also found that CD34 expression in human adipose-derived stem cells might correlate with self-renewing capacity, differentiation potentials, expression profiles of angiogenesis-related genes and immaturity of the cells [19]. Thus, we use the $CD34^+$ purified hMDCs to test the feasibility of smooth muscle differentiation.

The induction of smooth muscle differentiation from different kinds of progenitor cells is feasible. Transforming growth factor-β 1 activated Nox4 and promoted smooth muscle cell differentiation from mouse embryonic stem cells, which is important for vessel formation *in vivo* and vascular tissue engineering in the future [20]. Becker *et al.* found that the combined application of transforming growth factor-β 1 and epithelial-mesenchymal interactions promoted *in vitro* outgrowth of cells with a smooth muscle-like phenotype from a selected adherent murine bone-marrow-derived cell population [21]. A combination of transforming growth factor-β 1 and ascorbic acid is useful for the differentiation of human bone-marrow-derived mesenchymal stem cells into smooth muscle cells for use in tissue engineering [22]. Induction for smooth muscle differentiation of adipose-derived stem cells was successfully conducted by using SMIM [23].

In the present study, smooth muscle differentiation from the purified hMDCs is successfully achieved by using SMIM and low concentration serum medium for culture. The differentiated cells are excessively scarce using the low-serum medium. However, by the use of SMIM induction for 4 weeks, immunofluorescence study demonstrated the purified hMDCs expressed significant amount of MHC and ASMA, while the control group did not. Western blot studies also confirmed the immunofluorescence results with significant increase in the expression of these smooth muscle cell-specific genes at the protein level. The purified hMDCs are induced to express smooth muscle-specific genes. RT-PCR results justify the gene expression of smooth muscle cell-specific markers, such as ASMA, Calponin, SM22, Caldesmon, Smoothelin and MHC; the stably increased expressions are found after 2 and 4 weeks culture using SMIM. The data show two early smooth muscle labelling gene expression including ASMA and Calponin, and late smooth muscle labelling gene expression such as SM22, Caldesmon, Smoothelin and MHC, are not found in control group. Nevertheless, these above-mentioned gene expressions are identified obviously in the SMIM induction group which means the smooth muscle cell differentiation of purified hMDCs is feasible.

In our previous studies, we have reported that MDCs isolated from rat skeletal muscle are capable of forming myotubes and incorporate into small intestinal submucosa making contractile biomaterials with better compliance for the treatment of urinary incontinence and bladder reconstitution [8, 9]. Myotubes are formed *in vivo* or *in vitro* by the fusion of myoblasts and eventually

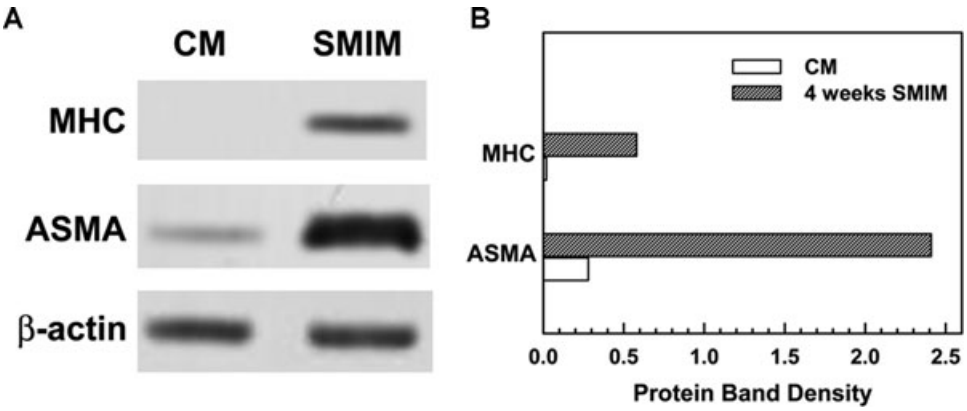

Fig. 4 Western blots showing the expression of smooth muscle-specific proteins, smooth muscle myosin heavy chain (MHC) (**A**) after 4 weeks in SMIM medium. β-actin is used as an internal control. There is an increased expression of MHC and ASMA in the cells cultured in SMIM medium for 4 weeks when compared to the controls (**B**).

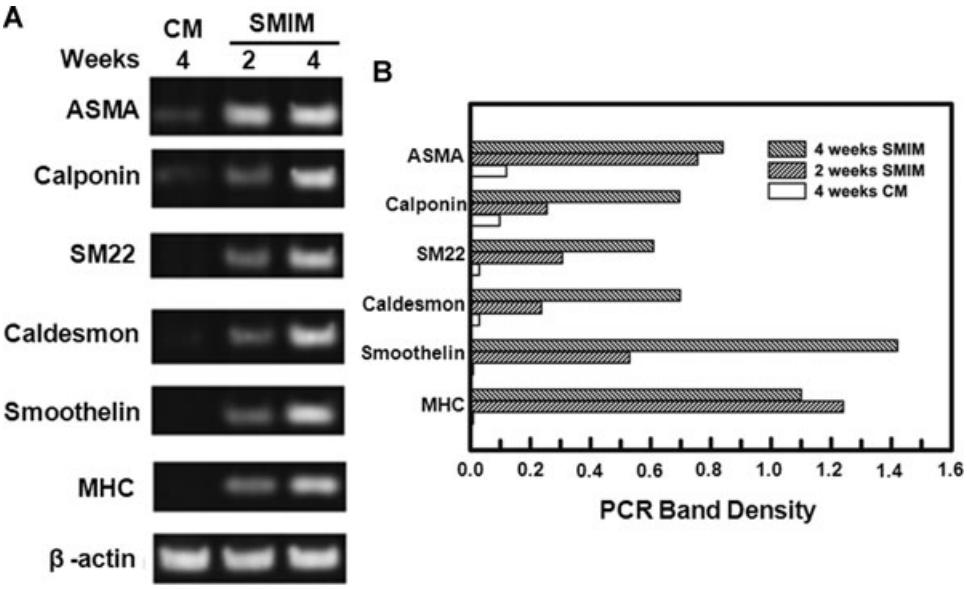

Fig. 5 Induction of smooth muscle differentiation when purified hMDCs are grown in SMIM. Gene expression of smooth muscle-specific markers such as ASMA, Calponin, SM22, Caldesmon, Smoothelin and MHC when purified hMDCs are exposed to SMIM for 2 and 4 weeks when compared to the control medium (CM) for 4 weeks as determined by RT-PCR. β-actin is used as a house-keeping gene (**A**). Increased gene expressions of smooth muscle-specific markers are determined by semi-quantitative RT-PCR using β-actin as a control gene (**B**).

develop into mature muscle fibres. In the present study, we further characterize smooth muscle differentiation from the purified hMDCs, which might be useful for the functional urological regeneration. Study using mouse bone-marrow-derived cells demonstrates that the cells can reconstruct layered smooth muscle structures in injured bladders to mediate urinary dysfunction. Cystometric investigations show that mice with implanted cells develop bladder contractions similar to normal mice [24]. In an *in vivo* study, the authors claim that the adipose-derived stem cells are a potential source for stem cell-based therapies, which would functionally recover the damaged erectile function [25]. MDSCs isolated from mouse skeletal muscle implanted into the corpora cavernosa of aged rats converted into smooth muscle cells and corrected ageing-related ED [26].

In the future, further functional studies need to be conducted to evaluate the functional property of adding these purified hMDCs into the bladder detrusor, urethral sphincter or penis with or without scaffolds. Our next experiments will be directed towards improving the differentiation rate, improving the growth stability of this smooth muscle cells, incorporate the hMDCs into 3D scaffolds and conduct functional studies *in vitro* and *in vivo*.

Conclusions

The differentiation of the purified hMDCs into smooth muscle cells is successfully conducted which gene expressions at the mRNA and protein levels are characterized. These cells might have the potential to be better biomaterials for tissue regeneration.

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