Genetic modification of human adipose-derived stem cells for promoting wound healing

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ARTICLE INFO

Article history:
Received 19 September 2011
Received in revised form 11 February 2012
Accepted 16 February 2012

Keywords:
Human adipose derived stem cells
v-myc
Genetic modification
Wound healing
Conditioned medium

ABSTRACT

Background: Diverse growth factors secreted from human adipocyte-derived stem cells (hASCs) that support or manage adjacent cells have been studied for therapeutic potentials to a variety of pathological models. However, senescent growth arrest in hASCs during in vitro culture and subsequent defective differentiation potential, have been technical barriers to further genetic modification of hASCs for functional improvement.

Objective: We investigated the feasibility of long-term hASC culture to enhance their therapeutic use.

Methods: We used a MYC variant to generate hASCs expressing v-myc and determined their growth potential and growth factor secretion profile. We further introduced an AKT variant to generate constitutively active (CA)-Akt/v-myc hASCs. Finally, we tested the ability of promoting wound healing of medium conditioned with CA-Akt/v-myc hASCs.

Results: The v-myc hASCs actively proliferated longer than control hASCs. Increased secretion of vascular endothelial growth factor (VEGF) by v-myc hASCs promoted the migration potential of hASCs and vasculogenesis in co-cultured endothelial cells. Additional genetic modification of v-myc hASCs using CA-Akt further increased VEGF secretion. In addition, injection of CA-Akt/v-myc hASCs-CM into wound-mice model promoted wound healing compared to normal hASCs-CM.

Conclusion: Genetic modification of hASCs to stimulate secretion of growth factors is a novel strategy to maximize their paracrine effect and improve their therapeutic potential.

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1. Introduction

Human adipose-derived stem cells (hASCs) have been widely studied as a promising stem cell source for future clinical application owing to their relatively high proliferating potential and broad differentiation potential not only to bone, cartilage, and muscle, but also to endothelial cells, cardiomyocytes [1], and neuron-like cells [2]. Moreover, hASCs are one of a few adult stem cells from which autologous cell therapy is achievable due to the possible rich supply of stem cells from extracted adipose tissue. Thus, autologous stem cell therapies with hASCs isolated from liposuction have advanced to clinical trials for a variety of conditions [3].

Recently, soluble factors secreted from hASCs have also been demonstrated to have a variety of biological activity, including anti-growth of cancer [4], immune modulation [5], promoting wound healing [6], and other functions [7]. Thus, a number of secreted growth factors in the conditioned medium of hASCs (hASCs-CM) have been identified and studied [8,9]. Among them, vascular endothelial growth factor (VEGF), one of the key factors that promotes vasculogenesis and angiogenesis [10], is considered to play important role in myogenic differentiation [11], wound healing, [12] and other processes [13]. However, hASCs cultured in vitro undergo permanent growth arrest in parallel with defective differentiation potential [14], similar to bone marrow-derived mesenchymal stem cells [15]. Decline in the regeneration potential of stem cells with senescence, which occurs with prolonged in vitro maintenance, is problematic considering the limited sources of stem cells. Thus, genetic modification of stem cells to maintain their stemness longer has been attempted and a few successes have been reported [15,16]. Moreover, additional genetic modification of longer maintainable
stem cells is desirable to improve their tissue regeneration potential [17,18].

In this study, we employed a similar strategy of genetic modification to promote the secretion of growth factors by hASCs. A MYC variant, showing successful immortalization of stem cells due to both its growth promoting capability as well as telomerase reverse transcriptase (TEKT) inducing capability, was utilized to generate hASCs expressing v-myc. The growth potential of v-myc hASCs was maintained for a longer period of time with less alteration in the profile of secreted factors compared to control hASCs. Using v-myc hASCs, we further introduced an AKT variant, generating CA-Akt/v-myc hASCs, which resulted in elevated VEGF secretion and enhanced vasculogenesis in co-cultured endothelial cells. Finally, we demonstrated that CM from CA-Akt/v-myc hASCs accelerated wound closure with less inflammation compared to that of normal hASCs, suggesting that genetic modification of longer maintainable stem cells would be able to extend the clinical usage of stem cells and secreted factors from stem cells.

2. Methods and materials

2.1. Cell culture and gene delivery

hASCs, of which characters have been demonstrated in number of studies [19–21] were cultured in alpha-MEM media (cat #32561, GlutaMAX™, Gibco®) with 10% fetal bovine serum (FBS) and 0.1% gentamycin. Cells were passaged every 4 days. Retrovirus encoding v-myc gene (courtesy provided by Dr. Seung U. Kim, ChungAng University, Korea) and CA-AKT1 genes (courtesy provided by Dr. Paul Shapiro, University of Maryland, Baltimore, MD, USA) was produced from 293GP cells and infected into parental cells with 4 μg/ml polybrene as described elsewhere [22]. CM, was prepared from culturing 1 × 10⁶ hASCs or genetically modified hASCs in 10 ml serum-free alpha-MEM media at 37 °C for 3 days and then filtered through a 0.22-μm microfilter (Millipore, Bedford, MA, USA). For cutaneous wound animal experiment, CM was concentrated by dissolving in 1 ml dPBS after freeze-drying.

2.2. Real-time PCR

Total RNA was extracted from cells and processed for cDNA synthesis using MaxiQuick® RT PreMix (Oligo [dT]₁₁, primer) (cat #25081, INTRON Biotechnological). The cDNA was amplified on the Light Cycler 480™ system (Roche Applied Science, Indianapolis, IN, USA) using gene-specific primers as follows: hGAPDH, 5’-GAAG TGAAAGTCCAGTGC-3’ and 5’-GAAGATCTGAT GGGATTTC-3’; hVEGF, 5’-AAGGAGGAGGGCAGATCAT-3’ and 5’-ATCTGCAITGAT GTTTGGA-3’; and c-Myc, 5’-TCCTGGCCAAAGGTCAGAG-3’ and 5’-GGAGTTTTATGTATTTCCA-3’. Data analysis was based on the ΔΔCt method with normalization to the housekeeping gene, GAPDH.

2.3. Immunoblotting and immunofluorescence staining

Immunoblotting and immunofluorescence staining were performed as described elsewhere using protein-specific antibodies as follows: Erk2 (cat #sc-154, Santa Cruz Biotechnology, Santa Cruz, CA, USA), c-myc (cat #sc-764, Santa Cruz Biotechnology), and AKT (cat #sc-5298, Santa Cruz Biotechnology).

2.4. Cytokine array

The CM from each type of hASCs was concentrated 5-fold using the Amicon Ultra-4 (Millipore, cat # UFC800324). Levels of paracrine factors were measured on the Human Growth Factor Array 1 (cat #AAH-GF-1, RayBiotech, Inc., Norcross, GA, USA) following the manufacturer’s protocol.

2.5. hVEGF enzyme-linked immunosorbent assay (ELISA)

Cells were cultured in alpha-MEM media containing 0.5% FBS for 3 days and then the CM were harvested. Protein levels of VEGF in CM without concentration were determined using the Human VEGF Quantiklo ELISA Kit (R&D Systems, Minneapolis, MN, USA, cat #QVE00B).

2.6. Fluorescence-activated cell sorting analysis

hASC cells were stained with anti-human/mouse CD44 PE antibody (cat #12-0441-81, eBioscience Inc., San Diego, CA, USA) and anti-human CD73 PE antibody (cat #550257, BD Pharmingen™, San Diego, CA, USA) and then analyzed with FACS caliber (BD Biosciences) and CellQuest software.

2.7. In vitro migration assay

For migration assay using the scratch method, cells were grown to confluence on 12-well plates and then serum-starved using alpha-MEM media with 0.02% FBS for 24 h. After serum starvation, a scratch was introduced with a yellow pipette tip as described elsewhere. Cells were further incubated in various CMs and areas were re-filled using hASCs or genetically modified hASCs. After 24 h, tube formation of HUVECs was assessed and images of non-overlapping areas were taken. Tube junction and length were analyzed for each condition using ImageJ software.

2.9. Cutaneous wound animal model

To investigate the effect of paracrine factors secreted by genetically modified hASCs on cutaneous wound healing, hairless mice were anesthetized by intraperitoneal injection of 300 mg/kg Avertin (cat #T48402-5G, Sigma–Aldrich, St. Louis, MO, USA). Two full thickness excisional wounds (left and right wound separated by 2 cm) were created on dorsolateral area using a standard skin biopsy punch (5 mm in diameter, Acuderm Inc., Ft. Lauderdale, FL, USA) and then 120 μl of concentrated CM from each hASC type was immediately injected at each of the three different intact dermis sites near the wounds on the same mouse to rule out the inter-animal variance. Each CM was injected two times every other day after surgery. The open wound area was measured on days 1, 3, and 6 after surgery using a method described previously [23]. All procedures were performed with the approval of Kangbuk Samsung Hospital Institutional Animal Care and Use Committee (No. 09-006-C5-N).

2.10. Immunohistochemistry

Wound tissues obtained on 7 day after wounding were fixed in 4% paraformaldehyde for 3 days and then embedded in paraffin. Wound tissue sections (2-μm thickness) were obtained using a Leica RM2065 microtome (Leica, Wetzlar, Germany) and stained with hematoxylin–eosin (H&E) and Masson’s trichrome. All tissue sections were carefully examined by one experienced dermatologist.
2.11. Statistical analysis

Graphical data are presented as mean ± standard error of the mean (S.E.M.). Statistical significance among groups and between groups was determined using one-way analysis of variance (ANOVA) following application of the Bonferroni multiple comparison post-test and Student’s t-test, respectively. Significance was assumed for p < 0.05. Statistical analysis was performed using SAS statistical package, v.9.13 (SAS Inc., Cary, NC, USA, http://www.sas.com/).

Fig. 1. Generation of hASCs expressing v-myc. Stable v-myc expression in hASCs was confirmed by (A) semi-quantitative real-time PCR analysis, (B) immunoblotting (ERK2 for equal loading control), and (C) immunofluorescence staining (DAPI, blue for nuclear counterstaining). (D) Growth rates of control hASCs and v-myc hASCs were determined by cell counting at each indicated time point and presented as a graph. (E) Immunophenotype of control hASCs and v-myc hASCs were determined by fluorescence-activated cell sorting analysis.
3. Results

3.1. Generation of hASCs stably expressing v-myc

Stable expression of MYC in stem cells has been used to either promote stem cell growth or achieve immortalization of primary somatic cells [24–26] or stem cells [16,27,28]. Therefore, we introduced v-myc into hASCs using a lentiviral gene transfer system to generate v-myc expressing hASCs (v-myc hASCs). Stable v-myc expression in hASCs was confirmed by semi-quantitative real-time RT-PCR, immunoblotting, and immunofluorescence staining analysis (Fig. 1A–C). Considering the critical role of MYC to induce a variety of cell cycle regulated genes and subsequently promote cell growth [29], accelerated cell growth of hASCs was predicted. As expected, v-myc hASCs proliferated more efficiently than normal hASCs (Fig. 1D). The promoted growth potential of v-myc hASCs remained up to 20 passage, when normal hASCs underwent senescent growth arrest (data not shown). v-myc hASCs also expressed a set of common surface markers of hASCs such as CD44 and CD73 (Fig. 1E).

3.2. Altered paracrine secretion of hASCs

It has been well documented that human mesenchymal stem cells (MSCs) that share close similarity to hASCs undergo premature senescence during in vitro culture after more than 10 passages and that post-senescence hMSCs lose their differentiation potential significantly [15]. Along with lowered differentiation potential of hMSCs with senescence, the profile of secreted cytokines and growth factors from hMSCs, which have both paracrine and autocrine activities [30,31] appeared to be altered in post-senescence hASCs (Fig. 2A). In particular, secretion of granulocyte macrophage colony-stimulating factor (GM-CSF) and hepatocyte growth factor (HGF) was significantly decreased while vascular endothelial growth factor (VEGF) was significantly increased in v-myc hASCs compared to normal hASCs (Fig. 2B, 2C).
reduced in post-senescence hASCs compared to pre-senescence hASCs (Fig. 2A). Of note, v-myc expression in hASCs apparently recovered the secretion of HGF, but failed to recover the secretion of GM-CSF. More interestingly, vascular endothelial growth factor (VEGF) expression was induced in v-myc hASCs compared to both pre and post-senescence hASCs (Fig. 2A). The induction of VEGF expression in v-myc hASCs was confirmed by both semi-quantitative PCR analysis at the mRNA level (Fig. 2B) and ELISA at the protein level (Fig. 3C). Consistent with this, VEGF protein level in the conditioned medium (CM) of v-myc hASCs was significantly higher than in the CM from pre-senescence hASCs (Fig. 2C).

3.3. Enhanced VEGF secretion by v-myc hASCs

Considering the important role of VEGF in promoting vascularization and migration that may contribute to the wound healing potential of hASCs [32], we next determined the cellular response of upregulated VEGF secretion of v-myc hASCs. Migration potential of hASCs appeared to be significantly enhanced in v-myc hASCs compared to normal pre-senescence hASCs (Fig. 3A). Similarly, tubule formation by human umbilical vein endothelial cells (HUVECs) appeared to be increased when HUVECs were co-cultured with v-myc hASCs compared to mock culture or normal hASCs, which clearly indicated that increased VEGF secretion from v-myc hASCs stimulates the tubule forming capability of co-cultured endothelial cells (Fig. 3B).

3.4. Generation of CA-Akt1 expressing hASCs

Using v-myc hASCs as a cellular platform, we next attempted further genetic modification. For this purpose, we took advantage of Akt1, the expression of which increases survival and tissue repair capability of mesenchymal stem cells (MSCs) [33] and neural stem cells [18]. To maximize the effect of Akt1, we utilized an attached myristoylation signal (myr-Akt: CA-Akt) [34], which is

**Fig. 3.** CM from v-myc hASCs enhanced wound healing and vasculogenesis. (A) Representative images of in vitro wound healing assay of hASCs (p14) and v-myc hASCs (p14) (left panel, 100×). Graphical presentation of the quantification data of in vitro wound healing assay (right panel, ***p < 0.0001). (B) Representative images of in vitro tubule formation by HUVECs co-cultured with, or without (mock), normal hASCs and v-myc hASCs (left panel). Graphical presentation of the quantified tubule length (right panel; ns, non-significant; ***p < 0.0001).
constitutively active due to proximal location to the upstream kinase of Akt at the plasma membrane. Introduction of myr-Akt to v-myc hASCs resulted in constant active phosphorylation (ser 473) in both exogenous Akt and endogenous Akt (Fig. 4A), implying that expression of CA-Akt promotes endogenous Akt signaling. Akt1 localization at the plasma membrane due to myristoylation and nuclear v-myc expression in CA-Akt/v-myc hASCs were confirmed by immunofluorescence staining (Fig. 4B). CA-Akt expression in v-myc hASCs did not add further growth advantage compared to that of v-myc hASCs (Fig. 4C). In addition, oxidative stress has been considered to be one of the main causes for cellular senescence during in vitro culture condition [15,35]. Under oxidative stress condition induced by H2O2, both ASCs stably expressing v-myc (v-myc ASCs and CA-AKT v-myc ASCs) appeared not to be positive to β-galactosidase staining, which is a typical assay to determine the cellular senescence (Supplemental Fig. 1). Thus, we concluded that both v-myc expressing ASCs are more resistant to senescence inducing condition.

3.5. Effect of increased VEGF secretion by CA-Akt/v-myc hASCs

Since Akt activity has been closely associated with not only survival or cell growth, but also VEGF expression in a HIF-1-dependent [36] and independent manner [37], we expected that CA-Akt/v-myc hASCs would have further increased VEGF expression compared to the v-myc hASCs. ELISA measuring VEGF protein levels in the conditioned medium clearly demonstrated that CA-Akt/v-myc hASCs had increased VEGF secretion compared to v-myc hASCs (Fig. 5A). Similarly, the cell migration capability of hASCs was further enhanced in CA-Akt/v-myc hASCs (Fig. 5B). Vasculogenesis by HUVECs was also significantly increased when co-cultured with CA-Akt/v-myc hASCs (Fig. 5C).

3.6. Enhanced wound healing by CM from CA-Akt/v-myc hASCs

Because co-culturing HUVECs with CA-Akt/v-myc hASCs promoted vasculogenesis (Fig. 5C), it would be readily inferred

Fig. 4. Generation of the CA-AKT v-myc-expressing hASCs. Stable expression of CA-AKT1 in v-myc hASCs was confirmed by (A) immunoblotting (ERK2 for loading control) and (B) immunofluorescence staining (DAPI for nuclear counter staining); (C) Growth rates of control v-myc hASCs and CA-Akt/v-myc hASCs were determined by cell counting at each indicated time point and presented graphically.
that the secreted protein(s) from CA-Akt/v-myc hASCs (e.g., VEGF) would be responsible for such an effect. Considering the important role of vasculogenesis in wound healing, we next examined whether the conditioned medium of CA-Akt/v-myc hASCs could accelerate wound healing by comparing the effect of CM from CA-Akt/v-myc hASCs to CM from normal hASCs in a standard nude mouse wound model. As shown in Fig. 6A, macroscopic observation of the wounds treated with CM from either normal or CA-Akt/v-myc hASCs at the indicated dates clearly showed accelerated wound closure in wounds treated with CA-Akt/v-myc hASCs CM, as determined by measurement of open wound area (Fig. 6A). On microscopic observation of wound tissue...
samples, clear improvement of epidermis and collagen formation was seen in CA-Akt/v-myc hASCs CM-treated wounds. Wound closure after treatment with CM from CA-Akt/v-myc hASCs showed well-organized epidermis, normal dermal collagen arrangement, and clear adnexal structure regeneration; whereas the wound areas treated with CM from hASCs showed premature epidermal regeneration and dense inflammatory cell infiltration (Fig. 6B). The anti-inflammatory effect of hASCs was proven in a number of studies [38–40], and fewer infiltrating inflammatory cells in wounds treated with CM from CA-Akt/v-myc hASCs may suggest enhancement of that anti-inflammatory effect. Masson's trichrome staining clearly demonstrated dense collagen regeneration in the wounds treated with CM of CA-Akt/v-myc hASCs (Fig. 6C). Four independent sections were analyzed, and overall scores of epidermis/collagen formation and inflammation are summarized (Fig. 6D).
4. Discussion

Cellular senescence of stem cells has been problematic in possible application of a variety of adult stem cells. Unlike embryonic stem cells, which are capable of unlimited proliferation, adult stem cells including hASCs, hMSCs, and human neural stem cells (hNSCs) undergo senescence during in vitro culture [15,27]. Due to limited supply of adult stem cells, it is critical to develop strategies that allow continuous proliferation of these cells for therapeutic applications. However, due to cellular senescence caused by either telomere erosion or onset of stress signaling, adult stem cells lose both proliferating potential and stemness during prolonged in vitro culture. In addition, genetic modification to improve the functionality of stem cells would be impractical owing to the relatively short-term maintenance. Therefore, a number of attempts have been made to delay or block senescence of stem cells by introducing various (proto)oncogenes, such as BM1 [41]. MYC variants [16,27,28], or TEKT [42]. Similarly, we have reported that the protein phosphatase, PPM1D was able to delay onset of cellular senescence in bone marrow mesenchymal stem cells induced by prolonged in vitro culture [15].

Among the known approaches, we took advantage of v-myc, which was proven to successfully immortalize bone marrow-derived MSCs and neural stem cells [16,28]. Because the MYC oncogene promotes cell proliferation through upregulating a number of cell cycle regulatory genes such as cyclin D1 [43], cyclin E [44], and cyclin A [45] with subsequent CDK activation, stable expression of MYC can accelerate cell proliferation. As predicted, introduction of v-myc, a viral homolog of c-myc into hASCs significantly promoted cell growth compared to the controls (Fig. 1D), while maintaining the expression of typical hASCs surface markers (Fig. 1E). We have not confirmed whether v-myc hASCs acquired immortalized properties due to the relatively short-term maintenance in culture so far (20 passage), which would be insufficient to observe telomere erosion. However we observed that v-myc introduction induced TERT expression in hASCs (data not shown).

Factors secreted by hASCs have been demonstrated to exhibit a variety of biological activities including suppressing immunoreaction [46], promoting tumor cell growth [47], and facilitating skin wound closure [21]. However, post-senescent hASCs appeared to have an altered profile of secreted factors that may be responsible for such variety of biological activity consistent with significant loss of stemness frequently observed in post-senescent stem cells [15,48]. As expected, expression of several cytokines, which appeared to be altered in post-senescent hASCs, was partly restored in v-myc hASCs (Fig. 2A). In particular, VEGF, one of the key secreted factors in hASC-mediating regeneration [13,49], was further upregulated in v-myc hASCs (Fig. 2). Due to increased levels of VEGF secretion from v-myc hASCs, HUVECs co-cultured with v-myc hASCs formed vessel more efficiently (Fig. 3B). Taking advantage of v-myc hASCs as a cellular platform, we introduced further genetic modification with constitutively active AKT mutant, since AKT plays important role in promoting both cellular survival and vasculogenesis [50,51]. CM from CA-Akt/v-myc hASCs was more efficient in accelerating wound healing than normal hASCs (Fig. 6), suggesting that further genetic modification of long-term maintainable stem cells by MYC variants would be important source of future cell therapy. Because the present study only utilized the CM that may be as active as stem cell therapy, at least in the wound repair process [23], the risk of tumorigenicity of hASCs genetically modified with (proto)oncogenes such as v-myc and CA-Akt would be negligible. Additionally, using the in vitro transformation assay in soft-agar, we did not observe any colony formation with CA-Akt/v-myc hASCs, suggesting that CA-Akt/v-myc hASC-CM is not tumorigenic (data not shown).

In summary, we generated v-myc-expressing hASCs as a long-term maintainable stem cell model for further genetic modification to achieve additional functional improvement. The well-controlled genetic modification of hASCs may create new important sources of paracrine factors for therapeutic applications.

Acknowledgments

This work was supported by the Scientific Research Center (SRC) program (M-2011-A0403-00080) and the Priority Research Centers Program (2010-0028297).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2012.02.010.

References


Birren SJ, Anderson DJ. A v-myc-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. Neuron 1990;4:189–201.


