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ORIGINAL ARTICLE

Influence of high glucose and AGE environment on the proliferation, apoptosis, paracrine effects, and cytokine expression of human adipose stem cells in vitro

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Abstract The incidence of delayed wound healing in patients with diabetes has increased in recent years. However, the reason of delayed diabetic wound healing and the changes of human adipose stem cells (hASCs) in the diabetic environment are still unclear. We simulated diabetic microenvironment with high glucose and glycation end products (AGEs) in vitro. CCK-8 and flow cytometry were used to study the proliferation and apoptosis of hASCs in the simulated diabetic microenvironment. The paracrine of hASCs was studied by transwell co-culture system. Protein chip was used to measure the expression of cytokines in hASCs. We found that high glucose and AGEs did not affect the proliferation of hASCs but arrested them in the S phase. More hASCs appeared early apoptosis in the simulated diabetic microenvironment. The promoting effect on the proliferation of fibroblasts and endothelial cells was weakened when hASCs were cultured in diabetic microenvironment for 6 days. The five cell factors, granulocyte colony-stimulating factor (G-CSF), transforming growth factor-α (TGF-α), hepatocyte growth factor (HGF), tissue inhibitor of metalloproteinases-1 (TIMP-1), and vascular endothelial growth factor (VEGF), were all downregulated in hASCs of AGEs and the high glucose group. In this study, we simulated diabetic microenvironment with high glucose and AGEs in vitro to evaluate the changes of proliferation, apoptosis, paracrine, and cytokine expression of hASCs in the diabetic environment and tried to find the possible reason of delayed diabetic wound healing.

Keywords hASCs · AGEs · Glucose · Apoptosis · Paracrine · Cytokine

Introduction

The advanced glycation end products (AGEs) are latter products of Maillard reaction or glycation and adversely affect the functional properties of proteins, lipids, and DNA [1]. In many clinical researches, AGEs in the diabetic skin are higher than normal tissue [2]. Also, positive correlations are found between the concentration of skin AGEs and age or incidence of foot ulcer [3]. A group of studies indicated that the AGEs inhibit the proliferation of endothelial cell [4], fibroblast [5], and nerve cell [6] and induce apoptosis. The former experiments indicated AGEs inhibit the proliferation and lead to human adipose stem cell (hASCs) apoptosis, decreasing the proliferation-promoting effect of hASCs on fibroblasts and endothelial cells. The formation of AGEs is closely related to the high concentration of tissue glucose, and AGEs accompany by high glucose can simulate a diabetic environment better. hASCs sited in skin follicles and subcutaneous fat may be important seed cells in wound repair [7]. The aim of our research was to study the changes of hASCs in the high glucose and AGE-simulated diabetic microenvironment, and refer another theoretical basis for diabetic wound healing and stem cell therapy.

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Material and methods

Material

Human adipose-derived stem cells were provided by the stem cell technology platform of Shanghai Biochemistry and Cell Biology Institute (commercial cell line, cell No.: SCSP-404). The hASCs are derived from adipose tissue of an adult female donor and are negative for CD14, CD31, CD34, and CD45 and positive for CD29, CD44, CD73, CD90, CD105, and CD166.

Human fibroblasts were provided by Dr. Ying-kai Liu of Shanghai Burn Institute of Rui Jin Hospital.

Human umbilical vein endothelial cells were provided by ATCC (strain, PCS-100-010).

Methods

Preparing and grouping of hASCs

The hASCs were cultured in a mesenchymal stem cell growth medium (serum and antibiotics free, ready-to-use, PromoCell, Heidelberg, Germany) and were detached by a low serum detach kit (0.04% trypsin, PromoCell, Heidelberg, Germany). The primary hASCs were passed to passage 7 (P7) before experiment. The primary, passage 7 and passage 10 (P10) hASCs was photographed under a microscope (200×).

Three groups were designed as follows: hASCs cultured in a medium contained 100 mg/L AGEs (AGE full length protein, Abcam, Cambridge, MA, USA) and 28 mmol/L high glucose as an experimental group (AG group), 100 mg/L BSA and 28 mmol/L mannitol as a control group (BM group) [8], and normal hASCs without any addition in the medium as a blank group (BK group).

Culturing the hASCs in simulated diabetic microenvironment

The hASCs were cultured in the mesenchymal stem cell growth medium with 100 mg/L AGEs and 28 mmol/L high glucose. The medium was changed every 48 h and remove the non-adherent cells. The hASCs cultured 2, 4, and 6 days were collected to be used in the following experiments. Three groups of hASCs were photographed after 6 days culture.

Proliferation and apoptosis of hASCs

The CCK-8 assay (Dojindo Laboratories, Tokyo, Japan) was applied to study the proliferation of hASCs cultured 2, 4, and 6 days. The CCK-8 test sample was measured three times, and the average OD value was taken as the final data. Ten repeat samples were collected from each group.

On day 2, day 4, and day 6, hASCs were stained by AnnexinV/PI (BD Pharmingen™, San Jose, CA, USA) to detect the percentage of early, late, and total apoptotic hASCs in each group by flow cytometry. Four repeat samples were collected from each group.

Cell cycle detection of hASCs

The hASCs cultured in simulated diabetic microenvironment for 6 days were fixed with 75% ethanol 30 min. The fixed hASCs were stained with PI/RNase staining buffer (BD Pharmingen™, San Jose, CA, USA) and detected by flow cytometry. Three repeat samples were collected in each group; the experiment was repeated three times.

Co-culture of hASCs with human fibroblasts and endothelial cells

Human fibroblasts (1×10^4) were seeded into the upper compartments of 24-well transwell inserts (Membrane pore size 0.4um, Corning, USA). 1×10^5, 0.5×10^5, and 0.25×10^5 hASCs suspended in a 500-μl stem cell growth medium were seeded into the lower compartments. Culture media was added into the lower compartment as the control group.

Endothelial cells (1×10^4) were seeded into the upper compartments. 1×10^5 hASCs in the 500-μl stem cell growth medium were seeded into the lower compartments. Culture media was added into the lower compartment as the control group.

Human fibroblasts (1×10^4) and endothelial cells (1×10^4) were seeded into the lower compartments. The upper compartments were seeded by 1×10^4 hASCs. The same volume of culture media was added into the lower compartment as the control group.

Human fibroblasts (1×10^4) and endothelial cells (1×10^4) were seeded into the upper compartments. 1×10^5 hASCs cultured in AGEs and high glucose for 6 days were seeded into the lower compartments (AG co-culture group). 1×10^5 hASCs cultured in BSA and mannitol for 6 days were seeded into the lower compartments as the control group (BM co-culture group). 1×10^5 hASCs were seeded into the lower compartments (BK co-culture group). Culture media were added into the lower compartment as the blank group (BK group).

All the co-culture systems (the upper and lower compartments) lasted for 4 days and we changed the medium on the second day. Then the cell proliferation of upper compartments was detected by CCK-8. Each co-culture system had 12 repeat samples.

Cytokine expression of hASCs in simulated diabetic microenvironment

Total cell proteins from 0, 2, 4, and 6 days intervened hASCs were extracted and the expressions were measured by using

QAH-ANG-1000-2 protein chip (RayBiotech, Norcross, GA, USA). Data of five interested cytokines were statistically analyzed. Each group on each time point had four repeat samples.

Data analysis

The cytokine data in the best confidence interval was chosen to be statistically analyzed. All data was identified by the normal distribution test (*W* test) and homogeneity test of variance (*F* test) with SPSS19.0. The data of each group were compared by *t* test using SPSS19.0. p value <0.05 indicates statistically significant difference.

Result

Cell culture and morphology of hASCs

Primary hASCs showed spindle, fusiform shape, 2–3 pseudopodia, large and oval nucleus and one to three nucleoli. The hASCs attached the culture dish firmly and distribution patterns were swirling or radial (Fig. 1a Primary). The shape and structure of P7 cells were not different from primary cells (Fig. 1a P7). P10 hASC volume increased, and the body became long and narrow with more pseudopodia. Some cells had intracellular clusters of vacuoles, transparent nuclei, and nucleolus pyknosis which were like "fried egg." Cell growth became slow and the number of floating cells increased (Fig. 1a P10).

The morphology of hASCs in simulated diabetic microenvironment

The AG group hASCs proliferated rapidly, reaching the peak in 2–4 days; cell count did not significantly increase in 4–6 days, and some cells became larger. The "fried egg"-like cells were more than the BM group and BK group (Fig. 1a).

The proliferation of hASCs cultured in simulated diabetic microenvironment

The OD values of three groups on days 2, 4, and 6 measured by CCK-8 are compared. The AG group is not different with the BM group on day 2 and day 6, but higher than the BM group on day 4. There were no significant differences between the BM group and BK group (Fig. 1b).

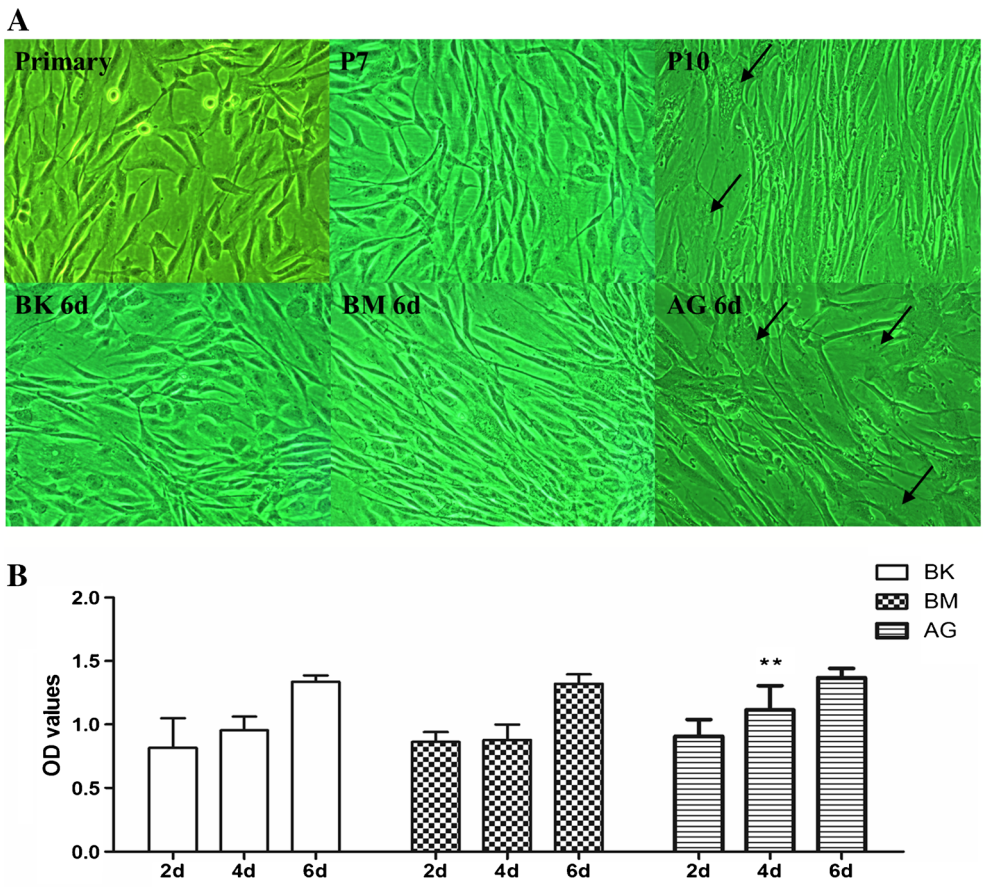
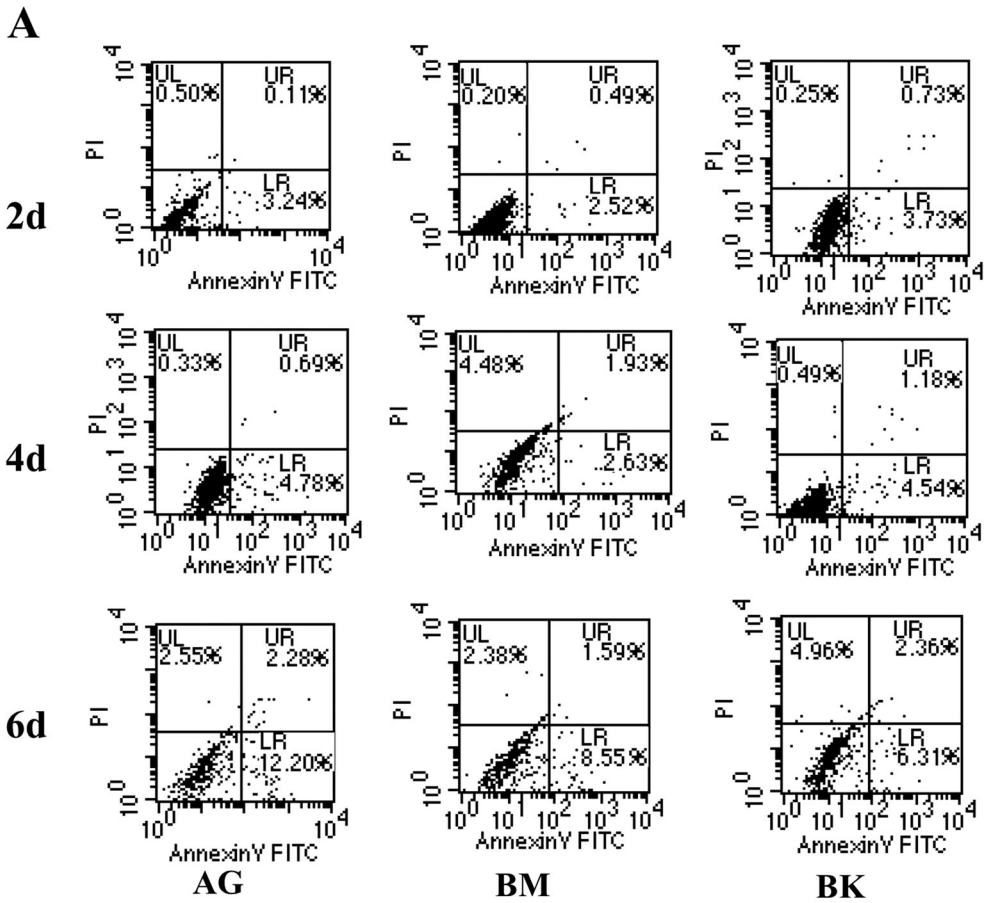


Fig. 1 The morphology and proliferation of hASCs in simulated diabetic microenvironment. The P10 hASCs contained more "fried egg" like cells than primary hASCs (*arrow*) (**a** *P10*) (200×). The hASCs in AG group could be found a few "fried egg" like cells on day 6 (*arrow*) (**a** *AG6d*) (200×).The differences of hASC proliferation between AG group and BM group (**b**) BM group and BK group (**c**). *$p < 0.05$, **$p < 0.01$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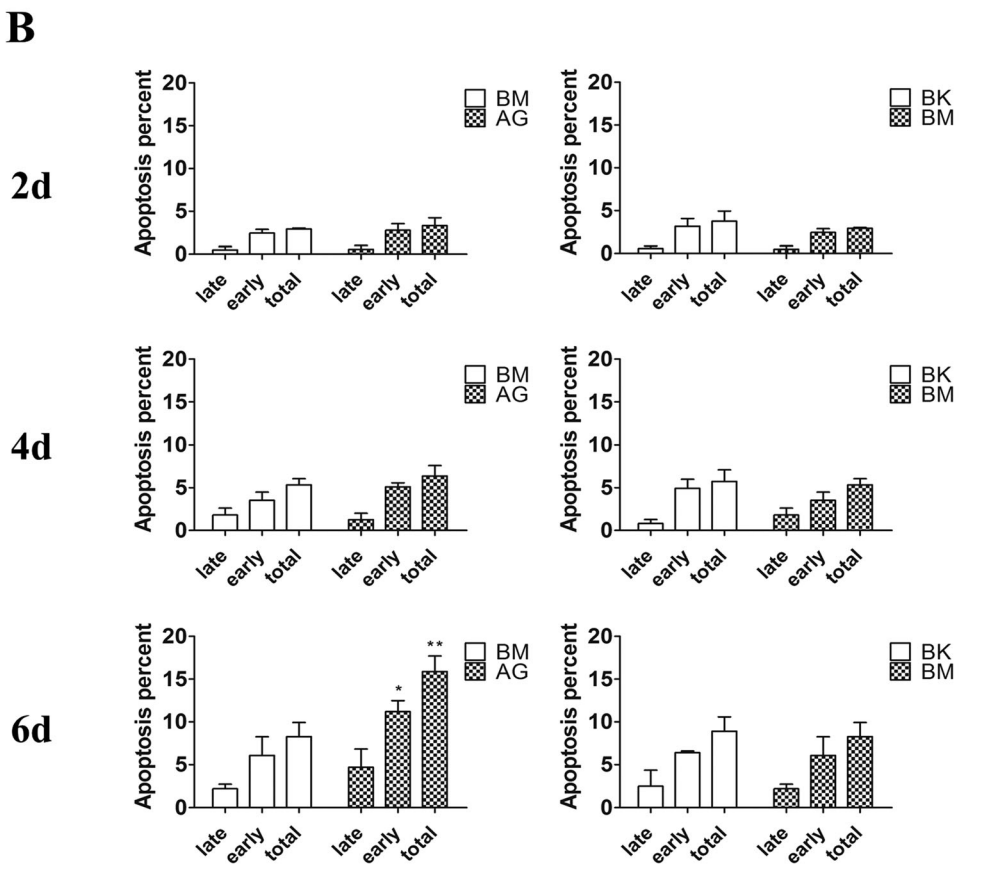
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◀ **Fig. 2** The apoptosis of hASCs cultured in simulated diabetic microenvironment. The flow cytometry results of the AG, BM, and BK groups on day 2, day 4, and day 6 (**a**) and the bar graph (**b**). *$p < 0.05$, **$p < 0.01$

The apoptosis of hASCs cultured in simulated diabetic microenvironment

The difference of apoptosis between the AG group and BM group was not significant on day 2 and day 4. The early apoptosis (AnnexinV+/PI-) and total apoptosis (early + late) of the AG group was higher than those of the BM group on day 6. The late apoptosis (AnnexinV+/PI+) of the AG and BM groups had no significant difference on day 6. The apoptosis of the BM and BK groups showed no significant difference (Fig. 2).

Cell cycle of hASCs in simulated diabetic microenvironment

The percentage of the G1 phase between the AG group and BM group showed no significant difference on day 6. The S phase percentage of the AG group was higher than that of the BM group, but the G2 phase percentage was lower than the BM group on day 6. The S/G2 value of the AG group was also higher than the BM group on day 6. The results of the BM and BK groups showed no significant difference (Fig. 3b).

The paracrine of hASCs in simulated diabetic microenvironment

The number of fibroblast in the 1×10^5 hASC co-culture group was significantly higher than that in the control group after 4 days (Fig. 4a). The number of fibroblast cultured with 0.5×10^5 or 0.25×10^5 hASCs was not significantly higher than that in the control group (Fig. 4b, c). The number of fibroblast in the 1×10^5 hASC group was significantly higher than that in the 0.5×10^5 hASC group and 0.25×10^5 hASC group. The number of endothelial cells co-cultured with 1×10^5 hASCs was significantly higher than that in the control group (Fig. 4d).

There were no significant differences between the number of hASCs in the upper compartments co-cultured with fibroblasts or endothelial cells and the number of hASCs in the control group (Fig. 4e, f).

The number of fibroblasts in the AG co-culture group was significantly lower than that in the BM co-culture group (Fig. 4g) but was still higher than that in the BK group (Fig. 4h).

The number of endothelial cells in the AG co-culture group was not different with that in the BM co-culture group (Fig. 4i). The endothelial cells in the AG co-culture group were no more than the BK group (Fig. 4j).

The number of endothelial cells and fibroblasts in BM co-culture group were not different with those in the BK co-culture group.

The protein expression of hASCs in simulated diabetic microenvironment

The expression of five interested cytokine, hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), transforming growth factor-α (TGF-α), tissue inhibitor of metalloproteinases-1 (TIMP-1), and vascular endothelial growth factor (VEGF), were measured in the AG, BM, and BK groups on day 2, day 4, and day 6.

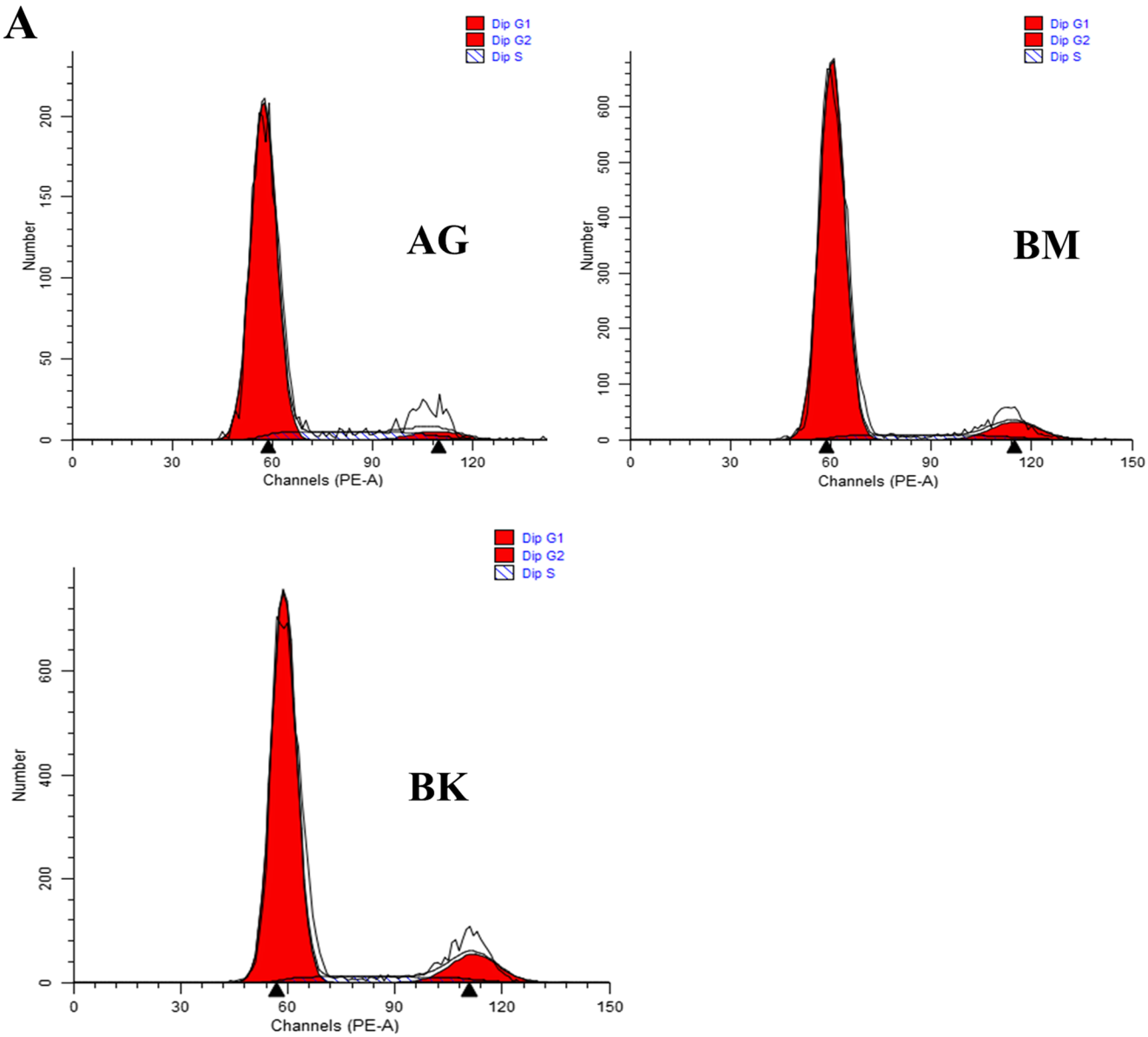
In simulated diabetic microenvironment, HGF was downregulated on day 2, day 4, and day 6 ($p < 0.01$). The expressions of G-CSF, TGF-α, and TIMP-1 in the AG group were not different from the BM group on day 2 and day 4. On day 6, the expressions of the three factors were downregulated ($p < 0.05$). VEGF expression was not different between the AG group and BM group on day 2, while the expression was downregulated on day 4 ($p < 0.05$) and day 6 ($p < 0.01$) (Fig. 5).

Discussion

The primary hASCs will grow old with time, morphology of P10 hASCs has changed, and the "fried egg"-like cells may no longer have stem cell properties [9]. P7 hASCs were chosen for experiments because they had enough number, morphology as primary cells, stable proliferation rate, and low apoptosis. The BK group was used to verify whether the BSA and mannitol environment has effect on hASCs.

The AGEs in our study were produced by the Maillard reaction of BSA and glucose which was similar to in vivo [10]. The combination of AGEs and high glucose in the culture medium can simulate the micro environment of diabetic tissues better than adding AGEs or high glucose only. The 28 mmol/L glucose can simulate the hyperglycemia of a diabetic patient [11]; while not reaching the blood glucose of ketoacidosis (33 mmol/L), 100 mg/L AGEs was chosen as former research [12].

The growth of hASCs in high glucose and AGEs was not inhibited; the simulated diabetic microenvironment might even promote the growth of hASCs on day 4. Many former researchers found that only high glucose would promoted the growth of hASCs [13, 14]. In our study, the promoting effect was not continuous for the number of hASCs in the AG group on day 6 and was no more than the BM group. In the cell cycle experiment, more hASCs in simulated diabetic environment were blocked in the S phase and unable to complete cell division. This may explain the low proliferation of hASCs on day 6.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◀ **Fig. 3** Cell cycle of hASCs in simulated diabetic microenvironment. The cell cycle results of the AG, BM, and BK groups on day 6. (**a**) and the bar graph contrast AG and BM group (**b**) and bar graph contrast BM and BK group (**c**) $*p < 0.05$, $**p < 0.01$

The percentage of early apoptotic hASCs increased in in vitro diabetic environment. Those early apoptotic hASCs could not complete differentiation and might not participate in wound healing. These results happened on hASCs just had been cultured in simulated diabetic environment for 6 days. The hASCs of diabetic patients may stay in the high glucose and AGE environment for several months or even years. It may lead to more severe cell cycle arrest and apoptosis [15, 16], may reduce the wound healing ability of hASCs, and may finally cause the delayed wound healing. AGEs and high glucose may also affect hASCs (local or transplanted) in the diabetic wound and weaken the efficacy of stem cell therapy.

The hASCs might promote the proliferation of fibroblasts and endothelial cells by paracrine [17]. The promoting effect of hASCs was positively correlated with cell number. In diabetic patients, apoptosis induced by AGEs in the diabetic microenvironment may decrease the number of hASCs and may weaken the paracrine promoting effect of hASCs on proliferation of fibroblasts and endothelial cells.

The HGF can upregulate the expression of VEGF and TIMP-1 by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor [18]. HGF plays a central role in angiogenesis and tissue regeneration. VEGF promotes the proliferation of vascular endothelial cells and angiogenesis [19]. The expression of HGF in hASCs of AG group was decreased, which may weaken the promoting effect of hASCs on the proliferation of endothelial cells. VEGF in hASCs was decreased on day 4 and day 6 after HGF which may further weaken the angiogenic promoting effect of hASCs and reduce the proliferative activity of endothelial cells.

The G-CSF can promote endothelialization of injured vascular. It may reduce expression of pro-inflammatory cytokines and enhance neurogenesis [20]. The low expression of G-CSF in the AG group may lead to decreased proliferation of endothelial cells and delayed repair of vascular injury.

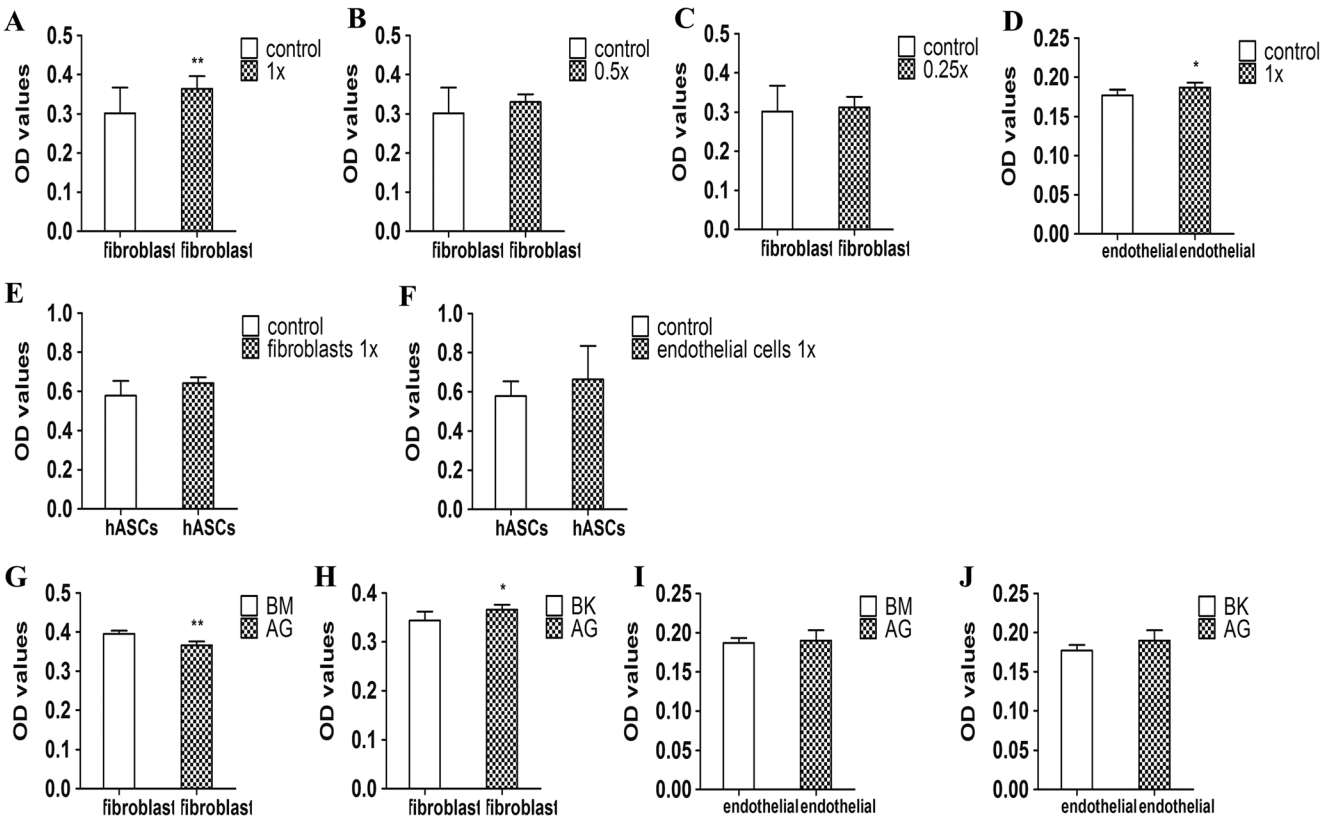

Fig. 4 The results of transwell co-culture system. The proliferation of fibroblasts co-culture with 1×10^5(**a**), 0.5×10^5(**b**), and 0.25×10^5(**c**) hASCs seeded in the lower compartments and with 1×10^5 hASCs of AG group in the lower compartments (**g**, **h**). The proliferation of endothelial cells co-culture with 1×10^5 hASCs seeded in the lower compartments (**d**) and with 1×10^5 hASCs of AG group in the lower compartments (**i**, **j**). The proliferation of hASCs co-culture with 1×10^5 \fibroblasts seeded in the lower compartments (**e**) and with 1×10^5 endothelial cells of the AG group in the lower compartments (**f**)

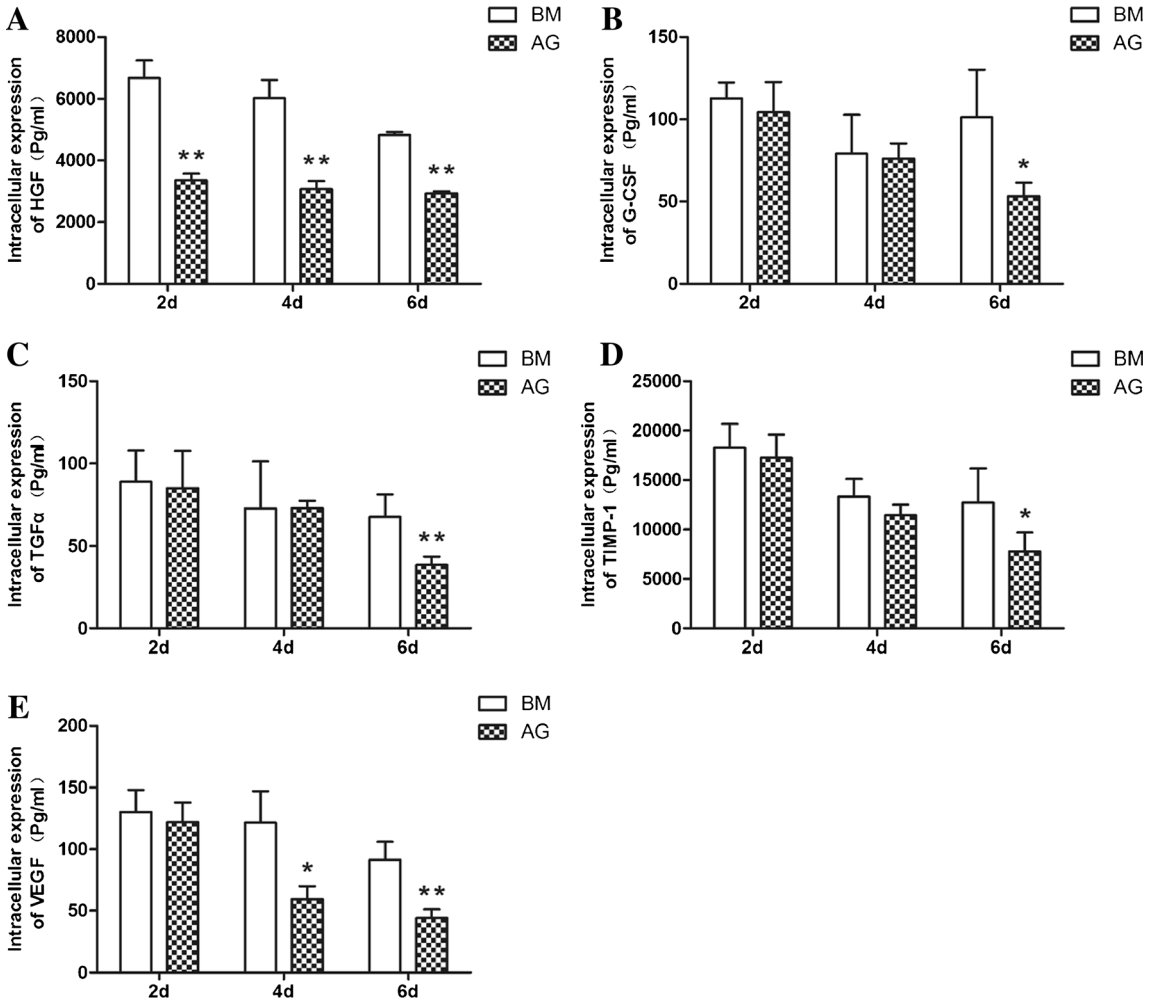

Fig. 5 Differences in protein expression of hASCs cultured in simulated diabetic microenvironment. The HGF (**a**), G-CSF (**b**), TGF-α (**c**), TIMP-1 (**d**) and VEGF (**e**) expression of hASCs cultured in the AG and BM groups. *$p < 0.05$; **$p < 0.01$

The TGF-α is a member of the epidermal growth factor (EGF) family, which activates cell proliferation and differentiation. TGF-α is also believed to promote angiogenesis and protect endothelial cell from apoptosis [21]. The TGF-α was downregulated in the AG group, which may decrease epithelial regeneration and may affect diabetic wound healing.

The TIMP-1 promotes cell migration and proliferation and is involved in degradation and remodeling of the wound matrix. Bullen et al. [22] found TIMP-1 levels were lower in chronic than in healing wounds. Imbalance of TIMPs and MMPs is a necessary factor for chronic wound formation [23]. TIMP-1 in AG group was decreased, which may accelerate the wound tissue degradation and aggravate the spread of inflammatory factors. The downregulation of growth factors such as G-CSF and TGF-α will reduce the proliferation of wound healing cells [24]. Fewer mesenchymal cells will lead to the decrease of HGF expression and further decrease the expression of VEGF and TIMP-1. A vicious cycle of wound healing factors was formed, and it may explain the causes of delayed diabetic wound healing.

Conclusions

High glucose and AGEs do not inhibit the proliferation but arrest the hASCs in the S phase. High glucose and AGEs lead to hASC apoptosis, decreasing the paracrine-promoting effect of hASCs on proliferation of fibroblasts and endothelial cells. The decrease of growth factors HGF, G-CSF, and TGF-α in hASCs may influence the proliferation of endothelial and epithelial cells. All the changes may lead to delayed diabetic wound repair and regeneration. The results of this study suggest that decreasing glucose and AGEs in the microenvironment of hASCs or increasing the expression of HGF and TGF

in hASCs may be a new therapeutic option in clinical to speed up diabetic wound healing. This study is not without limitations. We did not carry out these experiments on cells obtained from diabetic patients. More experiments are needed to study the changes of diabetic hASCs in the simulated diabetic microenvironment.

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Authors' contributions Conceived and designed the study: JHG, SLL, JYD. Performed the experiments: JHG. Analyzed the data: JHG. Contributed reagents/materials/analysis tools: JHG, SLL, TX. Wrote the manuscript: JHG, SLL. Experiment skill advising: JYD, SLL. All authors read and approved the final manuscript.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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