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HGF and IGF-1 promote protective effects of allogeneic BMSC transplantation in rabbit model of acute myocardial infarction

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Abstract

Objectives: To explore effects of hepatocyte growth factor (HGF) combined with insulin-like growth factor 1 (IGF-1) on transplanted bone marrow mesenchymal stem cells (BMSCs), for treatment of acute myocardial ischaemia.

Materials and methods: After ligation of the left anterior descending artery, rabbits were divided into a Control group, a Factors group (HGF+IGF-1), a BMSC group and a Factors+BMSCs group. Allogenous BMSCs (1×10^7) and/or control-released microspheres of 2 μg HGF+2 μg IGF-1 were intramyocardially injected into infarcted regions. Apoptosis and differentiation of implanted BMSCs, histological and morphological results, and cardiac remodelling and function were evaluated at different time points. *In vitro*, BMSCs were exposed to HGF, IGF-1 and both (50 ng/ml) and subsequently proliferation, migration, myocardial differentiation and apoptosis induced by hypoxia, were analysed.

Results: Four weeks post-operatively, the above indices were significantly improved in Factors+BMSCs group compared to the others ($P < 0.01$), although Factors and BMSCs group also showed better results than Control group ($P < 0.05$). *In vitro*, HGF promoted BMSC migration and differentiation into cardiomyocytes, but inhibited proliferation ($P < 0.05$), while IGF-1 increased proliferation and migration, and inhibited apoptosis induced by hypoxia ($P < 0.05$), but did not induce myocardial differentiation. Combination of HGF and IGF-1 significantly promoted BMSCs capacity for migration, differentiation and lack of apoptosis ($P < 0.05$).

Conclusions: Combination of HGF and IGF-1 activated BMSCs complementarily, and controlled release of the two factors promoted protective potential of transplanted BMSCs to repair infarcted myocardium. This suggests a new strategy for cell therapies to overcome acute ischemic myocardial injury.

Abbreviations

MI: Myocardial infarction

ANOVA: Analysis of variance

BMSCs: Bone marrow mesenchymal stem cells

DiI: 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate

cTnT: Cardiac Troponin T

EDV: End-diastolic volume

EF: Ejection fraction

FACS: Fluorescence activated cell sorting

FITC: Fluorescein isothiocyanate

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HGF: hepatocyte growth factor

IA: infarction area

IGF-1: insulin-like growth factor 1

LAD: left anterior descending artery

LV: Left ventricle

OD: Optical density

RT-PCR: Reverse transcription polymerase chain reaction

SPSS: Statistical Product and Service Solutions

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

VD: Vessel density

WT: Wall thickness

Introduction

Bone marrow mesenchymal stem cell (BMSC) transplantation has emerged to be a promising method for treatment of ischaemic heart disease, due to potential of these cells to promote myocardial regeneration after myocardial injury (1,2). However, some experimental and clinical studies have strongly suggested that effects of cell transplantation might be largely impacted by hostile microenvironment of the ischaemic region (3,4). Thus, it may be critical to improve the microenvironment of transplanted zones and potential of transplanted BMSCs from hypoxia, for enhancement of efficiency of such therapy.

It has been demonstrated that hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1) can increase angiogenesis of ischaemic regions and improve their hostile microenvironment, consequently promoting myocardial repair, following acute myocardial infarction (AMI) (5–8). However, it is unclear whether or not HGF and IGF-1 would enhance protective effects of BMSC transplantation after AMI. To explore the effects of these two factors on BMSCs, the present study set to assess the following parameters: proliferation, migration, differentiation and apoptosis of BMSCs *in vitro*, transplanted BMSC survival and differentiation *in vivo*, left ventricular function and remodelling after combined administration of HGF+IGF-1 and BMSCs.

Materials and methods

The project protocol was approved by the Liaoning Administrative Committee for Laboratory Animals. According to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health in 1996, all animals received humane care and all procedures were carried out strictly in accordance with best practice.

Isolation and identification of BMSCs

Rabbit BMSCs were prepared as described previously (9). Briefly, BMSCs were isolated from bone marrow using the method of whole BM adherent cultures, and subsequently incubated with DMEM/F12 medium containing 10% foetal bovine serum, 100 μg/ml penicillin and 100 U/ml streptomycin sulphate (Gibco Life Technologies, Paisley, UK) in a 95% humidified incubator at 37 °C and 5% CO_2. Forty-eight hours later, adherent cells were collected, maintained and cultured continuously.

Fluorescence-activated cell sorting (FACS) was performed for analysis of BMSC immunophenotype with anti-rabbit CD34, CD44 and CD45 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Results showed they positively expressed CD44 (92.15%) and did not significantly express CD34 (1.79%) or CD45 (1.94%). Before implantation, BMSCs were stained with cross-linkable membrane dye 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen Corporation, Carlsbad, CA, USA) for cell labelling.

Surgical procedures

The rabbit (2.5 ± 0.3 kg) model of AMI was established by ligation of the mid-third of the left anterior descending artery (LAD). Thereafter, animals were randomly assigned to four groups: Control group (no treatment, $n = 8$), Factors group (controlled release of HGF and IGF-1, $n = 8$), BMSC group (BMSC transplantation, $n = 12$) and Factors+BMSCs group (controlled release of HGF and IGF-1 and BMSC transplantation, $n = 12$).

In the Control group, 200 μl saline was injected by sterile microinjection at two sites within the infarcted region. For the other three groups, allogenous BMSCs (1×10^7) and/or biodegradable microspheres containing 2 μg IGF-1 and 2 μg HGF were administrated in the same way. Antibiotics were administrated intramuscularly for 3 days postoperatively. As described in our previous paper (10), the biodegradable microspheres (diameter 1–3 μm and release period of 2–3 weeks) were made from biodegradable polymer, poly D, L-lactic/glycolic acid (PLGA), which has no effect on activation of BMCSs (11–14); this has been widely used as delivery material in wide ranges of BMSCs studies (11,12,15,16).

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After assessment of left ventricular wall thickness (LVWT) of infarcted regions, end-diastolic volume (EDV) and ejection fraction (EF) by echocardiography, animals were sacrificed by overdose of potassium chloride. Tissues of ischaemic areas were harvested at different time points, for PCR, western blotting and histological analysis.

In vitro measurement of BMSC proliferation

To further explore mechanisms of effects of HGF and IGF-1 on BMSCs, the following *in vitro* experiment was designed to investigate the BMSC proliferation, migration, differentiation and apoptosis after direct exposure to HGF (50 ng/ml) and/or IGF-1 (50 ng/ml), not released from biodegradable microspheres.

Passage 3 BMSCs growing on glass slides were serum-starved in serum-free DMEM/F12 supplemented with 0.5% BSA (wt/vol) for 14 h. Then they were cultured in the above medium containing 0.25% BSA (Control group) and stimuli (in HGF, IGF-1 or HGF+IGF-1 groups) for 6 h. Before the end of experiment, the BMSCs were incubated with BrdU (30 μg/l) for 40 min, and anti-BrdU staining was performed to evaluate cell proliferation.

In addition, BMSCs were seeded on to 96-well plates at initial density of 500 cells/well. After 14 h serum starvation in serum-free DMEM/F12 supplemented with 0.5% BSA (wt/vol), the cells were cultured in the above medium containing 0.25% BSA, meanwhile 'stimuli' and Alamar blue (1:10 vol/vol ratio, Invitrogen, DAL1025) were added to the culture system. Absorbance was monitored at 570 nm and 600 nm for 5 days to evaluate proliferation rate.

In vitro determination of BMSC migration

Transwell migration chambers (Millipore, Billerica, MA, USA), pore size 8 μm, were used to assess BMSC migration. After serum starvation for 14 h, the cells were plated in upper chambers and stimuli were added to lower chambers. Eight hours after treatment, cells remaining on upper faces of Transwell chambers were removed carefully using cotton swabs, and subsequently crystal violet staining was performed. Quantitative analysis was performed with both cell counting and measurement of absorbance.

BMSC differentiation

After exposure to HGF and/or IGF-1 for 1 week and HGF+IGF-1 for 6 h, the BMSCs were continually cultured for the next 2 weeks. To evaluate their differentiation into cardiomyocytes, expressions of cardiac Troponin T (cTnT), GATA4, CX43, Nkx2.5 were determined, using β-actin as internal control, and immunofluorescence was performed with anti-cTNT antibodies (Abcam Ltd, Cambridge, UK).

Apoptosis induced by extreme hypoxia

BMSC apoptosis was induced by extreme hypoxia (0.2%), in a sealed GENbox hypoxic chamber (17), and serum deprivation for 24 h. Subsequently, apoptotic cells were evaluated using flow cytometry (Cytoron Absolute; Ortho) with fluorescein isothio-cyanate (FITC)-labelled annexin V and propidium iodide (PI, Roche Applied Science, Basel, Switzerland) staining.

Histological analysis

Immunofluorescence staining of lamella of migrating cells was carried out using anti-BrdU antibodies (Abcam Ltd, Cambridge, UK) to determine their proliferation, and with anti-cTNT antibodies to identify their differentiation. For tissues of ischaemic areas, vWF counterstaining was performed to observe angiogenesis, Masson's trichrome stain was used to assess MI, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) was carried out for analysis of apoptosis of implanted BMSCs, and anti-cTNT immunofluorescence staining was performed to identify their myocardial differentiation. For each sample, five non-overlapping fields at 100× magnification in transverse sections were randomly captured using a video camera, which were stored in TIFF format. New vessels (NV) and infarcted areas (IA) were quantified using Image Pro Plus (IPP) 6.0 software package (IPP, Media Cybernetics, Silver Spring, MD, USA). Positively stained regions were padded with single colour and converted to pixels by optical density (OD) calibration.

Statistical analysis

Blind methods were used to measure all offline data. Results are presented as mean ± SD. Statistical analysis was performed using SPSS 18.0 software package (SPSS Inc, Chicago, IL, USA) and Student's *t*-test and one-way or two-way analysis of variance (ANOVA) with Bonferroni *post hoc* correction, used for group-comparison. $P < 0.05$ was regarded as statistically significant.

Results

Of the 44 rabbits initially studied, four were excluded from further experiments due to intractable ventricular

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fibrillation after ligation of the LAD. No severe events such as malignant arrhythmias, embolization, bleeding, or haemodynamic abnormality occurred during the Factors and Cells implantation procedure.

Combination of HGF and IGF-1 inhibited implanted BMSC apoptosis and promoted their differentiation into cardiomyocytes

TUNEL assays (Fig. 1a,b) revealed many apoptotic BMSCs implanted into ischaemic regions in the BMSCs group 24 h after implantation (91.6 ± 3.3%, $n = 4$), but this was largely attenuated in the Factors + BMSCs group (77.4 ± 2.7%, $n = 4$, $P = 0.004$, Fig. 1c). OD of DiI was higher (33601 ± 824 pixels/hpf, $P < 0.001$, Fig. 1d) in the Factors+BMSCs group, compared to the BMSCs group (33.2 ± 4.8 cell/hpf and 16127 ± 248 pixels/hpf respectively). As shown in fluorescence images 4 weeks postoperatively (Fig. 1e,f), there were more surviving BMSCs within ischaemic regions in Factors+BMSCs group (OD = 26937 ± 1017 pixels/hpf) than BMSCs group (OD = 10931 ± 346 pixels/hpf, $P < 0.001$, Fig. 1g). The following anti-cTNT staining found more cTNT$^+$ BMSCs in Factors+BMSCs group (9.9 ± 2.4 cell/hpf) than BMSCs group (2.6 ± 2.2 cell/hpf, $P < 0.001$, Fig. 1h), revealing that HGF+IGF-1 promoted BMSC differentiation into cardiomyocytes.

Combined therapy increased angiogenesis, reduced IA and improved LV function

Representative images (Fig. 2a–d) of vWF-stained sections of different groups revealed that OD of NV was significantly higher in the Factors+BMSCs group (81328 ± 7729 pixels/hpf, $P < 0.001$), compared to other groups, although there also was an increase in Factors (59455 ± 5709 pixels/hpf, $P < 0.001$) and BMSCs groups (45456 ± 4319 pixels/hpf, $P < 0.001$)

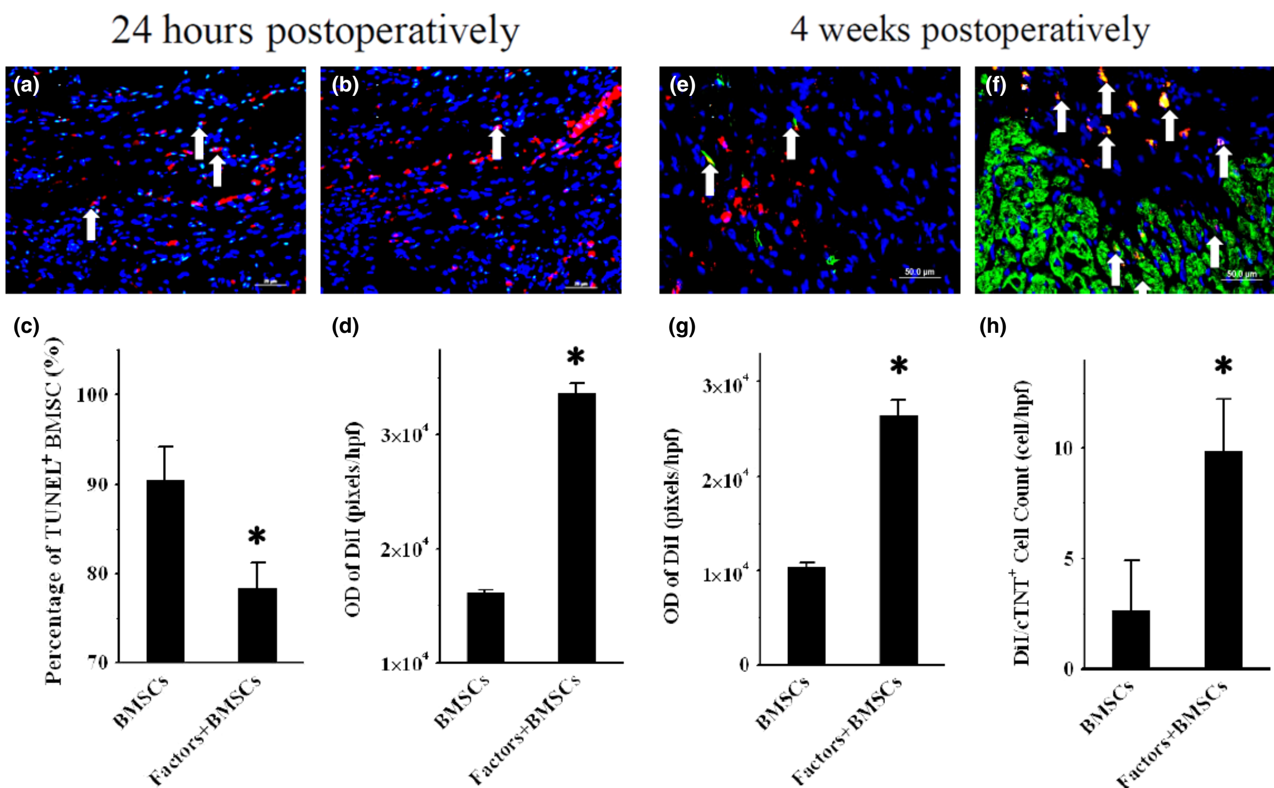

Figure 1. Survival and differentiation of transplanted cells. (a) and (b) representative TUNEL-staining images of 24-h postoperative myocardial frozen sections in BMSCs and Factors+BMSCs groups (200× magnification). DiI (Red fluorescence)- and TUNEL (green fluorescence)-positive cells indicate apoptotic transplanted BMSCs (white arrows). Comparisons of percentage of DiI$^+$/TUNEL$^+$ cells and OD of DiI in two groups are shown in (c) and (d). Four weeks postoperative anti-cTNT-staining images of the above two groups are shown in e and f (200× magnification). DiI (Red fluorescence)- and cTNT (green fluorescence)-positive cells indicate transplanted BMSCs differentiating into cardiomyocytes (white arrows). (g) and (h) respectively show comparisons of amounts of DiI$^+$/cTNT$^+$ cells and OD of DiI in two groups. TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; BMSCs, Bone marrow mesenchymal stem cells; DiI, 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; OD, Optical density; cTNT, Cardiac Troponin T. *$P < 0.01$ versus Control group.

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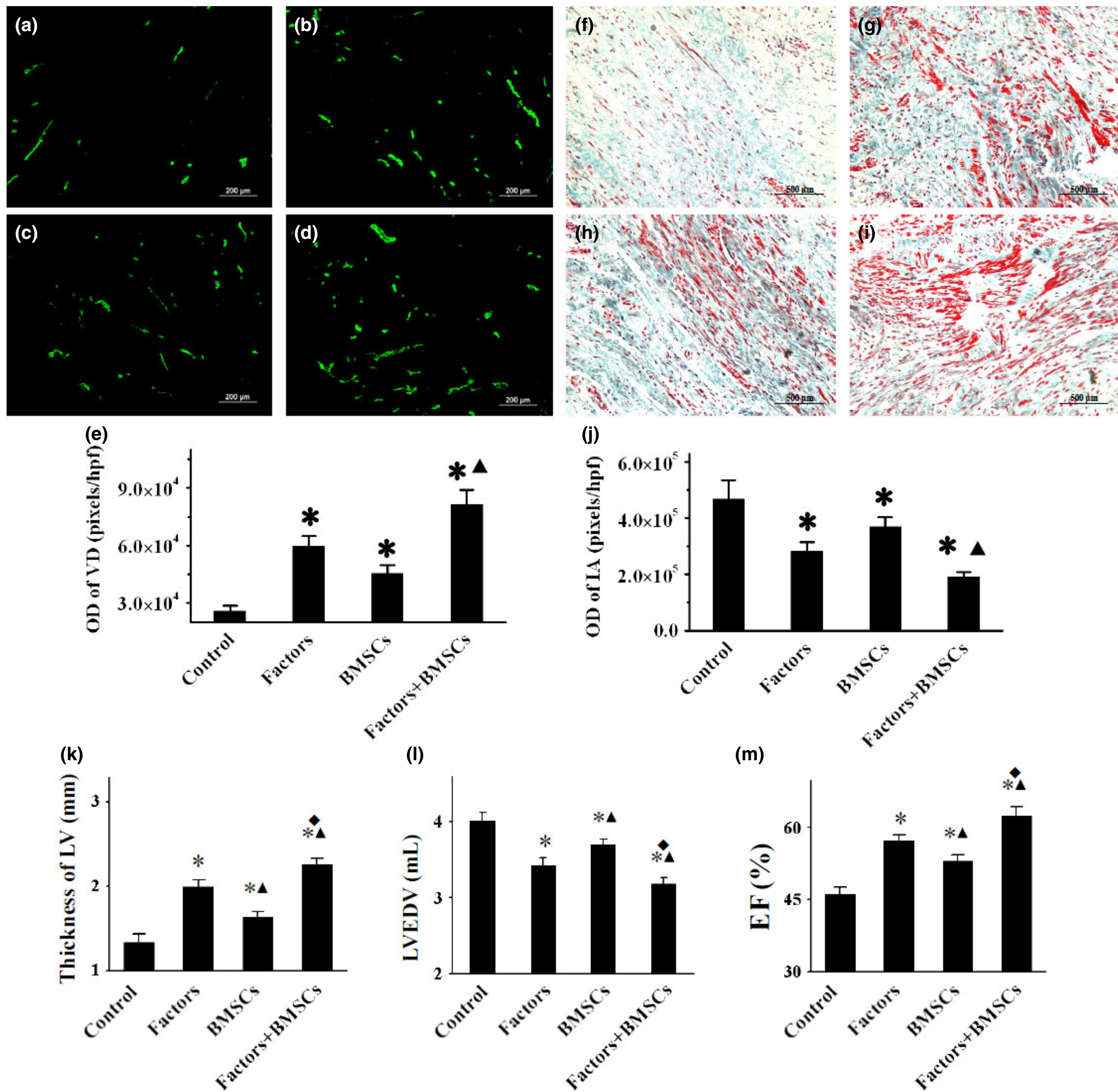

Figure 2. Analysis of angiogenesis, infarction and cardiac function. Representative anti-vWF and Masson's trichrome-stained images of myocardial sections (5 μm) within ischaemic territory from control, Factors, BMSCs and Factors+BMSCs groups are shown in (a)–(d) (200× magnification) and (f)–(i) respectively (100× magnification). With Masson's staining, red indicates viable myocardium, blue indicates collagen. Vessel density (VD) and infarcted area (IA) were quantified as OD values by using IPP software, then compared in (e) and (j) respectively. WT, EDV and EF of LV are compared in (k)–(m), respectively. VD, vessel density; IA, infarcted area; WT, wall thickness; EDV, end-diastolic volume; EF, ejection fraction; LV, left ventricle. *$P < 0.001$ versus Control group. ▲$P < 0.01$ versus Factors groups. ◆$P < 0.01$ versus BMSCs groups.

compared to controls (25820 ± 2934 pixels/hpf, Fig. 2e).

Representative Masson's trichrome staining images of the four groups are shown in Fig. 2f–i. Qualitative analysis (Fig. 2j) demonstrated significant reduction in IA in Factors+BMSCs group (OD = 190220 ± 18280 pixels/hpf) relative to Factors group (OD = 282323 ± 32672 pixels/hpf, $P = 0.001$) and BMSCs group (OD = 368295 ± 36314 pixels/hpf, $P < 0.001$), although the two latter also had smaller IA than control (OD = 468828 ± 66349 pixels/hpf, $P < 0.001$).

No differences in LVWT, EDV or EF were found between the four groups prior to treatment; mean EF value was 49%. Four weeks after treatment, Echo

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detected significant elevation of LVEF in Factors group (57 ± 2%, $P < 0.001$) and BMSCs groups (52 ± 2%, $P = 0.004$) compared to the Control group (47 ± 3%). Importantly however, there was further improvement in Factors+BMSCs group (64 ± 3%, $P < 0.001$) relative to Factors and BMSCs groups (Fig. 2m). Similar results were found in EDV and WT; the most significant improvement was in the Factors+BMSCs group ($P < 0.05$, Fig. 2k,l).

Assessment of BMSC proliferation in vitro

Representative anti-BrdU staining images of BMSCs in Control, HGF, IGF-1 and HGF+IGF-1 groups are respectively shown in Fig. 3a–d. Six hours after exposure to the factors, there was a significant increase in percentage of $BrdU^+$ BMSCs in the IGF-1 group (9.13 ± 0.41%, $P = 0.022$), and reduction in the HGF group (7.15 ± 0.25%, $P = 0.04$), but there was no difference in HGF+IGF-1 group (8.13 + 0.35%, $P = 1$), compared to the control (8.04 ± 0.22%, Fig. 3e). Growth curves showed similar changes ($P < 0.05$, Fig. 3f). These results suggest that IGF-1 promoted BMSC proliferation, but HGF inhibited it, resulting in an offsetting effect of each to the other.

Measurement of BMSC migration in vitro

Representative images of BMSC migration are shown, Fig. 4a–d. Quantitative analysis of absorbance (Fig. 4e) revealed significantly enhanced migration after exposure to HGF (OD = 0.29 ± 0.01, $P < 0.001$) and IGF-1 (OD = 0.37 ± 0.01, $P < 0.001$) compared to Control cells (OD = 0.21 ± 0.01). Combined exposure further increased BMSC migration (OD = 0.41 ± 0.02), relative to single exposure to HGF ($P < 0.001$) or IGF-1 ($P = 0.019$), indicating a superimposed effect.

Identification of BMSC differentiation into cardiomyocytes in vitro

Images of immunofluorescence staining (Fig. 5a–d) reveal that compared to control cells (2.37 ± 0.45%), HGF (11.52 ± 1.03%, $P < 0.001$) and HGF+IGF-1 (11.73 ± 1.06%, $P < 0.001$) significantly increased percentage of $cTNT^+$ BMSCs, without marked differences between the two groups ($P = 1$), but single IGF-1 did not increase cTNT expression of BMSCs (2.9 ± 0.4%, $P = 1$). A similar trend was found in mRNA and protein expressions of GATA4, cTNT, NKx2.5, CX43 ($P < 0.05$), as shown by RT-PCR (Fig. 5g) and western blotting (Fig. 5h,i).

Analysis of hypoxia-induced BMSC apoptosis in vitro

As shown by annexin-V/PI staining (Fig. 6a–d), there were fewer early- and late-phase apoptotic BMSCs (18.97 ± 1.16%) induced by hypoxia and more surviving BMSCs (80.58 ± 0.86%) after treatment with IGF-1 than controls (28.39 ± 0.82% and 68.31 ± 0.99% respectively, $P < 0.001$, Fig. 6e). However, there was no further improvement of BMSC apoptosis (17.74 ± 1.12%, $P = 1$) and survival (81.58 ± 1%, $P = 1$) after administration of IGF-1 combined with HGF. Single exposure to HGF did not inhibit BMSC apoptosis (28.55 ± 1.07%,

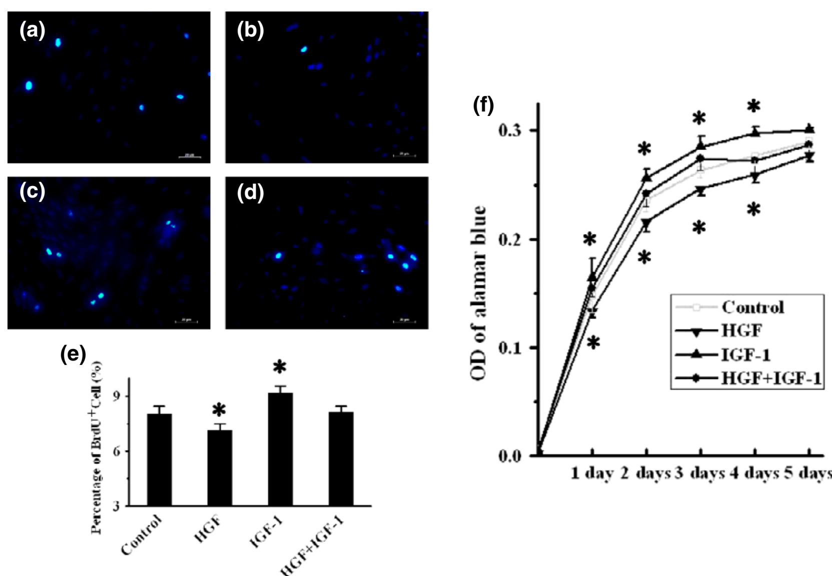

Figure 3. Assessment of BMSC proliferation *in vitro*. (a)–(d) Representative BrdU-staining images of BMSCs in control, HGF, IGF-1 and HGF+IGF-1 groups respectively. BrdU-positive cells (green fluorescence) in each group were counted and compared in (e). Cell proliferation measured by the Alamar blue assay, and growth curves for each group are shown in (f). BrdU, 5-bromo-2-deoxyuridine; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1. $^*P < 0.05$ versus Control group.

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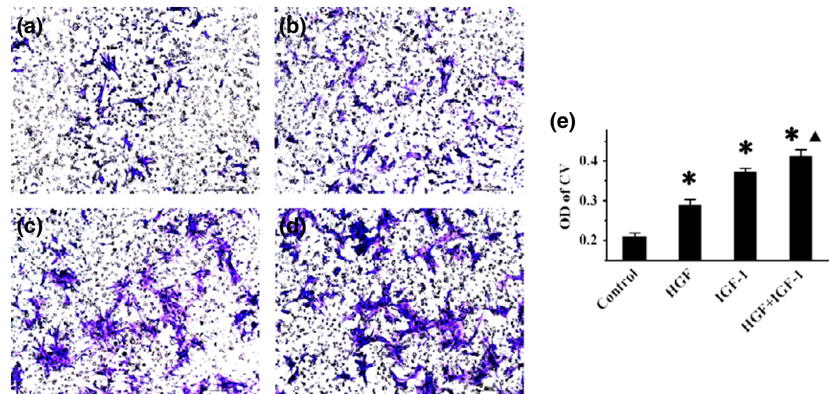

Figure 4. Effects of combined treatment on BMSC migration. (a)–(d) Representative crystal violet staining images of BMSCs in control, HGF, IGF-1 and HGF+IGF-1 groups respectively. (e) – comparison of OD value of crystal violet among all groups. *$P < 0.001$ versus Control group. ▲$P < 0.05$ versus HGF and IGF-1 groups.

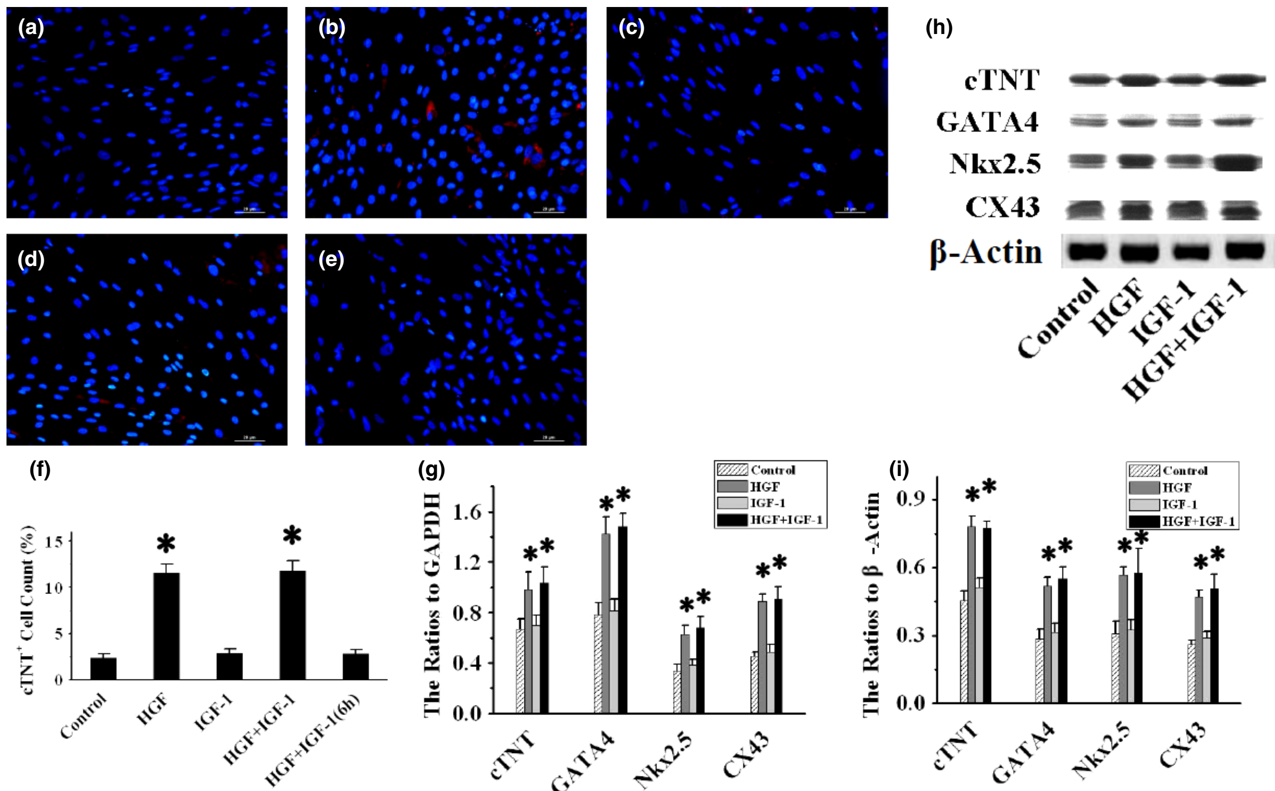

Figure 5. BMSC differentiation into cardiomyocytes. Representative anti-cTNT (red fluorescence) staining images of BMSCs exposed to blank control, HGF, IGF-1 and HGF+IGF-1 for 1 week, as well as HGF+IGF-1 for 6 h, are shown in (a)–(e) respectively. Numbers of cTNT-positive cells are compared in (f). Myocyte-specific factor cTNT, GATA4, Nkx2.5 and CX43 mRNA expressions were measured by RT-PCR, and ratios to GAPDH are compared in (g). Representative images of western blots are shown in (h), and ratios of these proteins to β-Actin are compared in (i). CX43, connexin43; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, Reverse transcription polymerase chain reaction. *$P < 0.001$ versus Control group.

$P = 1$) or enhance BMSC survival (70.62 ± 0.98%, $P = 0.112$), compared to controls.

Discussion

Although BMSC implantation has been advocated as a very promising therapeutic strategy for treatment of patients with MI (due to possible ability to improve cardiac function and reverse ventricular remodelling (3,18)), there are still two crucial problems – survival and differentiation of transplanted BMSCs (13,19), largely restricting their protective effects on ischaemic heart disease. It has been documented that most implanted BMSCs die under extreme hypoxic conditions

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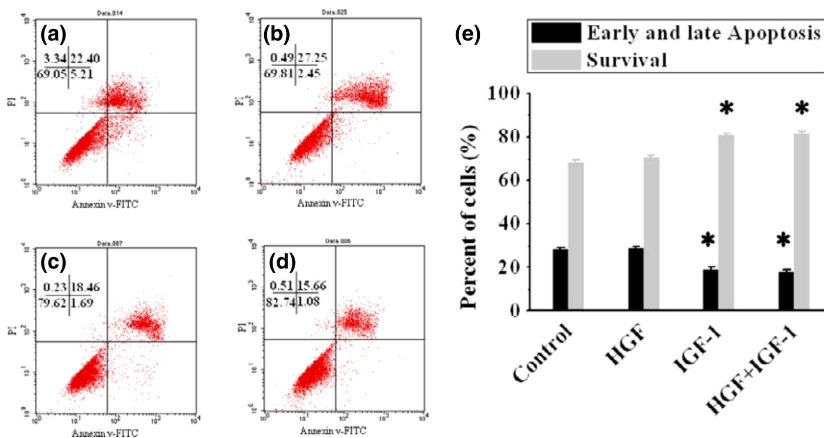

Figure 6. Analysis of BMSC apoptosis *in vitro*. (a)–(d) show BMSC apoptosis detected by flow cytometry with annexin-V/PI staining, in control, HGF, IGF-1 and HGF+IGF-1 groups respectively. Percentages of early and late apoptotic cells and those surviving, in all group are compared in (e). *$P < 0.01$ versus Control group.

experienced by ischaemic regions (20). Moreover, their very low intrinsic capacity to differentiate into cardiomyocytes impacts direct cell participation in myocardial regeneration.

Some *in vitro* experimental results have shown that HGF significantly promotes myocardial differentiation of BMSCs, but has not provided protective effects for apoptosis induced by hypoxia (21,22). IGF-1 has been certified to be an anti-apoptotic factor, while it is unclear whether it promotes BMSCs to differentiate into cardiomyocytes (12,23,24). Due to complementation of function, there have already been studies trying to apply combinations of the two factors for AMI therapy and yield expected results (7,8). Recently, Savi *et al.* reported continuous release of HGF and IGF-1 promoted repairing ability of adipose-derived stem cells, in healing myocardial infarction in rats (25). This strongly suggests that this combination may promote therapeutic effects of BMSC transplantation for treatment of ischemic myocardial injury. Our previous studies have revealed that HGF+IGF-1 promoted BMSCs co-cultured with myocardium, to express cardiac specific transcription factor GATA4, which indicated myocardial differentiation (26). In the present experiment, protective effects of HGF+IGF-1 on BMSCs were further investigated. The results show that controlled release of HGF and IGF-1 significantly increased angiogenesis, reduced apoptosis, and enhanced implanted BMSC survival and differentiation into cardiomyocytes, consequently improved LV remodelling and cardiac function. However, it has been identified that single intramyocardial injection did not provide these benefits (published in Chinese). The main reason to account for the different results is that intramyocardially injected cytokines have short biological half-life *in vivo* and may be rapidly washed out from large vascular structures in targeted sites. Thus, it is strongly suggested that slow release is very important for administration of HGF and IGF-1(6,8).

Following *in vitro* experiments revealed that HGF significantly enhanced myocardial differentiation of BMSCs and promoted their migration, but reduced their proliferation and did not inhibit apoptosis induced by hypoxia. However, IGF-1 significantly increased BMSC proliferation and inhibited hypoxia-inducing apoptosis. Price *et al.* demonstrated that IGF-1 is a comitogen for HGF in a rat model of hepatocellular carcinoma (27). However, it is disappointing that similar effects were not found in rabbit BMSCs. Although it is unclear whether there is any synergistic effect between HGF and IGF-1, complementary activity has been confirmed in the present study. Combination of HGF and IGF-1 not only promoted differentiation of BMSCs into cardiomyocytes but also inhibited apoptosis induced by hypoxia, which is very important for cells implanted in ischaemic areas, to survive and provide protective effects after AMI. It has also been revealed in the present study that effects of the two factors on differentiation were significantly time-dependent; short-term exposure (6 h) did not work, further emphasizing the importance of controled-release.

Despite encouraging results, there are still questions and considerations that need to be further addressed. For instance, in this study it has not been investigated whether there were ranges of effects of different doses of cytokines on BMSCs. In addition, due to dissimilarities between human and rabbit hearts, animal experiment results may not contribute precisely to clinical application.

In summary, it was demonstrated that in a rabbit model of AMI, controlled release of HGF and IGF-1 reduced implanted BMSC apoptosis and promoted their myocardial differentiation, consequently reducing IA, attenuating LV remodelling and improving cardiac function. *In vitro* experimentation revealed that combined administration of HGF and IGF-1 increased BMSC migration, inhibited hypoxia-induced apoptosis and

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time-dependently promoted differentiation into cardiomyocytes. It is strongly suggested that combination of HGF and IGF-1 may be a new therapeutic method in BMSCs transplantation.

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Conflict of interest

None declared.

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