The effect of angiotensin (1-7) on bone marrow stem cells
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Transplantation of angiotensin (1-7) stimulated bone marrow-derived progenitors does not additionally improve cardiac function

Cheng Qian; Anton J.M. Roks; Regien G. Schoemaker; Lili Yu; Heleen M.M. van Beusekom; Wim J. van der Giessen; Bo Yu & Wiek H. van Gilst

Submitted
Abstract

Background: We and others have recently demonstrated beneficial effects of angiotensin (1-7) [Ang-(1-7)] in different cardiovascular diseases. In addition, Ang-(1-7) can stimulate blood or bone marrow derived progenitor cells both in vivo and in vitro. However, whether the ex-vivo effects of Ang-(1-7) on bone marrow-derived mononuclear cells (BM-MNCs) would further benefit its therapeutic potential after transplantation in-vivo for cardiac disease, remains unknown.

Methods and results: Using cell culture and pharmacological tools, we examined the effects of Ang-(1-7) on BM-MNCs. To trace transplanted cells in-vivo, BM-MNCs were isolated from R26 human alkaline phosphatase (hPAP) transgenic rats (F344 background). After pretreatment with Ang-(1-7) for 7 days, transgenic BM-MNCs (5×10^5/200 µl) were intracoronary administrated to inbred F344 rats with acute myocardial infarction. Ang-(1-7) (10^-8 mol/L) significantly enhanced the proliferation and differentiation of the cultured BM-MNCs/EPCs during ex-vivo expansion, which could be antagonized by A-779 (10^-7 mol/L), but not by PD123319 (10^-7 mol/L). Intracoronary transplantation of either untreated or Ang-(1-7) pretreated BM-MNCs/EPCs significantly attenuated cardiac remodeling and partly improved cardiac function. Transplanted cells (hPAP+), expressing endothelial phenotype, were predominantly detected in the infarcted- and border zone. The recruitment of hPAP+ cells was inversely correlated with LVEDP (R^2=0.617, P<0.001). However, no difference in cardiac remodeling or function was observed between infarcted rats with untreated or Ang-(1-7) pretreated BM-MNCs.

Conclusions: These data indicate that Ang-(1-7) significantly enhances the percentage EPC in the BM-MNCs fraction during expansion, however without affecting their therapeutic potential, when used in in-vivo therapy for heart failure.
Introduction

Angiotensin (1-7) [Ang-(1-7)], a heptapeptide containing the initial seven amino acids of Angiotensin (Ang) II, has been identified as a cardiac protector counteracting some of the detrimental effects of Ang II [1-3], partly via a specific receptor in the cardiovascular system; the Mas receptor [4]. We and other groups have recently reported that apart from the beneficial effects in cardiovascular disease, Ang-(1-7) can stimulate bone marrow cells and hematopoietic progenitor cells in animal experiments and preclinical studies [3,5].

Acute myocardial infarction (AMI) evokes left ventricular (LV) remodeling characterized by cardiomyocyte hypertrophy, reduced capillary density and increased extracellular matrix deposition between myocytes [6]. There is increasing evidence that bone marrow-derived progenitor cells, especially endothelial progenitor cells (BM-EPCs) or circulating EPCs contribute to neovascularization after vascular injury in animal models [7,8]. Transplantation of bone marrow-derived mononuclear cells (BM-MNCs) or EPC has been proposed to restore cardiac function after ischemic cardiac disease, depending on various mechanisms [9,10]. Some studies show reduction in infarct size, but no improvement of regional cardiac contractile function [11,12]. Other studies show that transplantation of either unfractioned BM-MNCs or certain phenotype-selected cells gives rise to various benefits on cardiac functional recovery after AMI [13-15].

Bone marrow has been considered as a main reservoir of adult stem cells to be obtained for ex-vivo expansion. BM-EPCs, as a novel drug target, have been widely investigated. Amongst others it has been shown that pharmacological treatment with ACE inhibitors, of which the beneficial effects are partly ascribed to increase of Ang-(1-7) levels [16], is accompanied by increased numbers and functional improvement of EPC [17]. However, whether ex vivo-stimulation of EPCs could further potentiate cell-based therapeutic potential for ischemic cardiac disease is unknown yet.

In the present study, we explored whether stimulation of ex-vivo expanded BM-MNCs by Ang-(1-7) could enhance its therapeutic effect in a cell-based approach for cardiac remodeling. We compared the therapeutic potential of untreated BM-MNCs with Ang-(1-7) pretreated BM-MNCs after intracoronary application in a rat model of acute myocardial infarction.

Materials and methods

Animals

Three-month-old wistar rats were used to study the effects of Ang-(1-7) on BM-MNCs. For the in-vivo cell transplantation study, we used R26-hPAP rats as bone marrow donors (F344 background) and F344 rats as recipients (male, weight: 260-300 g, Harlan, Horst, the Netherlands). Rats were fed ad libitum, and housed in groups of 4-5 rats, at a 12:12 h light/dark cycle. All procedures performed on rats complied with the guideline of care and use
of laboratory animal. The experimental protocol was approved by Animal Ethical Committee of the University of Groningen.

Effects of Ang-(1-7) on EPC’s proliferation and differentiation
Bone marrow mononuclear cells (BM-MNCs) were isolated from femurs and tibias of 3-month-old Wistar rats with Lympholyte-rat (CEDARLANE, SANBIO b.v. Canada) by density-gradient centrifugation. The mononuclear cells (5×10^5/well) were cultured on 1% gelatin (225 bloom, Sigma-Aldrich, Zwijndrecht, The Netherlands) coated 96-well culture plate (Corning incorporated, Corning, NY, USA) in endothelial basal medium-2 (EBM-2) containing EGM-2™ SingleQuots supplement, 2% fetal bovine serum [Cambrex, Bio Science, Walkersville, Inc.]. After 2 days of culture, BM-MNCs were treated with Ang-(1-7) (Bachem, Bubendorf, Switzerland) at a concentration of 10^-8 mol/L, in the presence or absence of Mas receptor antagonist A-779 (10^-7 mol/L) [ISOGEN Bioscience B.V. Maarssen, the Netherlands] or AT2 receptor antagonist PD123319 (10^-7 mol/L) [Park Davis, USA.] based on pilot studies. The treatments were refreshed every second day for 7 days. Then, EPCs were detected by triple staining with 1,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-Ac-LDL, Molecular Probes™, OR, USA), FITC-conjugated lectin from bandeiraea simplicifolia (BS-I lectin, Sigma-Aldrich, Saint Louis, USA) and nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, molecular probes™, Eugene, OR, USA), as previously described [18]. The number of BM-MNCs and EPCs were counted in five high power fields (HPF) per treatment by a Leica DMIL inverted fluorescence microscope with Leica DC300F digital camera and software [Leica Microsystems, Bensheim, Germany]. Images were analyzed and recorded by Imagine-pro software (version 4.5.029; Media Cybernetics, Inc. USA)

To assess the effects of Ang-(1-7) on transgenic R26-hPAP BM-MNCs and EPCs, an abbreviated version of the above described protocol for the BM-MNCs of Wistar rats was performed.

Effect of Ang-(1-7) on EPC tube formation
In order to evaluate the potency of the cultured EPCs to form capillary-like tubes, after 7 days of culture with or without Ang-(1-7) in EBM-2 medium, BM-MNCs were collected and reseeded on growth factor reduced Matrigel™ (BD Biosciences, MA, USA) coated 24-well culture plates (10^5/well), and allowed to attach for 2 hours, followed by refreshment with EBM-2 medium. Tube formation was examined at 24 hours after reseeding.

Animal groups and surgical procedures
Inbred F344 rats were subjected to either left coronary ligation (n=22) or sham operation (n=10). After 30-40 minutes, rats with left coronary ligation were randomly allocated to one of the three groups: control (MI+medium, n=8) and two groups treated with cultured BM-MNCs: (MI+MNCs, n=7) or (MI+MNCs_Ang-(1-7), cells pretreated with Ang-(1-7) 10^-8 mol/L, n=7).
The initiation of anesthesia was induced with 4-5% isoflurane in a small chamber. After endotracheal intubation, all the surgical procedures were performed under anesthesia with 2% isoflurane at a flow rate 1.0 l/min and mechanically ventilated (Amsterdam Infant Ventilator, Hoek/Loos, Schiedam, The Netherlands; frequency 80/min) using 40% oxygen plus 60% N₂O.

Transplantation of ex vivo-expanded BM-MNCs
hPAP⁺ BM-MNCs were cultured in the presence or absence of Ang-(1-7) (10⁻⁸ mol/L) for 7 days before transplantation. The BM-MNCs containing enriched BM-EPCs fraction were then gently detached by trypsinization (0.05% trypsin-EDTA, invitrogen, Breda, the Netherlands) and filtered through a 40 µm strainer (Falcon™, BD Bioscience Europe, Belgium). After washing steps with Ca²⁺/Mg²⁺ free phosphate-buffered solution (PBS, Gibco, Invitrogen, Breda, the Netherlands), the EPC enriched BM-MNCs (5×10⁵) were thoroughly resuspended in 200 µl EBM-2 for transplantation.

The intracoronary administration of BM-MNCs has been described previously by our group [18]. After preparation for intracoronary delivery, the left anterior descending (LAD) coronary artery was permanent ligated by 6-0 silk suture just proximal to the bifurcation of LAD, beneath the left atrial appendage. Extensive MI size was verified by discoloration at the LV free wall and cardiac apex. Thirty to forty minutes post-MI, the aorta and pulmonary artery were clamped, immediately followed by a LV intracavity injection of BM-MNC suspension or equal volume of medium (control group), facilitating intracoronary delivery. By checking blood withdrawal, the possibility of introducing BM-MNCs directly into myocardium was avoided. The aorta and pulmonary artery were kept closed for 10 seconds, and then released to allow normal blood flow recovery. Sham-operated rats underwent the same procedure except for actual coronary artery ligation and received LV cavity injection of the same volume of medium.

Hemodynamic measurement
Four weeks posttransplantation, rats were anesthetized as described above. A microtip pressure transducer (Millar instr. Inc., Houson, Texas, USA) was inserted into the left ventricular cavity via the right carotid artery. After stabilization, heart rate (HR), left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were measured. As indices of contractility and relaxation, the maximal rates of increase and decrease in LVP (dP/dt_max and dP/dt_min) were determined. The catheter was retracted into the aortic arch and arterial systolic and diastolic blood pressure (SBP, DBP) were recorded.

Infarct size
After hemodynamic measurements, hearts were rapidly excised and placed in ice cold potassium chloride (KCl, 2 mol/L). Subsequently, hearts were weighted and mid-papillary slices were fixated in 4% paraformaldehyde and paraffin-embedded for immunohistochemistry. Infarct size was determined by planimetry at mid-ventricular level in
transverse slices on picrosirius red/fast green-stained sections. Infarct size was expressed as the percentage of scar length to total LV circumference, as described previously [3,19].

Myocyte hypertrophy, Capillary Density
In order to visualize individual cardiomyocytes, dewaxed 4-µm thick slices were stained with a Gomori’s silver staining. Digital pictures were taken at 400 magnifications under a microscope [Nikon, Elipse 50i, Japan] with Paxcam digital microscope camera (Pax-it, USA). Cardiomyocyte hypertrophy in the viable LV free wall was measured as the transverse cross-sectional area of myocyte profiles at the level of a nucleus. In a randomly chosen area of the slices (25% of total area) the actual number of myocytes was counted and divided by tissue area, as to present myocyte density. Capillary density was measured in the same area as used for myocyte size, by staining endothelial cells with biotin-labeled Lectin GSL (1:100; Sigma-Aldrich, Zwijndrecht, The Netherlands), using image-pro analysis software (Media Cybernetics, Silver Spring, Maryland, USA) and expressed as number of capillaries per mm². Tissue area was corrected by excluding the interstitial blank space. A diameter criterion of 10 µm was set up to exclude venules and arterioles. Actual neovascularization was determined by the ratio of capillary number to cardiomyocyte number, calculated as capillary density divided by myocyte density [20].

Retrieval and Phenotype Identification of the transplanted cells
The basal parts of the hearts were embedded in OCT compound [Tissue Tek™, Zoeterwoude, The Netherlands] and rapidly frozen in liquid nitrogen. The organ samples were then stored at -80°C until processed. To retrieve the transplanted cells (i.e. hPAP⁺ cells), hPAP staining was performed in cardiac cryosections. The detection of transplanted hPAP⁺ BM-MNC was confirmed by immunohistochemical staining using a rabbit-anti-human PAP antibody [SeroTec, Dusseldorf, Germany]. In order to detect the number of positive cells, at least 10 slices per heart were analyzed for hPAP⁺ cell detection by independent observer who is blinded for experiment design. Immunofluorescent staining for confocal microscopic analysis was performed on 4-µm cryostat sections. For co-staining of hPAP and RECA-1, a rat endothelial specific antigen [a generous gift from Dr.J.L.Hillebrands], primary antibodies were incubated for 1 h followed by development of RECA-1 with tyramide-TRITC labelled goat anti-mouse (Southern Biotechnology, Birmingham, AL) and hPAP with FITC-labeled goat anti-rabbit conjugate (Southern Biotechnology). Sections were mounted with mounting medium for fluorescence with DAPI (vector, Laboratories, Inc. Burlingame, USA). Fluorescence images were obtained using a Leica TCS SP2 three-channel confocal laser scanning microscope, equipped with lasers that provide 488-, 543-, and 633-nm laser lines. Tissue sections were observed using a 20/63 x 0.70 NA oil immersion objective lens and images were obtained at 1024 x 1024 pixel resolution with Leica confocal software, and the number of double stained cells was counted.
Statistical Analysis

All results are expressed by mean±SEM. Statistical significant differences between groups were compared with one-way analysis of variances (ANOVA, SPSS 12.02) followed by post-hoc analysis and corrected by LSD/Dunnett t-test. Differences were considered significant at P< 0.05.

Results

Ang-(1-7) stimulates BM-derived progenitor cells in-vitro via Mas receptor

The effects of Ang-(1-7) in the presence or the absence of Mas receptor antagonist A-779, or angiotensin II receptor antagonist PD123319 on Wistar rat BM-derived MNCs and EPCs were assessed after 7 days of treatment [Figure 1]. Immunofluorescent staining of Dil-Ac-LDL, BS-1 lectin and DAPI revealed markedly stimulative effect of Ang-(1-7) on the cultured BM-MNCs and BM-EPCs, as shown in Figure 1A. The number of BM-MNCs was significantly increased by Ang-(1-7), which was abolished by adding A-779 [Figure 1B]. Ang-(1-7) resulted in a 136% increase in absolute numbers of BM-derived EPC as compared to control. The relative BM-EPC percentage was 45% as compared to 25% in control, which was again completely inhibited by the Mas receptor blocker A-779, but not by PD123319 [Figure 1C and D]. The Matrigel tube formation assay [Figure 1E] shows that Ang-(1-7) (10^-8 M) pretreated cells are still capable of tube formation in vitro, similar to untreated controls.

Confirmation of effects of Ang-(1-7) on the transgenic hPAP^+ BM- MNCs/EPCs in vitro

Using the same treatment protocol, we studied the stimulative effect of Ang-(1-7) on cultured BM-MNCs from R26 hPAP bone marrow. Figure 2A shows representative photomicrographs of hPAP^+ and Dil-Ac-LDL(+) bone marrow derived cells. We observed a similar stimulative effect of Ang-(1-7) on BM-MNCs and EPCs [Figure 2B-C]. Ang-(1-7) significantly increased the number of BM-MNCs and EPCs up to 1.4 folds and 3.5 folds as compared to control, respectively. In addition, Ang-(1-7) treatment profoundly increased BM-EPCs percentage of the total cultured BM-MNCs up to 1.6 folds as compared to control.

General Characteristics of experimental animals

After 7 days of culture, in the presence or absence of Ang-(1-7) treatment, we transplanted the ex vivo-expanded BM-MNCs into the hearts of myocardial infarcted inbred F344 rats. General characteristics of the experimental rats are presented in Table 1. There were no
Figure 1: Effects of Angiotensin-(1-7) on proliferation, differentiation and tube formation after 7 days of culture of BM-MNCs. (A) BM-EPC were defined as DAPI (blue), Dil-Ac-LDL (red) and BS-1 lectin (green) triple-stained BM-MNCs. Left row: controls; right row: Ang-(1-7) (10^8 mol/L) treatment; inset: typical double staining for Dil and lectin; scale bar= 50 µm ; (B-D) effect of 7-day treatment with Ang-(1-7) and specific receptor antagonists on BM-MNC number (B), BM-EPC number (C) and EPC percentage (D) observed per 100x magnification microscopical field (HPF). (E) Matrigel network formation at 24 hours following seeding of cultured mononuclear cells that were pretreated for 7 days with vehicle (control: 0 or 10^-8 M Ang-(1-7). bar=200 µm. Results are mean±SEM of 5 experiments. *: P<0.05 vs. vehicle baseline, student t-test; §: P<0.05 vs. Ang-(1-7) control, Dunnett t-test.
significant differences between the MI groups on infarct size, heart rate, blood pressure and initial body weight. However, cell therapy significantly prevented the loss of body weight and markedly reduced the corrected heart and spleen weight at 4 weeks after MI, as compared to the MI group.

**Intracoronary transplantation of BM-MNCs attenuates HF via improvement of neovascularization in the heart**

As shown in Table 1, the ratio of heart weight to body weight was significantly increased in the MI compared to sham-operated group. Both BM-MNCs and BM-MNCs \( \text{Ang}-(1-7) \) treatment significantly prevented this increase. As total ventricular weight includes right- as well as left ventricular (LV) weight, specific LV hypertrophy was further studied by histology and immunohistochemistry [Figure 3]. Data show that the cross-sectional area of myocytes was markedly increased in MI+medium group as compared to sham [Figure 3A and C]. The transplantation of both treated and untreated BM-MNCs significantly inhibited myocyte

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**Figure 2:** Effect of \( \text{Ang}-(1-7) \) on ex vivo-expanded hPAP+ BM-MNCs and EPC numbers (A,B) and EPC percentage (C) used for cell transplantation. Before cell transplantation, the non-treated (left) or \( \text{Ang}-(1-7) \) pretreated BM-MNCs (right) were identified by hPAP (green) and Dil-Ac-LDL (red) staining, scale bar= 50 µm. Results are mean ± SEM. *: P<0.05 vs. control.
hypertrophy compared to MI, but values were still elevated as compared to sham (P< 0.05 vs. sham). Capillary density was significantly reduced in MI+medium as compared to sham. Transplantation of both BM-MNCs and BM-MNCs$_{Ang-(1-7)}$ normalized this reduced capillary density [Figure 3 B and D].

Table 1: Characteristics of the experimental animals

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<td></td>
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<td>8</td>
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Data were presented by means±SEM; BW indicates body weight; DBP, diastolic blood pressure; LV, left ventricle; N, number of animals; SBP, systolic blood pressure; *: P< 0.05 vs. sham; †: P< 0.05 vs. MI

Figure 3: Effects of intracoronary transplantation of untreated or Ang-(1-7) pretreated BM-MNCs on cardiac remodeling after AMI at 4 weeks posttransplantation
THERAPEUTIC POTENTIAL OF BONE MARROW PROGENITORS

Since capillary density can decrease as a direct consequence of increased myocyte size, actual angiogenesis is represented as increased capillary-to-myocyte ratio [Figure 3E]. This ratio indeed did not change due to MI, indicating hypertrophy-induced decreased capillary density. Transplantation of both treated and untreated BM-MNCs increased this ratio up to 20% as compared to MI (P< 0.01 vs. MI), indicating true angiogenesis. However, no significant therapeutic outcomes in capillary density, myocyte hypertrophy and actual angiogenesis were observed between untreated and Ang-(1-7) pretreated BM-MNCs.

Transplanted BM-MNCs were retrieved and localized in the hearts
Four weeks posttransplantation, transplanted hPAP⁺ cells were detectable in heart tissue. As noted, hPAP⁺ cells were clearly visible as individual cells in the infarcted area, the border zone and the spared myocardium [Figure 4A]. Figure 4B shows that co-staining of hPAP⁺ cells with RECA-1, a rat pan-endothelium monoclonal antigen confirmed the endothelial
phenotype of hPAP-expressing cells in the infarcted zone. The hPAP+/RECA-1+ cells were either individual or in small cellular clusters, but did not appear as vascular structures. The total number of hPAP+ cells per tissue area per heart in BM-MNCs treated rats did not differ from that in BM-MNCsAng-(1-7) rats [Figure 4C]. The transplanted hPAP+ cells were localized in the infarcted area, the infarct border and the spared myocardium, but predominant in or close to the infarct area as compared to the spared myocardium [Figure 4D].

Figure 4: Recruitment and localization of the transplanted BM-MNCs in the hearts. (A) hPAP+ cells (arrows) are localized in infarcted cardiac tissue, border zone and spared heart, magnification: 40x. (B) Confocal microphotographs of transplanted hPAP+ (green) BM cells with and endothelial (RECA1+: red) phenotype. Magnification: 200x. The right lower photo with DAPI staining (blue) to visualize nuclei is the magnified view (630x) of the framed area, arrows show small BM-derived endothelial cell clusters (arrows) co-stained for hPAP/RECA-1. The recruitment of hPAP+ cells presented in entire cardiac tissue (C) and in special areas (D). (Data are mean±SEM). *: P<0,05 vs. spared myocardium; **: P<0,001 vs. spared myocardium; scale bar= 50 µm.
In order to relate the number of hPAP\(^+\) cells to functional changes, we correlated the number of retrieved hPAP\(^+\) cells with LVEDP; a strong inverse correlation was observed between recruitment of hPAP\(^+\) cell in the heart and LVEDP [Figure 5].

**Figure 5:** Correlation between hPAP\(^+\) transplanted BM-MNCs and LV end-diastolic pressure (LVEDP)

**Ex-vivo beneficial effects of Ang-(1-7) on BM-MNCs are not translated in an increased therapeutic potential on cardiac function in vivo**

Hemodynamic data were obtained 4 weeks posttransplantation by a left ventricle pressure transducer catheter. LVSP was decreased in MI compared to sham rats [Figure 6A], while LVEDP in MI rats was significantly elevated compared to sham [Figure 6B]. Notably, both BM-MNCs and BM-MNC\(_{Ang-(1-7)}\) significantly prevented the elevation of LVEDP compared to MI group (both P< 0.01 vs. MI), but not the reduced LVSP [Figure 6A and 6B]. The accelerated rate of myocardial contractility (dP/dt\(_{max}\)) was significantly impaired in all MI groups compared to sham (P< 0.005 vs. Sham). Transplantation of either BM-MNCs or BM-MNC\(_{Ang-(1-7)}\) did not give rise to a significant improvement on contractility rate [Figure 6C]. Although we observed a trend towards increased relaxation rates in the BM-MNCs group, and further pronounced in the BM-MNC\(_{Ang-(1-7)}\), this did not reach statistical significance [Figure 6D].

**Discussion**

Objective of the present study was to evaluate the effects of Ang-(1-7) on BM-MNCs/EPCs *in vitro*, and explore whether these effects could contribute to a better therapeutic effect of cell transplantation *in vivo*. Our main findings were that: 1. Ang-(1-7) through *Mas* receptor
significantly increases BM-MNCs/EPCs’ proliferation and differentiation in vitro, and that these cells are capable of tube formation in vitro. 2. Intracoronary transplantation of untreated or Ang-(1-7) pretreated BM-MNCs markedly attenuates LV remodeling, but only partially improves cardiac function. 3. Pretreatment of healthy BM-MNCs by Ang-(1-7) to enrich the EPC subpopulation in vitro does not further enhance the therapeutic potential on cardiac remodeling and function in vivo. 4. The transplanted BM-MNCs at least partially express an
endothelial marker and are predominantly localized in the infarcted/border zone at 4 weeks posttransplantation. 5. the number of incorporated cells correlates with cardiac function.

Rat myocardial infarction by left anterior coronary ligation has been well established and is shown to provide a relevant model to study post-MI remodeling, including myocardial neovascularization [3,19]. The intracoronary delivery of therapeutic cells has been used in both large animal experiments and human clinical investigations [11,13]. We recently developed an approach to intracoronary transplanted stem/progenitor cells into the rat heart with high cell density and homogenous distribution [18]. In the present study, intracoronary delivery of BM-MNCs differentiated towards EPCs resulted in a substantial neovascularization. It has been postulated that homogeneous distribution of angiogenic factors is crucial to accurately control angiogenesis during vascular development [21]. Therefore, we anticipated that homogeneously targeted cell delivery may lead to the uniform distribution of angiogenic factors emanated from the transplanted cells in the entire heart. Whether this angiogenic effect could be enhanced by pharmacological pretreatment of the cells during culture was addressed in the present study.

Ang-(1-7) increased the total number of surviving cells as well as the percentage of cells that differentiated to EPCs in-vitro. Results show that only inhibition with A-779 led to a significant decrease of the Ang-(1-7) effects on cultured MNCs, whilst Angiotensin II type 2 receptor (AT$_2$R) antagonist PD123319 exerted no significant effect. Therefore, we concluded that the observed effect of Ang-(1-7) on total number of MNCs is mainly involving mechanisms mediated by the Mas receptor. Instead of A-779, similar results were observed when using Mas receptor knock-out BM-MNCs compared with wild-type BM-MNCs after Ang-(1-7) treatments (Qian C. et al. submitted for publication). However, the loss of effect of Ang-(1-7) compared to vehicle in the presence of PD 123319 suggests partial involvement of AT$_3$R as well. Although at the given concentration (10$^{-8}$ mol/L) of Ang-(1-7) an effect through direct binding of Ang-(1-7) to AT$_2$R seems unlikely [22], the involvement of this receptor subtype in Ang-(1-7) effects has been reported repeatedly, amongst others in endothelial cells [23,24], and can hypothetically be explained by AT$_2$R -mediated potentiation of Akt-dependent endothelial nitric oxide synthase (eNOS) activation and nitric oxide production via Mas receptor stimulation [25]. The present data show that A-779 , but not PD 123319, completely block the Ang-(1-7) induced differentiation to EPCs, implicating that also this effect predominantly involves the Mas receptor[25]. When cultured BM-MNCs were reseeded on matrigel, they displayed a pronounced capacity to form tubes. This supports the anticipated capacity of these cells to exert a functional potency for angiogenesis.

Given the fact that a cell population consisting of single type of cells is less efficient in stimulation of neovascularization [26,27], we did not use specifically purified EPC as therapeutic cells for neovascularization but instead enriched the heterogeneous population through ex-vivo culture. By using a conventional EPC culture system at low serum
concentration (2%), the EPC fraction was augmented up to 20-30% of total cultured BM-MNCs. When stimulated by Ang-(1-7), the EPC population was further expanded up to 60% of total attached MNCs. However, despite the in-vitro functional and numeric improvement of BM-MNCs/EPCs, none of the in-vivo effects on remodeling, angiogenesis and function are different between pretreated and untreated cells, suggesting that transplantation of BM-MNCs per se already generated the optimal therapeutic potential.

To avoid the stimulating effects of Ang-(1-7) on autologous BM-MNCs, we pretreated BM-MNCs with Ang-(1-7) in-vitro instead of systemically treating animals with Ang-(1-7). Four weeks post-transplantation, the hPAP* cells (i.e. transplanted cells) were rarely observed in the spared myocardium and predominantly localized in the infarcted zone or peri-infarcted (border) zone. However, at the time of cell transplantation, the arterial bed in the ischemic zone was occluded, suggesting that the cells have not entered these zones by perfusion and subsequent local migration over the coronary artery wall. This implies that the cells have passed the arterial wall in the spared myocardium and subsequently migrated to the ischemic zones. This hypothesis is consistent with the paradigm that the hypoxic gradient regulates progenitor cell trafficking to the skin wound [28]. It is conceivable that a gradually hypoxic gradient exists between the infarcted zone and spared heart. Therefore, the present hypothesis is the most reasonable explanation for the observation that hPAP* cells are predominantly found in the ischemic zones. Importantly, the major part of hPAP* cells expressed in infarcted cardiac tissue displays an endothelial phenotype at 4 weeks post-MI (Figure 4B).

In the present study, the number of hPAP* cells was much too low in comparison with the overt increase of capillary to myocyte ratio. This result supports that cell-based therapy benefits cardiac function through paracrine cytokine effects emanating from the transplanted cells, which might improve autologous angiogenesis or resident stem cell niches in the cardiac tissue [29,30].

Cell therapy decreased LVEDP without significant effects on systolic pressure, suggesting that diastolic rather than systolic function in MI rats is improved. As diastolic function is strongly associated with cardiac perfusion, this finding clearly indicates a functional role for EPC induced neovascularization. Interestingly, the correlation between recruitment of hPAP* cell and LVEDP indicates that survival or maintenance of the transplanted cells in the infarcted cardiac tissue plays a pivotal role on cardiac function salvage. Of note, Sano et al[31].reported that regardless of increasing levels of homing signals, such as hypoxia inducible factor-1 and stromal-derived factor-1, in hypertrophied myocardial tissue the sustained LV pressure overload inhibited activity of those factors and thereby impaired cardiac angiogenesis. Thus, the reduced LVEDP by BM-MNCs therapy might increase autologous cardiac responses to angiogenesis in the spared cardiac tissue. The precise mechanism of these beneficial effects of BM-MNCs on cardiac remodeling remains open to further elucidation.
At 4 weeks posttransplantation, despite apparent ex-vivo effects of Ang-(1-7) on BM-MNCs and EPCs, there were no additional in-vivo consequences on either cardiac angiogenesis or function after cell transplantation when compared to untreated BM-MNCs. Rational explanations are that: 1. the therapeutic potential of untreated BM-MNCs already reaches to the optimal outcome. 2. Ang-(1-7) causes a transient functional improvement on BM-MNCs/EPCs in vitro, which is rapidly eliminated under in-vivo conditions post-transplantation. 3. Most likely, singly targeting one aspect of the function of transplanted BM-EPC is only partly efficient in correcting the deteriorated microenvironment in the failing heart. The presence of (relatively undifferentiated) cells may be the major determinant of therapeutic outcomes; increasing the homing signals together with improving autologous cardiac responses to angiogenesis may lead to further improvement.

To summarize, in our previous study, we showed that intravenous administration of Ang-(1-7) significantly improved cardiac function and rescued endothelial dysfunction after myocardial infarction [3]. In the present study, Ang-(1-7) pretreated BM-MNCs did not add further beneficial effects on cardiac remodeling when compared to non-treated BM-MNCs. Thus, Ang-(1-7) might improve cardiac function by increasing total number or function of endogenous bone marrow cells only in-vivo. The elevated formation of Ang-(1-7) in failing cardiac tissue found in previous study [32], might in fact be the signal to improve regeneration by stimulating endogenous stem/progenitor cells. The results suggest that only targeting the improvement of transplanted cells might be insufficient. The maintenance of the transplanted cells in the cardiac tissue may be the major determinant of therapeutic outcomes; increasing the homing signals together with improving autologous cardiac responses to angiogenesis may lead to further improvement.
CHAPTER 4

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Reference List


