The effect of angiotensin (1-7) on bone marrow stem cells
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Chapter 2

Angiotensin (1-7) stimulates bone marrow-derived progenitor cells \textit{in vitro} and \textit{in vivo}


*Both authors equally contributed to this work
Abstract

**Backgrounds:** Angiotensin (Ang) II is an octapeptide that plays a critical role in cardiac and blood-pressure control. However, also other Ang peptides, particularly Ang-(1-7), have important biological activities. The cardiovascular actions of Ang-(1-7) counteract pathogenic effects of Ang II and attenuate the development of heart failure. Apart from direct effects on cardiovascular tissue, Ang-(1-7) stimulates bone marrow, which harbors cells that might support the therapeutic effect of Ang-(1-7). Therefore, we studied effects of Ang-(1-7) on bone marrow-derived hematopoietic and endothelial progenitor cells.

**Methods and Results:** Ang-(1-7) stimulated the proliferation of human CD34+ and mononuclear cells in vitro. Under in vivo conditions monitoring proliferation and differentiation of human cord blood mononuclear cells in NOD/SCID mice, the number of HLA-I+ and CD34+ cells was increased by 42 fold and 600 fold, respectively. In rodents, Ang-(1-7) stimulated bone marrow-derived mononuclear cells and especially endothelial progenitor cells in vitro and in vivo. Consequently, in a murine infarct model, treatment with the heptapeptide improved cardiac function and elevated the number of c-kit and VEGF-positive cells.

**Conclusions:** this suggests Ang-(1-7) has crucial role in regenerative processes after myocardial infarction in part through progenitor stimulation, thus characterizing it to be a promising new tool in stimulating the cardiovascular regeneration under pathophysiological conditions.

**Nonstandard abbreviations:** Ang, angiotensin; AT1, angiotensin II type I receptor; BFU-E, burst-forming units-erythroid; EPC, endothelial progenitor cells; IMDM, Iscove's modified Dulbecco's medium; MNC, mononuclear cells; MI, myocardial infarction; LVEDP, left ventricular end-diastolic pressure; LAD, left descending coronary artery; HR, heart rate; LVESP, LV end-systolic volume.
Introduction

Among the mediators involved in cardiovascular remodeling, Ang II, the first known acting peptide of the renin-angiotensin system, appears to be one of the most important. Ang II modulates vasomotor tone, cell growth, apoptosis, cell migration and extracellular matrix deposition in many cardiovascular tissues (1, 2). Interestingly, these proliferative effects of Ang II are not restricted to these tissues but have also been found for hematopoietic cell lineages (3-5). Apart from its role in cardiovascular homeostasis, Ang II can be detrimental, and inhibition of its activity is one of the most important treatment modalities against hypertension, heart failure and diabetic nephropathy.

Besides Ang II, other Ang peptides, such as Ang IV [Ang-(3-8)] and Ang-(1-7) also have cardiovascular activities. Interestingly, Ang-(1-7) counteracts the majority of Ang II effects and could therefore be an endogenous antagonist of the detrimental Ang II actions during the manifestation of a variety of diseases (6). Our group recently identified the G protein-coupled receptor Mas encoded by the Mas protooncogene (7) to be associated with the Ang-(1-7) signaling (8). Mice deficient of the Mas protooncogene present beside sustained long-term potentiation in hippocampal neurons and sex-specific alterations in exploratory behavior (9, 10), alterations in heart rate and blood pressure variability (11). Notably, Tallant et al. showed for cardiomyocytes that Ang-(1-7) has growth inhibitory effects that are transmitted by Mas (12).

However, Ang-(1-7) effects are not limited to direct manipulation of cardiovascular tissue. In vivo studies have shown that Ang II and Ang-(1-7) increased hematopoietic recovery after myelosuppression and progenitor engraftment (13, 14). The increases in cell numbers were most profound and long-lasting in the bone marrow, consistent with the observed effects on early progenitors, and exhibited effects on multiple blood cell lineages. In addition to stimulating bone marrow regeneration in vivo, Ang II was also shown to stimulate the proliferation of hematopoietic progenitors in vitro in cells isolated from both mouse bone marrow and human cord blood (13, 15). In this report, we expand these observations to show that Ang-(1-7) stimulates the proliferation and differentiation of CD34+ and mononuclear cells isolated from human cord blood.

It is, however, not known how these stimulatory effects of Ang-(1-7) relate to stimulation of specific subspecies of bone marrow-derived mononuclear and progenitor cells in vivo and which receptors may mediate these effects. Moreover, progenitors that might contribute to cardiovascular regeneration, such as the endothelial progenitor cell, have not been explored. We therefore set also out to study the in vivo effects of Ang-(1-7) on human bone marrow-derived blood cell types and explored those on cardiovascular progenitor cells in animal models for myocardial infarction.
Results

**In-vitro Ang-(1-7) effects on human mononuclear cells**

To show whether the Ang-(1-7) effects on bone marrow regeneration shown in *in vivo* studies were through direct effects on hematopoietic progenitors and the translation of these effects into human cells, we initially conducted studies with human cord blood cells stimulated with Ang-(1-7) as previously described for Ang II. CD34+ cells isolated from human cord blood were expanded *in vitro* in the presence of Ang-(1-7). After the expansion phase, the cells were transferred to semi-solid medium for assessment of colony formation. This experiment showed that Ang-(1-7) increased the number of large colonies (Figure 1a) and burst-forming units-erythroid (BFU-E) (Figure 1b) in these *in vitro* studies.

![Figure 1: Ang-(1-7) treatment on cultured human bone marrow-derived mononuclear cells](image)

Human cord blood cells were processed to isolate CD34+ cells. The CD34+ cells were exposed to Ang-(1-7) in suspension culture and then transferred to semi-solid medium. The number of large colonies (a) and BFU-E formed (b) were assessed at various times after initiation of culture. The bars marked with (*) were significantly increased from control (= 0 mcg/ml Ang-(1-7)).

**In-vivo Ang-(1-7) effects on human mononuclear cells in mice**

To investigate whether these effects of Ang-(1-7) could also be observed under *in vivo* conditions and to identify the cell populations that are stimulated, a study was designed to evaluate human cord blood progenitor engraftment into NOD/SCID mice after application of two different concentrations of Ang-(1-7) by daily s.c. injection. Administration of PBS alone resulted in approximately 0.5% of the bone marrow cells (Figure 2a and Table 1) and 0.3% of the splenocytes (Figure 2b and Table 1) being of human origin. Administration of the lower dose of Ang-(1-7) did not have significant impact on the stimulation and recruitment of human cells in bone marrow and spleen, respectively, whereas administration of the high dose of peptide increased the number of human cells (HLA-I positive cells) in the bone marrow by approximately 42 fold (Figure 2a and Table 1) and in the spleen by approximately 7 fold (Figure 2b and Table 1). CD34+ cells were also identified in the bone marrow and spleen of placebo-treated mice. Both doses of Ang-(1-7) increased CD34+ cells.
in the bone marrow in a dose-dependent manner (20 and 600 fold for low and high dose, respectively) (Table 1). At the high dose of Ang-(1-7), the percentage of splenocytes that expressed human CD34 was increased 6 fold over placebo (Table 1). Differentiated cells of human origin (myelomonocytic [CD15, CD11a, HLA DR] and B cell[CD19, HLA DR] lineage) were found at very low levels in the bone marrow (0.19-0.41%) and spleen (0-0.11%) of vehicle-treated mice (Figure 2 and Table 1). While administration of low-dose Ang-(1-7) did not affect the number of differentiated cells of the hematopoietic human lineage, administration of the higher dose of the heptapeptide highly significantly increased the number of CD19-, CD15-, CD11a-, and HLA DR-positive cells in the bone marrow and spleen of treated animals at day 30 (Figure 2, Table 1).

**Ang-(1-7) stimulates mononuclear cells and endothelial progenitors**

Initial experiments focussed on bone marrow isolated from rats, an animal species for which heart failure models have been well validated, to determine the effects of Ang-(1-7) on rat bone marrow mononuclear cells (MNC) and the receptor involved (e.g. blockade by A779). MNC of Wistar rats were isolated, cultured for 2 days and subsequently treated during 7 additional days of culture with $10^{-11}$ to $10^{-7}$ mol/L Ang-(1-7) in the absence or presence of A779 ($10^{-7}$ mol/L). Ang-(1-7) increased the number of MNC (Figure 3a and b) reaching maximal efficacy at $10^{-9}$ mol/L ($p \leq 0.05$, t-test: control vs. $10^{-9}$ mol/L Ang-(1-7), n=5; Figure 3b), while higher concentrations of Ang-(1-7) did not further sustain the stimulation of the number of MNC. This is consistent with previous findings that Ang-(1-7) mediates its
physiological actions at very low concentrations (16, 17). Importantly, the Mas receptor antagonist A779 (10^{-7} \text{ mol/L}) abolished the effect of Ang-(1-7) (Figure 3b).

Table 1: Percentage of human cells and subpopulations in bone marrow and spleen of NOD/SCID mice, treated for 30 days post injection of CD34+ cells with A: daily injection of PBS s.c.; B: daily injection of 1.08 µg/mouse Ang-(1-7) s.c.; C: daily injection of 10.8 µg/mouse Ang-(1-7) s.c.

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<tr>
<th>Organ</th>
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<th>B</th>
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The broad spectrum of beneficial actions observed with Ang-(1-7) may be due to effects on early progenitor cells. We investigated whether this is restricted to hematopoietic progenitors or also occurs with endothelial progenitor cells (EPC), a cell type that is of particular importance in vessel regeneration (18). Thus, we investigated the number of EPC, cells characterized by triple positive staining for DiI-acLDL uptake, lectin and DAPI, in the MNC fraction we characterized before. At day 9 of culture, Ang-(1-7) did not increase significantly the number of non-EPC (data not shown) but had increased the absolute
number of EPC reaching maximal efficacy at $10^{-8}$ mol/L ($p<0.05$, t-test: control vs. $10^{-8}$ mol/L Ang-(1-7), n=5) (Figure 3c). Notably, A779 (10-7 mol/L), an antagonist of the Mas receptor, abolished this effect of Ang-(1-7) on EPC (Figure 3c). As Ang-(1-7) and its antagonist were influencing both total MNC as well as EPC numbers, the effect specifically on EPC was estimated by correcting for the effect on MNC. Therefore, EPC numbers were expressed as percentage of total MNC. As shown in Figure 3d, there was a significant dose-dependent increase in EPC percentage displaying a pEC50 of -8.8 mol/L and a maximal response at $10^{-7}$ mol/L ($p<0.05$, t-test control vs. Ang-(1-7) $10^{-7}$ mol/L) implicating that the increase in MNC (Figure 3b) is mostly mediated by an increase in EPC. A779 abolished the Ang-(1-7) responses whereas A779 alone did not have any effect (Figure 3d).

![Image of Figure 3](image)

**Figure 3: Ang-(1-7) treatment on cultured, isolated rat bone marrow-derived MNC:** (a) representative pictures of cultured MNC, treated for 7 days with either $10^{-8}$ mol/L Ang-(1-7) or vehicle (control). Arrows point to Dil-AcLDL+/lectin+ EPC. Top pictures: without DAPI nuclear staining; bottom pictures: with DAPI staining. Inset: enlarged EPC picture. (b) Effect of a 7-day Ang-(1-7) treatment on cultured, isolated rat bone marrow-derived MNC numbers per high power field (HPF: 200x) in the presence or absence of Mas receptor antagonist A779. (c) Effect on EPC number per high power field, and (d) relative EPC amounts (as % of total MNC population). * $P<0.05$, Dunnett-test (the group without Ang-(1-7) treatment was taken as control group); n=5.

**Mas deficiency prevents stimulatory properties of Ang-(1-7)**
To finally prove whether Ang-(1-7) requires the Mas receptor to stimulate EPC proliferation, we isolated bone-marrow cells from Mas-deficient mice and their age-matched wild-type controls to test Ang-(1-7) on cultured MNC (Figure 4a). As shown in Figure 4b, Ang-(1-7)
stimulated dose-dependently MNC in wild-type mice as demonstrated for rat MNC before (Figure 3b). This effect was blocked by co-treatment with A779 and was completely abolished in MNC isolated from Mas-deficient bone marrow cells (Figure 4b). In contrast to wild-type EPC, the Mas-deficient EPC fraction also lost the ability to respond on Ang-(1-7) with a stimulation of proliferation (Figure 4c and d).

![Figure 4: Ang-(1-7) treatment on cultured, isolated mouse bone marrow MNC: (a) representative pictures of wild-type (wt) and Mas receptor knockout (Mas-KO) after 7-day treatment without and with 10^{-8} mol/L Ang-(1-7). Arrows point to Dil-AcLDL+/lectin+ EPC. Inset: enlarged EPC picture. (b) Dose-dependent effect of Ang-(1-7) on number of MNC per high powerfield (HPF: 200x). (c) Dose-dependent effect on EPC number. (d) Effect on relative amount of EPC as compared to the entire MNC population. * $P \leq 0.05$, Dunnett t-test (the group without Ang-(1-7) treatment was taken as control group); # $P \leq 0.05$, t-test vs. Ang-(1-7) 10^{-8} mol/L; n=5.

Effects of Ang-(1-7) in vitro on EPC are preserved after myocardial infarction

The stimulatory effects of the heptapeptide on EPC may also implicate regenerative properties of Ang-(1-7) on vascular and cardiac repair under pathophysiological conditions. To test this hypothesis we firstly addressed the question whether Ang-(1-7) could stimulate isolated MNC and EPC from bone marrow of rats with or without heart failure. MNC from infarcted Sprague-Dawley rats 12 weeks after operation and sham-operated animals were isolated and characterized in our in vitro assay. Cardiac function measurements and histological parameters are listed in Figure 5a, showing that 12 weeks after myocardial infarction (MI), rats had heart failure, as most importantly witnessed by elevated left ventricular end-diastolic pressure (LVEDP). Sham-operated rats showed normal cardiac function.
As shown before, Ang-(1-7) significantly stimulated MNC isolated from sham-operated rats (Figure 5b) and A779 fully inhibited this effect, whilst being without effect by itself. In cultured MNC from heart failure rats, Ang-(1-7) still significantly increased the number of bone marrow-derived MNC but not by co-treatment with A779. Notably, the total number of MNC derived from heart failure rats was significantly lower than that cultured from sham-operated animals (Figure 5b). However, the relative increase in cell number promoted by Ang-(1-7) treatment was still comparable (increase by 41% in sham vs. 54% in MI; n.s.). Focussing on the subpopulation of EPC within the MNC, we found numbers significantly increased in bone marrow-derived MNC cultures from both sham and heart failure rats after Ang-(1-7) treatment (Figure 5c). This significant increase was prevented by co-treatment with A779. As for total MNC, the relative effect of Ang-(1-7) on EPC was similar in sham vs. MI rats (96% vs. 128% increase respectively; n.s.).

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<td>471.6 ± 5.8</td>
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<td>Infarct size (%)</td>
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<td>44.3 ± 1.9 *</td>
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<td>HW/BW (mg/g)</td>
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<td>3.0 ± 0.1 *</td>
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<td>LVSP</td>
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<td>LVEDP</td>
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<tr>
<td>dp/dt max</td>
<td>13268 ± 476</td>
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<tr>
<td>dp/dt min</td>
<td>-11472 ± 522</td>
<td>-8180 ± 556 *</td>
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Figure 5: Effects of Ang-(1-7) on cardiac function after induction of myocardial infarction: (a) General characteristics in heart failure rats 12 weeks after coronary ligation; Effect of 7-day Ang-(1-7) treatment $10^{-7}$ mol/L on cultured, isolated rat bone marrow-derived MNC number (b) and EPC number (c) in the presence or absence of A779. *: p≤0.05 vs sham (a: student t-test) or control group (b: Dunnett t-test vs. group without treatment), n=5.
Ang-(1-7) effects on cardiac remodeling after myocardial infarction

We investigated in a former study the impact of the heptapeptide on cardiac outcome in rats with cardiac failure (19) and could identify a significant improvement of a variety of parameters characterizing cardiac function. Since our actual results clearly point to stimulatory properties of Ang-(1-7) on EPC, implicating a potential role of progenitor cells in cardiac repair under pathophysiological conditions, we added a further in vivo experiment. In contrast to Loot et al. (19) we started to deliver the peptide to an earlier time point to infarcted animals to better visualize the potency of Ang-(1-7) in stimulating cardiac repair after induction of MI. Furthermore, as in contrast to rats, there are murine markers available for primitive, regenerative cell populations, and thus we switched to this species for the MI experiments.

Figure 6: Effects of Ang-(1-7) infusion post myocardial infarction in mice: (a) Left ventricular pressure significantly impaired post MI in mice only infused with PBS (MI PBS), while the 18-day treatment with 50ng Ang-(1-7)/mouse/day starting 2 days post MI induction partly reversed this impairment. (b) The number of c-kit+ cells increased by 8 fold in the MI group treated with Ang-(1-7). (c) The number of VEGF+ cells increased due to cardiac infarction but was further 2.5 times more stimulated by Ang-(1-7) treatment post MI. * P≤0.05 vs. sham (student t-test); # P≤0.05 vs. MI PBS (student t-test); n≥5.

Three weeks after induction of myocardial infarction, mice had a significant impairment in cardiac function (Figure 6a). Low-dose treatment with 50 µg/day/mouse of Ang-(1-7) starting two days after induction of myocardial infarction already led to a mild but significant improvement of left ventricular pressure in the infarcted mice (Figure 6a). Ang-(1-7), however, significantly increased the number of c-kit+ cells (a marker for progenitor cells) (Figure 6b), implicating higher cardiac regenerative potential by increasing the number of cardiac stem cells (20) and/or recruiting c-kit positive bone marrow-derived hematopoietic stem/progenitor cells (21), whereby the latter ones are predicted to be the crucial source for cardioprotection by c-kit positive cells (22). Furthermore, VEGF, a marker for angiogenic cells (23), has been stained on significantly more cells in Ang-(1-7)-treated infarcted hearts (Figure 6c). This clearly points to the ability of Ang-(1-7) to stimulate the generation of new capillaries within the remodeling phase and thus demonstrates the impact of the heptapeptide on cardiac regeneration under pathophysiological conditions.
Discussion

The contribution of primitive hematopoietic progenitors to the restoration of hematopoietic lineages after injury has been well documented and utilized clinically for decades. Using hematopoietic chimeras to distinguish the contribution of bone marrow cells to neovascularization, circulating graft cells were shown to incorporate into neovasculature associated with wound healing, myocardial ischemia and corpus luteum formation (24, 25). These observations were the basis for our hypothesis that effects of Ang-(1-7) on bone marrow progenitors contribute to a stimulation of coronary capillaries and cardiac regeneration after myocardial infarction.

As Ang-(1-7) influences hematopoiesis (14) and increases neovascularization in skin wounds (26) and, as shown in the present paper, after cardiac ischemia, it is further hypothesized that this peptide may be a component of the regulatory system for angioblasts. Our results support this hypothesis and show that the EPC fraction of bone marrow-derived MNC is preferentially stimulated. This implies that a new pathway for the beneficial actions of chronic treatment with Ang-(1-7), or stimulators thereof, e.g. angiotensin converting enzyme inhibitors, has now been opened for investigation. We here show that regenerative cells are already stimulated by low-dose Ang-(1-7) to homing into the myocardium 3 weeks after infarction and that this may participate in the improvement of function. Further research on the importance of progenitor stimulation by Ang-(1-7) and its importance for the better cardiac remodeling and function mediated by Ang-(1-7) infusion post MI (19) should be initiated. Importantly, the protective Ang-(1-7) effects are not limited to MI models but can also include therapeutic effects of chronic Ang-(1-7) administration on restenosis (27, 28) and endothelial function (19, 29), considering especially the putative involvement of progenitor cells in endothelial repair (30, 31).

Furthermore, we could demonstrate that the Mas receptor is involved in the stimulation of EPC by Ang-(1-7). As we have shown under other circumstances for Mas/Ang-(1-7) before (8), the peptide effects take place already at low doses of Ang-(1-7), in vitro as well as in vivo. This emphasizes the likelihood that the presently observed Ang-(1-7) effects take part in the physiological function of the heptapeptide and thus not only in the therapeutic potency observed in our previous studies when delivered at pharmacological doses. Strikingly, Ang II, acting at the AT1 receptor, with comparable potency by which Ang-(1-7) involves the Mas receptor, has now been described as a stimulator of blood-derived human EPC proliferation and network formation (32). Although this would be the opposite of the assumed function of Ang-(1-7) to oppose Ang II effects, our data implicate that, at least for the stimulation of hematopoietic cells and endothelial progenitor cells, Ang-(1-7) and Ang II may also mediate similar functions. It may be interesting to investigate whether the blockade of the Ang-(1-7)/Mas axis may inhibit the effects described for Ang II. This could be hypothesized, since angiotensin converting enzyme 2 may rapidly metabolize Ang II to Ang-(1-7) that thus may
mediate indirectly the Ang II effects. On the other hand, we could demonstrate a direct effect of Mas on AT1 signaling (33) that may here cause an equally directed effect of both Ang metabolites.

However, beside these similarities, the actions of Ang-(1-7) and Ang II on MNC are partly distinct from each other. While Ang II suppresses the differentiation of mesenchymal stem cells into adipocytes (5), suggesting that both Ang II and Ang-(1-7) display effects protective to the cardiovascular system, Ang II, given in vivo for two weeks, suppresses further in vitro culture of BM-EPC (34), a finding that seems in contrast with the concomitant increase of c-kit+ and VEGF-expressing cardiac cells observed after a 3-week Ang-(1-7) delivery in the mouse MI model described here. However, for conclusive comparisons predestined studies have to be performed soon.

Our data point to the conclusion that a broad spectrum of beneficial activities observed with Ang-(1-7) may be due to actions on early progenitor cells. Since stem cells derived from adults may be capable of a great deal of versatility or plasticity, transplantation of bone marrow or activation of endogenous bone marrow cells could result in donor/activated cells possessing the added benefit of mediating the healing of injuries to the CNS, muscle, liver, and heart. Therefore, an agent that stimulates the proliferation and differentiation of stem cells has the potential for similar versatility. Given our present results, defining Ang-(1-7)’s versatility with respect to progenitor cell stimulation, it seems worthwhile to explore this heptapeptide as a regenerative agent beyond the scope of bone marrow repopulation and cardiovascular repair.
**Methods**

**Effects of Ang-(1-7) on human mononuclear cells in vitro**

Cord blood was obtained from the Los Angeles County Obstetrics Service immediately after delivery by vacutainers containing 10 mM of EDTA. The red blood cells were lysed by a hypotonic ammonium chloride solution and the nucleated cells were collected by centrifugation. The pellet from this centrifugation was resuspended to $10^7$ cells/ml. Ten µl of an antibody cocktail was added per ml of cells. The antibody cocktail contained antibodies to glycophorin A, CD2, CD3, CD45, CD24, CD19, CD66b, CD14, and CD16 was purchased from Stem Cell Technologies and used according to manufacturer's instructions. After isolation of the CD34$^+$ cells, they were resuspended at $5\times10^4$ cells/ml in serum free StemSpan [Stem Cell Technologies] containing the following human recombinant factors: 3 IU/ml human erythropoietin, 20 ng/ml stem cell factor (SCF), 20 ng/ml interleukin 3 (IL-3) and 20 ng/ml granulocyte-macrophage CSF (GM-CSF). These cells were cultured in 25 cm$^2$ flasks for 6 days at 37°C in a humidified atmosphere of 5% CO$_2$ in air. After 6 days, the cells were washed, counted by hematocytometer and resuspended at $5\times10^5$ cells/ml. One hundred µl aliquots were added to individual cells of a 96-well microtiter plate. These plates were then incubated for 3 days at 37°C in 5% CO$_2$ in air. After this incubation, the cells were washed and placed in semi-solid medium containing 0.9% methyl cellulose in Iscove's modified Dulbecco's medium (IMDM), 30% fetal calf serum (FCS), 1% bovine serum albumin (BSA), 10 µM 2-mercaptoethanol, 2 mM L-glutamine, 10% agar leukocyte conditioned medium with and without 3 IU/ml erythropoietin (Stem Cell Technologies, Vancouver, BC). At various times after initiation of culture, the number and size of colonies as well as the number of BFU-E per well were counted.

**Animal studies**

Wistar (naive) and Sprague-Dawley (sham or MI surgery) rats, Mas knockout mice (9), C57Bl/6, or NOD/SCID mice were used in the experiments. The animals were maintained under standardized conditions with an artificial 12-h dark-light cycle, with free access to food and water. Animals were killed either by cervical dislocation (mice) or exsanguination under O$_2$ – isoflurane anesthesia (rats); tissue samples were collected and immediately snap frozen in liquid nitrogen.

All animal studies were performed according to national guidelines and approved by the institutional animal care committees. This research was in compliance with the Guide for the Care and Use of Laboratory Animals published by the OPRR (Office for Protection against Research Risks) of the US National Institutes of Health, Washington, D.C. (NIH Publication No. 85-23, revised 1985).
CHAPTER 2

Effects of Ang-(1-7) on human mononuclear cells in NOD/SCID mice

Human Cord Blood: Human cord blood cells were harvested from umbilical cords at the time of delivery at the Charité, Campus Benjamin Franklin, Department of Obstetrics and Gynecology and HELIOS Klinikum Berlin-Buch. Umbilical cord blood samples from full-term deliveries were collected by gravity into sterile 50ml tubes containing 5000 I.E. heparin. All collections were performed with informed consent and with approval by the local hospital ethics committee.

Immediately after delivery to the laboratory, mononuclear cells were harvested from the cord blood by density gradient centrifugation. Cord blood mononuclear cells were prepared from 40-50 ml cord blood by density gradient separation using Lymphocytes separation medium (PAA Laboratories, Cölbe, Germany). Cells were centrifuged at 400 x g for 30 minutes at room temperature. The MNCs at the interface were washed with PBS and $5 \times 10^7$ resuspended in 1ml PBS containing 2mM EDTA.

Mice: NOD-SCID mice were used from the breeding stock of Dr. I. Fichtner (MDC, Berlin-Buch, Germany). Breeding pairs of NOD/LtSz-SCID/SCID mice (originally obtained from Dr. Leonard Schultz, Jackson Laboratories, Bar Harbor, Maine, USA) were expanded and maintained under pathogen-free conditions in the animal facility of the Max Delbrück Center of Molecular Medicine (Berlin, Germany). They were fed with a sterilized standard diet purchased from Sniff GmbH (Soest, Germany) and acidified drinking water ad libitum. Mice were irradiated sublethally at 6-8 weeks of age with a dose of 1.6 Gy of a $^{137}$Cs-gamma source before cell transplantation.

Treatment procedure: After isolation of human cord blood mononuclear cells, $1 \times 10^7$ cells were injected intravenously into each mouse. Starting two days after injection, the mice received daily injections s.c. of PBS (Group A), 1.08 µg/d Ang-(1-7) (Group B) or 10.8 µg/d Ang-(1-7) (Group C). At day 30, the mice were euthanized and the spleen and bone marrow harvested to assess the engraftment of human progenitors and mature cells by flow cytometry.

Flow cytometry: Two-colour immunofluorescence cytometry was used to quantify the expression of cell surface molecules on suspended bone marrow and spleen cells. The cells were stained with PE or fluorescein isothiocyanate (FITC) labeled monoclonal antibodies against anti-human: anti-CD34 (clone 581), anti-CD11a (clone G43-25B), anti-CD19 (clone H1 B19), anti-CD 15 (clone H 198), anti-HLA-DR (clone G46-6), anti- HLA-I (clone G46-2.6) from Becton Dickinson. FITC and PE conjugated isotype-matched mouse IgGs (clone X40) from Becton Dickinson were used as controls. After 25 min of incubation in the dark, cells were washed in PBS and analyzed immediately in a dual-laser FACSCalibur (Becton Dickinson, Heidelberg, Germany) flow cytometer with Cell-Quest-Software. Each measurement contained 10,000 events and cell debris was excluded by threshold. Background levels of staining were measured using isotypic controls.
The effect of Ang-(1-7) on mononuclear cells and endothelial progenitor cells from bone marrow of healthy rats and of Mas-deficient and wild-type mice

1. Dose-finding experiments: In order to assess direct effects on bone marrow-derived mononuclear cells under normal physiology and during heart failure, we chose to perform tests in cultured MNC from Wistar rat bone marrow. First, we assessed the Ang-(1-7) concentration needed for stimulation of MNC and the involvement of Mas receptors in the observed effects in bone marrow-derived cells from normal rats. This was done by generating dose-response curves in the presence or absence of Mas receptor antagonist A779. Later (see below), we proceeded with tests on MNC of heart failure rats using a suitable, single concentration of Ang-(1-7).

2. Experiments on cultured bone marrow-derived mononuclear cells
For each test group of rats or mice, bone marrow was isolated immediately after sacrificing from the left and right femurs by flushing bone marrow cavity with sterile phosphate-buffered saline at room temperature (PBS, Gibco, Invitrogen, Breda, The Netherlands). From each individual animal, MNC were obtained by density gradient centrifugation at 2000 rpm (MSE Mistral 3000i, UK) for 20 minutes at room temperature on lympholyte-rat or lympholyte-mouse according to manufacturer’s instruction (Cedarlane Laboratories ltd., Hornby, Canada). Cells from the individual rats were resuspended in endothelial cell basal medium-2 (EGM; Cambrex Bioproducts, Clonetics, New Jersey, USA) containing supplemented 2% fetal bovine serum and EGM-2 SingleQuots (Clonetics). At the first day, 5x10^5 cells/well were seeded in 1% gelatin (225 bloom, Sigma-Aldrich, Zwijndrecht, The Netherlands) precoated 96-well culture plates (Costar, Corning, New York, USA) at 37°C in a humidified incubator in the presence of 5% CO2. At day 3 of culture, medium with non-attached cells was removed, and was replaced by fresh culture medium containing Ang-(1-7) with or without A779 (referred to as ‘treatment medium’). Ang-(1-7) and A779 were purchased from Bachem, Bubendorf, Switzerland. From then, cells were grown until day 9, changing treatment medium at day 5 and day 7. At day 9, treatment medium was removed and culture medium supplemented with 10 μg/mL 1,1’-dioctadecyl-3,3’,3’-tertamethylindocarbocyanine-labeled acetylated low-density lipoprotein (Dil-Ac-LDL; Molecular Probes™, Invitrogen, Oregon, USA) was added to the cells for 4 h. Thereafter, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.5) at 4°C for 10 minutes. Then, cells were stained with BSI-lectin staining (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 4’,6-diamidino-2-phenylindole nuclear staining (DAPI, Molecular Probes, Leiden, The Netherlands).

Detection of the 3 stainings was performed with the use of fluorescence microscopy (Leica, Wetzlar, Germany) at a magnification of 200x. From each well, pictures from high power fields at 5 random locations that formed an imaginary “x” were taken, taking in consideration a comparable distribution of the detection loci between all wells. Cells were identified and counted by computer-assisted, automatic quantification with the use of ImagePro software.
(Media Cybernetics, Silver Spring, USA). In the occasion that the presence of cell clusters was hindering proper digital quantification, cell numbers were assessed through personal observation by a single experimenter who was unaware of the type of treatment. Triple-stained cells were defined as EPC whilst all DAPI-stained mononuclear cells were defined as MNC.

Effects of Ang-(1-7) on infarcted rats and mice

1. Myocardial infarction in rats and hemodynamic characterization

Induction of infarction and sham surgery: Sprague-Dawley rats were purchased (Harlan, Zeist, The Netherlands), and housed group-wise with free access to food and drinking water. Rats weighing 225-250 g were anesthetized with isoflurane (5% in O$_2$), intubated and mechanically ventilated. During surgery animals were kept on a homeothermic blanket. MI was induced by left coronary artery ligation as described previously (19). Briefly, an incision was made in the skin on the fourth intercostal space with the overlying muscles dully separated and kept aside. Thoracotomy was performed by cutting the intercostal muscles. MI was induced by ligation of the proximal portion of the left coronary artery with a 6-0 silk suture beneath the atrial appendage. Subsequently, the thorax was closed and when spontaneous respiration was sufficiently restored the rats were extubated and allowed to recover on a homeothermic blanket. Sham-operated rats underwent identical surgery, but did not undergo an MI.

Hemodynamic measurements: 12 weeks after MI or sham operation, the rats (n=5 per test group) were anesthetized with isoflurane. A microtip pressure transducer (Millar instruments Inc. Houston, Texas USA) was placed in the right external carotid artery to measure arterial blood pressure. The catheter was then advanced into the left ventricle, and intraventricular systolic and end-diastolic pressures, and the maximal rates of pressure increase (dP/dt$_{max}$) and decrease (dP/dt$_{min}$) were recorded. Subsequently, rats were heparinized and the hearts were excised.

Histology: After excision, the hearts were arrested in diastole in a 2 M KCl solution (4°C), the atria and right ventricles were removed, and the left ventricles weighed. A mid-ventricular slice was fixated in 4% paraformaldehyde in PBS. The slices were dehydrated and embedded in paraffin. Transverse sections (4 µm) were cut. To determine infarct size, sections were stained with Sirius red/fast green. Infarct size was expressed as the percentage of scar length of total left ventricular circumference. Saline-treated rats with infarct sizes less than 10% were considered shams.

2. Induction of infarction and sham surgery and hemodynamic characterization in mice

MI was induced by permanent ligation of the left descending coronary artery (LAD), as described by Tschöpe et al. (35). Briefly, mice were anesthetized with thiopental (125 µg/g; ip), intubated and artificially ventilated (200 strokes/min; tidal volume 8 µl/g-body weight;
FiO$_2$: 21%). After thoracotomy, the LAD was occluded. In mice that underwent sham surgery, ligatures were placed beside the LAD.

**Hemodynamic measurements:** The animals were anesthetized with thiopental (125µg/g; ip), intubated and artificially ventilated. In the sets of animals analyzed 48 h post-MI, a 1.4F Millar Tip-pressure catheter (Millar, Houston, TX, USA) was placed in the LV via the apex (open chest) to analyze maximal LV pressure (LVP), dP/dt min and dP/dt max, and heart rate (HR) after thoracotomy (adapted from Tschöpe et al.; 35). In short, in animals analyzed 3 weeks post-MI, a 1.4F micro-conductance catheter (ARIA SPR-719; Millar Instruments Inc., Houston, Texas, USA) was positioned in the LV via the right carotid artery to register mean arterial pressure and LV pressure volume loops. Calibration of the recorded volume signal was obtained by hypertonic saline (10%) wash-in technique. All measurements were performed while ventilation was turned off momentarily. Furthermore, LV end-systolic volume, LVEDV, cardiac output, ejection fraction, and HR were determined by customized software (IOX V 1.5, Emka, Paris, France).

**Statistical analysis**
Data were expressed by mean ± SEM. Statistical analysis was performed either by GLM-repeated measures or by one-way ANOVA followed by post-hoc multicomparisons. Statistical significance was assumed when $P\leq0.05$. 
References List


