Circulating Rather Than Cardiac Angiotensin-(1-7) Stimulates Cardioprotection After Myocardial Infarction

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Background—Angiotensin (Ang)-(1-7) attenuates the development of heart failure. In addition to its local effects on cardiovascular tissue, Ang-(1-7) also stimulates bone marrow, which harbors cells that might complement the therapeutic effect of Ang-(1-7). We studied the effects of Ang-(1-7) either produced locally in the heart or subcutaneously injected during the development of heart failure induced by myocardial infarction (MI) and explored the role of cardiovascular progenitor cells in promoting the effects of this heptapeptide.

Methods and Results—Effects of Ang-(1-7) on bone marrow–derived mononuclear cells in rodents, particularly endothelial progenitor cells, were investigated in vitro and in vivo in rats, in mice deficient for the putative Ang-(1-7) receptor Mas, and in mice overexpressing Ang-(1-7) exclusively in the heart. Three weeks after MI induction through permanent coronary artery occlusion, effects of Ang-(1-7) either produced locally in the heart or injected into the subcutaneous space were investigated. Ang-(1-7) stimulated proliferation of endothelial progenitor cells isolated from sham or infarcted rodents. The stimulation was blunted by A779, a Mas receptor blocker, or by Mas deficiency. Infusion of Ang-(1-7) after MI increased the number of c-kit– and vascular endothelial growth factor–positive cells in infarcted hearts, inhibited cardiac hypertrophy, and improved cardiac function 3 weeks after MI, whereas cardiomyocyte-derived Ang-(1-7) had no effect.

Conclusions—Our data suggest circulating rather than cardiac Ang-(1-7) to be beneficial after MI. This beneficial effect correlates with a stimulation of cardiac progenitor cells in vitro and in vivo. This characterizes the heptapeptide as a promising new tool in stimulating cardiovascular regeneration under pathophysiological conditions. (Circ Heart Fail. 2010;3:286-293.)

Key Words: angiotensin ■ blood cells ■ myocardial infarction ■ revascularization

Angiotensin (Ang)-2 is a potent modulator of blood pressure and fluid balance.1,2 Other peptide products of the renin-angiotensin system, such as Ang IV [Ang-(3–8)] and Ang-(1-7), also have cardiovascular activities.3 Indeed, we have shown that infused Ang-(1-7) attenuates the development of heart failure in a rat model of myocardial infarction (MI).4 We recently identified the G protein–coupled receptor Mas encoded by the Mas proto- oncogene5 to be associated with Ang-(1-7) signaling.6 Mice deficient in the Mas proto-oncogene exhibit sustained long-term potentiation in hippocampal neurons, sex-specific alterations in exploratory behavior,7,8 and alterations in heart rate and blood pressure variability.9 Tallant et al10 also reported that Ang-(1-7) has growth-inhibitory effects transmitted by Mas in cardiomyocytes.

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However, the beneficial effects of Ang-(1-7) may not be limited to direct action of the peptide on cardiovascular tissue. In vivo studies have shown that Ang-(1-7) increased hematopoietic recovery after myelosuppression and progenitor engraftment.11,12 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. However, progenitors that might contribute to cardiovascular regeneration, such as the endothelial progenitor cells (EPCs), have not been explored. Therefore, we studied the in vivo effects of Ang-(1-7) on bone marrow–derived mononuclear cells (MNCs) by focusing on cardiovascular progenitor cells and investigated whether these effects could mediate the cardioprotective effect of the heptapeptide in animal models of MI.

Methods

Animal Studies

Wistar and Sprague-Dawley rats, Mas-knockout mice,7 Ang-(1-7) transgenic (TG) mice specifically overexpressing Ang-(1-7) in cardiomyocytes,13 and their wild-type (WT) C57BL/6 controls were used in experiments. All animals were maintained under standardized conditions with an artificial 12-hour dark/light cycle, with free access to food and water. All animal studies were performed according to Dutch and German 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 Office for Protection against Research Risks of the US National Institutes of Health, Washington, DC (NIH publication No. 85-23, revised 1985).

Effect of Ang-(1-7) on MNCs and EPCs From Bone Marrow of Healthy or Infarcted Rats and of Mas-Deficient and WT Mice

To assess direct effects on bone marrow–derived MNCs under normal physiology and during heart failure, we chose to perform tests in cultured MNCs from Wistar rat bone marrow. First, we assessed the Ang-(1-7) concentration needed for stimulation of MNCs 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 the Mas receptor antagonist A779. Rats or mice were euthanized, and bone marrow was isolated from the femurs by flushing the bone marrow cavity with sterile phosphate-buffered saline (PBS). MNCs were obtained from lymphohematopoietic rat or lymphohematopoietic mouse (Cedarlane Laboratories Ltd, Hornby, Canada) by density gradient centrifugation at 2000 rpm for 20 minutes at room temperature. Then the cells were resuspended in endothelial cell basal medium-2 (EGM; Clonetics, East Rutherford, NJ) supplemented with 2% fetal bovine serum and endothelial cell basal medium-2 SingleQuots (Clonetics). On the first day, 5×10⁶ cells/well were seeded on 1% gelatin-precoated, 96-well culture plates at 37°C in a humidified CO2 incubator. On day 3 of culture, the medium was replaced by fresh medium containing Ang-(1-7) or without A779, and the treatment medium was changed every 2 days. On day 9, the treatment medium was removed, and culture medium was supplemented with 10 μg/mL 1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (Dil-Ac-LDL; Invitrogen, Eugene, Ore) for 4 hours. Then the cells were fixed in 4% paraformaldehyde and stained with bisbenzimide H 33258 (Sigma, St. Louis, Mo). Fluorescein isothiocyanate–tetramethylrhodamine B isothiocyanate–Cy5–conjugated specific secondary antibodies were used. Simultaneously, myocytes were identified by monoclonal mouse anti-sarcomeric actin antibody (Dako, Glostrup, Denmark). Nuclei were recognized by staining with bisbenzimide H 33258 (Sigma, Taufkirchen, Germany).

Immunohistochemical Analysis of c-kit and VEGF Expression in Hearts

LV sections embedded in paraffin were analyzed by immunofluorescence in the peri-infarct area to determine the cells expressing c-kit or vascular endothelial growth factor (VEGF). For this purpose, LV sections were incubated with rabbit polyclonal primary antibodies against c-kit or VEGF (Santa Cruz Biotechnology, Santa Cruz, Calif). Fluorescein isothiocyanate–tetramethylrhodamine B isothio-
cyanate–Cy5–conjugated specific secondary antibodies were used.

Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed with the iCycler (Bio-Rad, Hercules, Calif) to quantify molecular markers of heart failure, B-type natriuretic peptide, and collagen type I specific primers from Tebu-Bio, Peterborough, United Kingdom. Total RNA was isolated from the noninfarcted LV, and cDNA was generated according to the manufacturer’s instruction (Tebu-Bio). Gene expression was normalized in relation to the expression of the endogenous housekeeping gene hypoxanthine phosphoribosyltransferase 1.

Statistical Analysis

Data were expressed as mean±SEM. Statistical analysis was performed by the Student or Dunnnett t test to compare the difference between 2 groups. Kruskal-Wallis 1-way analysis was used to analyze the overall effect among 3 or more groups, which was followed by a Dunnnett post hoc test. Statistical significance was assumed when P<0.05.
Results

Ang-(1-7) Stimulates MNCs and EPCs

Initial experiments focused 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 MNCs. MNCs of Wistar rats were isolated, cultured for 2 days, and subsequently treated during 7 additional days of culture with \(10^{-9}\) to \(10^{-7}\) mol/L Ang-(1-7) in the absence or presence of the Mas receptor blocker A779 (10\(^{-7}\) mol/L). Ang-(1-7) increased the number of MNCs (Figure 1A and 1B), reaching maximal efficacy at \(10^{-9}\) mol/L (Figure 1B), whereas higher concentrations of Ang-(1-7) did not further sustain the stimulation of the number of MNCs. This is consistent with previous findings that Ang-(1-7) mediates its physiological actions at low concentrations\(^{15,16}\). Importantly, the Mas receptor antagonist A779 (10\(^{-7}\) mol/L) abolished the effect of Ang-(1-7) (Figure 1B).

To determine the nature of the progenitor cells stimulated by Ang-(1-7), we investigated the number of EPCs, cells characterized by triple-positive staining for Dil-Ac-LDL uptake, lectin, and 4',6-diamidino-2-phenylindole nuclear staining. At day 9 of culture, Ang-(1-7) did not significantly increase the number of non-EPCs (data not shown) but had increased the absolute number of EPCs, reaching maximal efficacy at \(10^{-8}\) mol/L (Figure 1C). Notably, the Mas antagonist A779 (10\(^{-7}\) mol/L) abolished this effect of Ang-(1-7) on EPCs (Figure 1C). Because Ang-(1-7) and its antagonist influenced both the total MNC and the EPC numbers, the effect specifically on EPCs was estimated by correcting for the effect on MNCs. Therefore, EPC numbers were expressed as the percentage of total MNCs. As shown in Figure 1D, there was a significant dose-dependent increase in EPC percentage, displaying a **** median effective concentration of \(-8.8\) mol/L and a maximal response at \(10^{-7}\) mol/L, implying that the increase in MNCs (Figure 1B) is mostly mediated by an increase in EPCs. A779 abolished the Ang-(1-7) responses, whereas A779 alone did not have any effect (Figure 1D).

Because macrophages can also uptake BSI-lectin and Dil-Ac-LDL, we cultured macrophages with or without Ang-(1-7) treatment for 7 days to exclude the stimulatory effect of Ang-(1-7) on macrophages. Ang-(1-7) failed to prompt macrophage proliferation compared with untreated macrophages (data not shown), confirming that the proliferative effects of Ang-(1-7) are specifically related to EPCs. Furthermore, we could demonstrate that all Dil-Ac-LDL–positive MNCs were also

Figure 1. Effects of Ang-(1-7) treatment on cultured, isolated rat bone marrow–derived MNCs. A, Representative pictures of cultured MNCs treated for 7 days with either 10\(^{-9}\) mol/L Ang-(1-7) or vehicle (control). Arrows point to Dil-Ac-LDL+/lectin+ EPCs. Upper panels: without 4',6-diamidino-2-phenylindole nuclear staining; lower panels: with 4',6-diamidino-2-phenylindole 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: \(\times200\)) in the presence or absence of Mas receptor antagonist A779. C, Effect on EPC number per high-power field. D, Relative EPC amount (as % of total MNC population). \(*P<0.05\), Dunnett t test (the group without Ang-(1-7) treatment was taken as the control group and compared with each concentration of Ang-(1-7) as indicated with brackets); \(n=5\) observations per concentration.
positive for VEGF receptor type 2, a commonly used marker for endothelial cells (data not shown).

Effects of Ang-(1-7) In Vitro on EPCs Are Preserved After MI in Rats
The stimulatory effects of the heptapeptide on EPCs may also implicate regenerative properties of Ang-(1-7) on vascular and cardiac repair under pathophysiological conditions. To test this hypothesis, we tested whether Ang-(1-7) could stimulate isolated MNCs and EPCs from bone marrow of rats with or without heart failure. MNCs from infarcted Sprague-Dawley rats 12 weeks after operation and from sham-operated animals were isolated and characterized by in vitro assay. Cardiac function measurements and histological parameters are listed in Figure 2A, showing that 12 weeks after MI, rats had heart failure, as evidenced by increased LV end-diastolic pressure. Sham-operated rats showed normal cardiac function.

As expected, Ang-(1-7) significantly stimulated MNCs isolated from sham-operated rats (Figure 2B), and A779 fully inhibited this effect while being without effect by itself. In cultured MNCs from rats with heart failure, similar results were obtained. Notably, the total number of MNCs derived from rats with heart failure was significantly lower than that cultured from sham-operated animals (*P<0.001). 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; *P=0.87).

We also found that EPC numbers significantly increased in bone marrow–derived MNC cultures from both sham-treated rats and rats with heart failure after Ang-(1-7) treatment (Figure 2C), and this increase was prevented by cotreatment with A779. Again, as for MNCs, the number of EPCs derived from rats with heart failure was significantly lower than that cultured from sham-operated animals (*P<0.001). As for total MNCs, the relative effect of Ang-(1-7) on EPCs was similar in sham versus MI rats (96% vs 128% increase, respectively; *P=0.64).

Mas Deficiency Prevents Stimulatory Properties of Ang-(1-7)
To determine 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 WT controls to test the effect of Ang-(1-7) on cultured MNCs (Figure 3A). As shown in Figure 3B, Ang-(1-7) dose-dependently stimulated MNCs in WT mice, as previously demonstrated for MNCs from rats (Figure 1B). This effect was blocked by cotreatment with A779 and was completely abolished in MNCs isolated from Mas-deficient bone marrow cells (Figure 3B). In contrast to control animals, the EPC fraction in Mas-deficient mice was not stimulated by Ang-(1-7) treatment (Figure 3C and 3D).

Effects of Elevated Levels of Circulating Ang-(1-7) on Cardiac Remodeling After MI in Mice
In a previous study, we identified that infusion of Ang-(1-7) conferred a significant improvement on cardiac outcome in rats with cardiac failure. Because our current results indicate that Ang-(1-7) stimulates EPCs involving Mas, we tested whether this could be correlated with a role for progenitor cells in cardiac repair under pathophysiological conditions. The availability of Mas-knockout strains and of well-identified markers for primitive, regenerative cell populations and their respective antibodies prompted us to use mice.

Three weeks after induction of MI, infarcted mice with or without Ang-(1-7) treatment had a normal heart rate but lower mean arterial pressure and a significant impairment in cardiac function. However, treatment with 50 µg/mouse per day of Ang-(1-7) beginning 2 days after induction of MI led to a mild but significant improvement of LV function, as shown for LV pressure in the infarcted mice in Figure 4A. Furthermore, there was a significant increase in cells staining for VEGF (a marker of angiogenic cells) in the infarcted
hearts of Ang-(1-7)-treated mice (Figure 4B). Moreover, Ang-(1-7) significantly increased the number of c-kit–positive cells in the heart (a marker for progenitor cells; Figure 4C through 4F), implicating higher cardiac regenerative potential by increasing the number of cardiac stem cells\(^{18}\) and/or recruiting c-kit–positive bone marrow–derived hematopoietic stem/progenitor cells\(^{19}\) (predicted to be the crucial source for cardioprotection by c-kit–positive cells\(^{20}\)). These results suggest that Ang-(1-7) stimulates the generation of new capillaries within the remodeling phase and thus demonstrates the impact of the heptapeptide on cardiac regeneration under pathophysiological conditions.

**Effects of Increased Cardiac Ang-(1-7) on Cardiac Remodeling After MI in Mice**

To better discriminate between cardioprotective properties that may be mediated by either cardiac or circulating Ang-(1-7) on cardiac remodeling after MI, in an independent set of animals, MI was induced in Ang-(1-7) TG mice\(^{13}\) specifically overexpressing Ang-(1-7) in cardiomyocytes and WT mice, which were treated daily with Ang-(1-7) or PBS subcutaneous bolus injection. The TG animals had increased Ang-(1-7) concentrations in the heart and lungs but not in blood. Thus, it is an ideal model to discriminate the effects of cardiac and circulating Ang-(1-7) on cardiac remodeling after MI. Both heart weight and cardiac function were comparable in WT and TG sham-operated mice. MI induced significant LV and right ventricle hypertrophy in both WT mice and Ang-(1-7)–overexpressing mice, whereas exogenously infused Ang-(1-7) prevented MI-induced cardiac hypertrophy (Figure 5A and 5B). In addition, an increase of circulating but not cardiac Ang-(1-7) improved cardiac function, as illustrated for contractility and relaxation (Figure 5C and 5D). These findings provide strong evidence that circulating Ang-(1-7) is more effective than the locally produced peptide in preventing pathophysiological remodeling after MI.

In agreement with hemodynamic parameters, upregulation of cardiac B-type natriuretic peptide and collagen type I observed in infarcted WT mice was almost completely suppressed by infusion with Ang-(1-7) (Figure 5E and 5F). In contrast, overexpression of Ang-(1-7) in cardiomyocytes did not impact collagen type I expression compared with control infarcted mice and had a less pronounced effect on cardiac B-type natriuretic peptide mRNA than shown for circulating Ang-(1-7).

**Discussion**

The contribution of primitive hematopoietic progenitors to the restoration of hematopoietic lineages after injury has been
well documented and used clinically for decades. With the use of hematopoietic chimeras to distinguish the contribution of bone marrow cells to neovascularization, circulating graft cells were shown to incorporate into the neovasculature associated with wound healing, myocardial ischemia, and corpus luteum formation. These observations were the basis for our hypothesis that effects of Ang-(1-7) on bone marrow progenitors could enhance cardiac recovery after MI.

Because Ang-(1-7) influences hematopoiesis, increases neovascularization in skin wounds, and improves cardiac function after MI, it is possible that this peptide is a component of the regulatory system for angioblasts. Our results support this hypothesis and show that the EPC fraction of bone marrow–derived MNCs is preferentially stimulated by Ang-(1-7) treatment. Taken together, our results identify a new pathway to explain the beneficial actions of Ang-(1-7) or of treatments that stimulate its accumulation (eg, angiotensin-converting enzyme inhibitors).

Our results clearly demonstrate that treatment of mice subjected to MI for 3 weeks with Ang-(1-7) causes increased progenitor cell homing to the myocardium and correlates with an improvement of cardiac function. Our study indicates that Ang-(1-7) induces mechanisms that are pivotal for regenerative therapy, and future studies on the signal transduction pathways activated by Ang-(1-7) in progenitor cells are warranted. Importantly, the protective Ang-(1-7) effects do not only apply to MI models, but also might extend to therapeutic effects of Ang-(1-7) infusion on restenosis and endothelial function. It is interesting to note that these may be related to the involvement of progenitor cells in endothelial repair.

Figure 4. Effects of Ang-(1-7) infusion after MI in mice. A, LV pressure is significantly impaired after MI in mice infused with PBS only (MI PBS), whereas the 18-day treatment with 50 μg Ang-(1-7)/mouse per day starting 2 days after MI induction partly reversed this impairment. B, The number of VEGF-positive cells increased because of cardiac infarction but was further 2.5 times more stimulated by Ang-(1-7) treatment after MI. C, The number of c-kit–positive cells increased by 8-fold in the MI group treated with Ang-(1-7). Representative immunofluorescence images of c-kit–positive cells from sham mice (D) and MI mice receiving PBS (E) or Ang-(1-7) (F). *P<0.05 vs sham (Student t test); #P<0.05 vs MI PBS (Student t test); n=7 per group.

Furthermore, we demonstrated that the Mas receptor is involved in the stimulation of EPCs by Ang-(1-7). Because the peptide effects in vitro and in vivo occur at low doses of Ang-(1-7), it is likely that the observed effects are in the physiological range of function of the heptapeptide. 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. However, prolonged exposure of EPCs to Ang II induces AT1 receptor–dependent reactive oxygen species–mediated senescence. This finding is in contrast to the concomitant increase of c-kit–positive and VEGF-expressing cardiac cells observed after a 3-week Ang-(1-7) delivery in the mouse MI model described in this study. These observations point out that Ang-(1-7) and Ang II may mediate similar functions during physiological modulation of hematopoietic cells and EPCs. However, the peptides act differently under pathological conditions, in which Ang-(1-7) in general opposes the detrimental effects of excessive AT1 receptor stimulation. We have previously reported a direct effect of Mas on AT1 signaling that may suggest that the similar acute effects of these 2 Ang peptides may result from convergence at this level; further studies will be needed to establish this in EPCs.
It is interesting that Ang-(1-7) produced directly in the heart did not display the beneficial effects of the systemically delivered peptide on recovery from MI. Our results suggest that in recovery from MI, Ang-(1-7) plays a greater role as a stimulant of bone marrow progenitor cell proliferation than as a protective agent acting directly on cardiomyocytes or as a chemoattractant for progenitor cell homing. This is not in contrast to the recent publication by Mercure et al,13 in which they demonstrated that cardiac overexpression of Ang-(1-7) blunts cardiac hypertrophy induced by Ang II. It is well documented that many signaling pathways are activated under MI conditions in addition to the renin-angiotensin system. Our results identified that cardiomyocyte-restricted overexpression of Ang-(1-7) is insufficient to inhibit MI-induced cardiac hypertrophy, whereas it is effective in countering Ang II-induced cardiac hypertrophy in another experimental approach.

That the effect of the heptapeptide is more related to EPC generation than EPC homing is also supported by the finding that after MI, Ang-(1-7) almost doubled the number of circulating EPCs in our MI model (data not shown). Because 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 central nervous system, 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 the versatility of Ang-(1-7) 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.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Accumulating evidence recently arose that stem and progenitor cells are a linchpin in cardiac regeneration after myocardial infarction. Thus, factors stimulating these cell populations or promoting their homing to injured areas gain increasing attention in cardiovascular research. We identified angiotensin (Ang)-(1-7), a heptapeptide that counteracts detrimental cardiovascular actions of Ang II and has been shown to attenuate the development of heart failure, as a stimulator of mononuclear cells and endothelial progenitor cells in vitro and in vivo. Furthermore, we showed that treatment with this heptapeptide improves cardiac remodeling and function in a murine infarct model and increases the number of c-kit+ and VEGF-positive cells. Importantly, we identified circulating rather than cardiomyocyte-specific overexpressed Ang-(1-7) to stimulate cardioprotective effects after myocardial infarction, and these beneficial effects correlate with a stimulation of cardiac progenitor cells in vitro and in vivo. This characterizes the heptapeptide as a promising new tool in stimulating cardiovascular regeneration under pathophysiological conditions and identifies its mechanisms of action. Further studies are immediately needed to investigate the overall effects of Ang-(1-7) on human progenitor endothelial cells and other bone marrow–derived cell populations. It will be a promising long-term objective to investigate the effects of this heptapeptide on cardiac remodeling after myocardial infarction in human subjects.
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