Apelin Enhances Cardiac Neovascularization After Myocardial Infarction by Recruiting Aplnr+ Circulating Cells

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Rationale: Neovascularization stimulated by local or recruited stem cells after ischemia is a key process that salvages damaged tissue and shows similarities with embryonic vascularization. Apelin receptor (Aplnr) and its endogenous ligand apelin play an important role in cardiovascular development. However, the role of apelin signaling in stem cell recruitment after ischemia is unknown.

Objective: To investigate the role of apelin signaling in recruitment after ischemia.

Methods and Results: Aplnr was specifically expressed in circulating cKit+/Flk1+ cells but not in circulating Sca1+/Flk1+ and Lin+ cells. cKit+/Flk1+/Aplnr+ cells increased significantly early after myocardial ischemia but not after hind limb ischemia, indicative of an important role for apelin/Aplnr in cell recruitment during the nascent biological repair response after myocardial damage. In line with this finding, apelin expression was upregulated in the infarcted myocardium. Injection of apelin into the ischemic myocardium resulted in accelerated and increased recruitment of cKit+/Flk1+/Aplnr+ cells to the heart. Recruited Aplnr+/cKit+/Flk1+ cells promoted neovascularization in the peri-infarct area by paracrine activity rather than active transdifferentiation, resulting into cardioprotection as indicated by diminished scar formation and improved residual cardiac function. Aplnr knockdown in the bone marrow resulted in aggravation of myocardial ischemia–associated damage, which could not be rescued by apelin.

Conclusions: We conclude that apelin functions as a new and potent chemoattractant for circulating cKit+/Flk1+/Aplnr+ cells during early myocardial repair, providing myocardial protection against ischemic damage by improving neovascularization via paracrine action. (Circ Res. 2012;111:585-598.)

Key Words: Apelin □ myocardial infarction □ stem cells □ cardiovascular disease □ angiogenesis

Several studies have reported the existence of circulating progenitor/stem cells originating from the bone marrow (BM). These circulating cells play a central role in postembryonic vasculogenesis, in analogy to angioblasts during embryonic development.1 Several reports have shown that in animal models of ischemia, progenitor/stem cells are mobilized and recruited to sites of ischemic neovascularization, including hind limb ischemia and myocardial infarction (MI), where they enhance the formation of new blood vessels by paracrine release of chemokines and angiogenic factors and differentiation into endothelial cells.2-4 Together with the observation that bone marrow–derived progenitor/stem cell numbers are increased in patients after an acute MI,5,6 this suggests that these cells may play an essential role in the repair response mechanism after ischemia. The therapeutic potential of these cells in tissue preservation and/or regeneration after ischemia has also been demonstrated by several studies in which incorporation of these stem cells into the ischemic heart improved capillary density and residual contractile function.7-9

Additionally, several molecular mediators of the ischemic repair response, including vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stromal cell–derived factor (SDF-1), and angiopoietin-1 (Ang-1), were shown to enhance the recruitment of circulating progenitor/stem cells and neovascularization in ischemic tissue.10-13 and some have already been used in clinical studies.14 Although the effectiveness of stem cell
mobilization shows great promise for therapy, the process has proven to be extremely complex involving several cell subtypes and molecular signals that are not explained by the current identified mediators. Further characterization of the biology of these cells and the molecular mechanism that activates and recruits them is needed to design new therapeutic strategies.

Recently, we performed a genome-wide expression profile study in order to identify new genes involved in the regulation of vasculogenesis. Gene expression profiles of isolated angioblasts during early murine development were compared with the nonangioblast cells. Apelin receptor (Aplnr) was specifically expressed in angioblasts in the early phase of embryogenesis, which suggests a potential regulatory role in vasculogenesis in development. Apelin, a member of the G-protein-coupled receptor family, and apelin, its natural ligand, are highly expressed in the cardiovascular system during development and adulthood. Loss of apelin or Aplnr in zebrafish and Xenopus larvae resulted in defective intersomitic vessel formation and cardiac development during embryogenesis.

Apelin signaling has been shown to be involved in the regulation of blood pressure, cardiac contractility, and cardiac differentiation. In addition, apelin/Aplnr signaling is associated with retinal blood vessel formation and tumor angiogenesis. The marked upregulation of both apelin and Aplnr after hypoxic insult in adult animals also suggest a role in ischemia-driven vasculogenesis.

Beneficial effects of apelin administration after MI have been reported previously, but this was performed in ischemia-reperfusion models and attributed to the direct cardioprotective actions involving the RISK pathway. A direct positive inotropic effect after MI has also been reported.

In the present study, we sought to test the hypothesis that apelin and Aplnr are important regulators in the early mobilization and recruitment of cKit+/Flk1+ cells, specifically after MI. For this purpose, we used a permanent ligation model to exclude an effect of apelin on ischemia-reperfusion damage. In the first days after MI, apelin was shown to enhance the recruitment of specifically cKit+/Flk1+/Aplnr+ cells into the heart after MI. Apelin-recruited Aplnr+ cells were shown to improve myocardial neovascularization by a paracrine function and not by transdifferentiation. The presence of recruited cKit+/Flk1+/Aplnr+ cells after MI promoted a local proangiogenic cytokine profile, which resulted in neovascularization, less adverse remodeling, and subsequent preservation of cardiac function after MI.

**Methods**

This study was performed in accordance with the Council of Europe Convention (ETS123)/Directive (86/609/EEC) for the protection of vertebrate animals used for experimental and other scientific purposes and with the approval of the National and local Animal Care Committee.

**Animals**

Eight-week-old female C57BL/6-Tg(CAG-EGFP)1Osb/J mice (Jackson laboratories, Bar Harbor, ME). These mice express EGFP under the control of a chicken β-actin promoter and cytomegalovirus enhancer in all cells. Wild-type female C57BL/6J mice (age, 8 weeks) were obtained from Jackson laboratories and randomly assigned to the experimental groups.

**In Vitro Viral Transduction of Donor Bone Marrow Cells**

C57BL/6-Tg(CAG-EGFP)1Osb/J donors were euthanized by cervical dislocation and tibias and femurs were harvested and flushed with PBS to retrieve bone marrow. Single cell suspensions were transduced with sh.Aplnr or sh.sham (moi=15). After 48 hours of incubation, Aplnr protein and mRNA expression were verified by quantitative PCR and Western blot analysis as described below.

**Bone Marrow Transplantation**

C57BL/6 recipients were irradiated (2×4.5 Gy) and injected intravenously with 2.5×10⁶ bone marrow cells, transduced with either sh.Aplnr, sh.sham, or EGFP as described.

The recipient mice were allowed to recover for 4 weeks under sterile conditions. The efficiency of BM repopulation was verified by flow cytometric analysis (BD FACSCanto, BD Biosciences, Erembodegem, Belgium) of EGFP expression in circulating and BM aspirate cells. BM functionality after transplantation was assessed using a Hemavet Mascot Multispecies Hematology System Counter 1500R (CDC Technology, Oxford, United Kingdom).

**Surgical Procedures**

Mice (age, 12 weeks) were weighed, sedated with 4% isoflurane, intubated, and pressure-control ventilated with O₂-N₂ (1:2, vol/vol) containing ~2.5% isoflurane for anesthesia. MI was induced by permanent ligation of the left anterior descending coronary artery, as described before, with a 7-0 silk suture (BBraun, Aesculap AG&CO.KG, Tuttingen, Germany). Hind limb ischemia (HLI) was induced by a permanent ligation of the femoral artery with a 6-0 silk suture (BBraun). Sham animals were subjected to the operation without arterial ligation. Every day for 5 days, animals were euthanized and tissues were harvested for subsequent analysis.

The mice were injected in the ischemic gastrocnemius muscle or myocardium with either 8 ng apelin-13 (Sigma) in 10 μL saline, or...
with saline only. Every day for 5 days, animals were euthanized and tissues were harvested for analysis.

In a different set of animals, we intravenously injected VEGFA, SDFα, apelin (all with a concentration of 8 ng/10 μL), or saline to study the effect on mobilization between the subsets. To further assess the function of apelin/Aplnr signaling after myocardial ischemia, bone marrow from C57BL/6-Tg(CAG-EGFP)1Osb/J was harvested and infected by sh.sham or sh.Aplnr, as described above and subsequently transplanted into irradiated C57BL/6J wt mice. After the induction of MI, transplanted animals received either an intramyocardial injection of 8 ng apelin in 10 μL saline, saline, or no injection. Animals were euthanized at 2, 6, or 14 days after MI. At 2 days, the animals were euthanized and the recruitment of cKit+/Flk1+ cells into heart was assessed by flow cytometry. At 5 days, the local production of angiogenic cytokines in the infarcted hearts was assessed. Other animals were used to isolated cKit+/Flk1+/Aplnr+ cells from the heart to study if these cells were producing angiogenic growth factors. At 14 days MI, hemodynamic measurements were performed under general anesthesia, as described before.19 In brief, short- and long-axis view M-mode LV echocardiography (ALOKA, Prosound SSD-4000: Tokyo, Japan) was performed by a single blinded observer. From these images left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameter were quantified, and fractional shortening calculated (FS=LVEDD–LVESD/ LVEDD×100%) by 2 independent blinded observers. In a subset of animals a 1.4F micropipetted pressure transducer catheter (Millar Instruments, Houston, TX) was inserted into the LV. All data were recorded, digitized, and analyzed with a software program written in MatLab for determination of maximum rate of rise of LV pressure (LVdP/dt max) and maximum rate of fall of LV pressure (LVdP/dt min).

Left ventricular weight including septum and lung fluid weight were determined as surrogate markers for LV hypertrophy and manifest heart failure. Tibia length was used as a measure of age/growth of the mouse.

Flow Cytometric Analysis
Mononuclear cell suspensions of BM and blood were obtained by ficoll density centrifugation and subsequent washing in RPMI-medium. Single cell suspensions of ventricles and gastrocnemic muscles were isolated by homogenization, washing, and filtration over a 40-μm Falcon cell strainer. Isolated cells were incubated with antibodies against Flk1 (Biotin-conjugated, BD Biosciences), cKit (APC-Cy7-conjugated, eBiosciences, Hatfield, United Kingdom), Sca1 (APC-conjugated, eBiosciences), Ter119, B220, CD11b, Gr-1, CD3, CD4, CD8 (PE-conjugated, eBiosciences), and Aplnr (LifeSpan Biosciences, Seattle, WA) for 15 minutes at 4°C. After washing, cells were incubated with goat anti-rabbit antibodies labeled with Alexa 488 (Biorad, Hercules, CA) for 15 minutes at 4°C. After washing, cells were incubated with goat anti-rabbit antibodies labeled with Alexa 488 (Biorad, Hercules, CA) for 15 minutes at 4°C.

Results
Apelin Expression on Circulating Cells and Apelin Expression After Ischemia
To investigate the role of Aplnr and apelin in cell recruitment, Apelin expression was assessed in different cell populations from the circulation by flow cytometry and quantitative PCR. Apelin was highly expressed in all cKit+/Flk1+ circulating cells, showing a 9-fold higher mRNA expression and a 15-fold higher protein expression of Apelin when compared with other circulating cell populations (Figure 1A through 1C). Serum levels of apelin were increased during the first 2 days after MI and gradually lowered during the following days. However, apelin levels failed to rise after HLI (Figure 1H). Myocardial apelin mRNA and protein expression were upregulated in the early days after the induction of MI, as compared with sham-operated animals (Figure 1D and 1F). In contrast, no increase of apelin mRNA or protein was noted in ischemic skeletal muscle after HLI (Figure 1E and 1G). These data suggest a possible role for Apelin and apelin in the recruitment of the cKit+/Flk1+ cells specifically after MI.

Cell Recruitment After Ischemia
To test if cKit+/Flk1+ cells were indeed specifically recruited to the infarcted heart, cell subsets were assessed by flow cytometry 3 days after the induction of MI and HLI. After HLI, Sca1+/Flk1+ cells but not cKit+/Flk1+ cells were increased 1.9-fold in the skeletal muscle and 1.7-fold in the circulation, when compared with control and sham-operated animals (Figure 2D and 2F). However, this subset was not recruited to the myocardium and/or the circulation after myocardial ischemia (Figure 2I and 2K). The cKit+/Flk1+ cells showed opposite effects. After MI, cKit+/Flk1+ cells but not Sca1+/Flk1+ cells were increased by 2.1-fold in the myocardium as well as in the circulation compared with sham-operated animals and control animals (Figure 2D and 2E) but failed to respond to HLI (Figure 2F). As Apelin was predominantly expressed in the cKit+/Flk1+ cell population, these findings point to a possible involvement of Aplnr/apelin signaling in this myocardial-specific repair response after ischemia. Interestingly, these cells were preferentially recruited to the borderzone of the infarct and not to other regions of the heart (Online Figure I).

Apelin Promotes Mobilization and Recruitment of cKit+/Flk1+ Cells
To investigate if apelin is capable of mobilizing and recruiting cKit+/Flk1+ cells, C57BL/6J mice received a single...
injection of apelin or saline in the ischemic myocardium or gastrocnemic muscle. During the following days, animals were euthanized and blood and tissues were analyzed for recruited cKit+/H11001+/Flk1+/H11001+ cells using flow cytometry. Apelin injection after MI resulted in an accelerated mobilization of cKit+/Flk1+ cells into the circulation, as compared with saline-injected or noninjected animals (Figure 3A). After apelin injection, cKit+/Flk1+ cell numbers were already enhanced from day 1 on and the peak values were reached at day 2, whereas in saline-injected or noninjected animals a

Figure 1. Aplnr expression on circulating cells and apelin expression after ischemia. A, Aplnr mRNA expression in circulating cell populations (n=6 per group). B, Aplnr protein expression on circulating cell populations, expressed as mean fluorescent intensity (MFI) obtained by flow cytometry (n=4 per group). C, Representative flow cytometry data of Aplnr expression on circulating cells. Red colored dots represent cKit+/Flk1+ cells and black dots represent other circulating cells. The blue line indicates the maximum signal of unstained cells. D, Apelin mRNA was increased in the heart 2 days after MI in wild-type mice (n=6 per group). E, Apelin mRNA levels in striated muscle were not upregulated 2 days after HLI (n=6 per group). F, Two days after MI, apelin protein expression in the heart increased (n=6 per group). G, Apelin protein was not upregulated in skeletal muscle 2 days after HLI (n=6 per group). H, Circulating levels of apelin protein increased after MI but not after HLI (n=6 per group). *P<0.05 compared with sham-operated animals.
Figure 2. Specific cKit+/Flk1+ cell recruitment and mobilization after myocardial and hind limb ischemia. A, B, and C, Representative flow cytometry data of blood (A), myocardium (B), and striated muscle (C). cKit+/Flk1+ cells were mobilized into blood and recruited to the myocardium 3 days after MI. D, E, and F, Quantification of cKit+/Flk1+ cells in blood (D), myocardium (E), and striated muscle (F) (n=8 per group). G, H, and I, Representative flow cytometry data of blood (G), heart (H), and striated muscle (I) for Sca1+/Flk-1 cells. Sca1+/Flk1+ cell numbers were specifically increased in the circulation and striated muscle after hind limb ischemia. J, K, and L, Quantification of Sca1+/Flk1+ cells in blood (J), myocardium (K), and striated muscle (L) (n=8 per group). *P<0.05 versus naive animals.
similar peak level was reached after 3 days. After apelin injection, an increase of 54% in mobilized cKit+/Flk1+ cells in the circulation was noted at day 2 when compared with saline-injected or noninjected animals (Figure 3B and 3E). Intramyocardial injections of apelin increased infiltrating cKit+/Flk1+ cells by 52% when compared with saline-injected animals (Figure 3C and 3F). Likewise, cKit+/Flk1+ cells in gastrocnemius muscle increased after local apelin injection, although this was not statistically significant ($P=0.071$) when compared with controls (Figure 3D and 3G). Interestingly, Sca+/Flk1+ cells seem not responsive to apelin, whereas VEGFA and SDFα were able to mobilize these cells to the circulation (Online Figure II). However, whereas apelin could induce a mobilization response of the cKit+/Flk1+ cells, VEGFA and SDFα seem to have no effect on the mobilization of these cells. These data suggest that apelin functions in addition to VEGFA and SDFα by selectively promoting mobilization of cKit+/Flk1+ cells but...
not the Sca1+/Flk1+ cells to the circulation and promotes recruitment into ischemic tissue.

**Apelin Recruits cKit+/Flk1+/Aplnr+ Cells Into the Ischemic Heart After MI**

To assess the effect of apelin-recruited cKit+/Flk1+ cells to the ischemic myocardium, unfractionated BM was harvested from C57BL/6-Tg(CAG-EGFP)1Osbi/J mice. This BM, which ubiquitously expresses EGFP, was infected with a lentivirus encoding a shRNA targeting Aplnr (sh.Aplnr) or sham lentivirus, encoding nontargeting scrambled shRNA (sh.sham). Infected BM cells were subsequently transplanted into irradiated C57BL/6J wild-type recipients. Aplnr mRNA expression was decreased by 73% and protein expression was decreased by 71% in BM cells treated with sh.Aplnr, compared with controls (Figure 4A and 4B). After a repopulation period of 8 weeks, MI was induced and subsequently the animals either received an intramyocardial injection with apelin or saline into the infarcted heart, or no injection was given. To assess the effect of the Aplnr knockdown on the recruitment of cKit+/Flk1+ cells, animals were euthanized 2 days after the induction of MI. In sham-silenced animals, apelin injection resulted in an increase of cKit+/Flk1+ cell numbers (Figure 4C). Sca1+/Flk1+ and Lin+ cell numbers did not change after apelin injection in sham-silenced animals (Figure 4G and 4H). Aplnr knockdown in BM cells resulted in a failure to recruit cKit+/Flk1+ cells into the myocardium after MI (Figure 4D). Sca1+/Flk1+ and Lin+ cell numbers did not differ between sh.sham- and sh.Aplnr-treated animals (Figure 4G and 4H), showing that endogenous apelin does not recruit these cells into the myocardium after a MI. Accordingly, in these animals, apelin injection with Aplnr-silenced BM failed to induce recruitment of cKit+/Flk1+ cells into the ischemic heart (Figure 4D).

**Apelin Recruitment of Aplnr+ Cells Preserves Left Ventricular Function After MI**

Next, we assessed the effects of recruitment via Aplnr/apelin signaling after MI. As Aplnr has been shown to affect heart function via direct stimulation of cardiomyocytes and local ECs, we proceeded with the BM transplantation setup to exclude possible confounding effects. Four weeks after transplantation, BM transplant efficiency and functionality were assessed. Both transplantation groups showed that more than 99% of cells from the BM and around 97% of the circulating cells were green fluorescent protein–positive (Online Figure III). The numbers WBCs, lymphocytes, neutrophils, and monocytes proved also to be not different between the 2 groups (Online Figure III). To assess the effect of recruited cKit+/Flk1+/Aplnr+ cells on myocardial function after an infarct, another 78 BM hybrid animals were subjected to an MI and subsequently received an intramyocardial injection of either apelin or saline, or no injection was given. One day after induction, 36 animals were euthanized to assess infarct size using Evans blue dye. Initial infarct size was not different between all experimental groups (Online Figure IV).

At 2 weeks after MI, left ventricular remodeling and function were measured by echocardiography, including left ventricular end-diastolic diameter (EDD) and fractional shortening (FS). All animals showed an expected decrease in FS on comparison with historical sham-operated control animals (39±2%). In sham-silenced animals, apelin injection resulted in a 48% improvement in FS, compared with saline-treated mice or control mice (Figure 5A and 5D), whereas EDD was similar between these groups (Figure 5C).

Aplnr-knockdown in the BM resulted in a significant reduction of FS by -41%, compared with their sham-silenced controls. In Aplnr-silenced animals, apelin failed to improve FS compared with saline-injected animals and noninjected animals (Figure 5B and 5D), providing clear evidence that the improved cardiac function can fully attributed to the recruited BM derived cells and not to confounding effects of Aplnr activation in cardiomyocytes and resident endothelial cells (ECs). In addition, in sham-silenced mice, left ventricular weight and lung fluid accumulation was reduced in apelin-treated animals compared with control animals (Online Table I), suggesting an attenuation of myocardial hypertrophy with less pulmonary congestion. In contrast, Aplnr-knockdown animals without apelin treatment showed a trend toward an increase of left ventricular weight and lung fluid compared with their sham virus counterparts. Apelin injection failed to improve left ventricular and lung fluid weights in the Aplnr-silenced animals (Online Table I).

A subset of animals was used to measure hemodynamic parameters, to assess cardiac function. In sham-silenced animals, apelin injection resulted in an significant increase of maximum rate of rise of LV pressure (LVDp/dtmax) and maximum rate of fall of LV pressure (LVDp/dtmin) compared with saline-treated mice (6596±367 mm Hg · s⁻¹ versus 5654±265 mm Hg · s⁻¹ and -6012±527 mm Hg · s⁻¹ versus -5049±375 mm Hg · s⁻¹; n=6 per group; P<0.05). Aplnr-knockdown in the BM resulted in a significant reduction of LVDp/dtmax and LVDp/dtmin (4792±466 mm Hg · s⁻¹ and -3898±515 mm Hg · s⁻¹; n=6 per group; P<0.05) as compared with saline-treated sh.sham animals. Apelin injection failed to improve these functional parameters (4920±247 mm Hg · s⁻¹ and -3765±539 mm Hg · s⁻¹; n=6 per group; P>0.05).

Taken together, these findings indicate that the contractile function after MI was improved by a repair mechanism initiated by apelin, by recruitment of cKit+/Flk1+/Aplnr+ cells from the BM and circulation.

**Aplnr+ Cells Diminish Scar Formation After Myocardial Ischemia**

The Aplnr-silenced BM recipients and control groups were further assessed on parameters of myocardial repair. Progressive left ventricular dysfunction after MI is caused by a combination of left ventricular dilation, compensatory cardiomyocyte hypertrophy, and scar formation. LV dilation was not attenuated by recruitment of cKit+/Flk1+/Aplnr+ cells after apelin treatment (Figure 5C). Myocyte cross-sectional area, as an indicator of cardiac hypertrophy, did not differ among the different groups at 14 days after MI (Figure 6E). However, apelin recruitment of Aplnr+ cells resulted in a reduction in epicardial infarct length of 22% and an increase of infarct thickness of 47% when compared with sh.sham virus-treated controls (Figure 6C and 6D). Silencing of endogenous Aplnr in the BM resulted in more
Figure 4. Apelin is important in the recruitment of cKit+/Flk1+/Aplnr+ cells. A, Aplnr mRNA expression was reduced in BM after sh-Aplnr infection (n=4 per group). B, Aplnr protein expression in BM was downregulated after sh-Aplnr infection. C and D, Representative flow cytometry data of heart from a sham (C) and an Aplnr-silenced animal (D), 2 days after MI. C, Apelin enhanced the recruitment of cKit+/Flk1+ cells after MI in sham-silenced animals. D, Aplnr-silenced animals failed to promote recruitment of cKit+/Flk1+ cells after MI or apelin treatment. E and F, Representative flow cytometric analysis of myocardium for Sca+/Flk1+ (E) cells and Lin+ cells (F). Apelin treatment or Aplnr silencing did not change Sca+/Flk1+ and Lin+ cells recruitment. G and H, Quantification of Sca+/Flk1+ (G) and Lin+ cells (H) in the myocardium (n=8 per group). *P<0.05 versus control.
extensive scar formation than their sham-silenced counterparts, suggesting that endogenous apelin and Aplnr also contributed to myocardial repair under normal conditions. Concordantly, in Aplnr knockdown animals, apelin treatment did not change epicardial infarct length or infarct thickness (Figure 6C and 6D), indicating that the remodeling effects of apelin requires Aplnr BM cells. Infarct measurements using Evans blue dye supported these findings. Apelin recruitment of Aplnr+ cells resulted in a reduction in infarct area compared with sh.sham virus–treated controls (Figure 6F). In contrast, animals in which Aplnr was silenced in the BM proved to have an increase of infarct area as compared with their sham-silenced counterparts (Figure 6F).

Taken together, these findings suggest that apelin-recruited Aplnr+ cells diminish myocardial scar formation early after MI, subsequently resulting in a persistent improvement of myocardial function.

**Apelin-Recruited Aplnr+ Cells Increase Neovascularization After Myocardial Ischemia**

Apelin recruitment of cKit+/Flk1+/Aplnr+ cells increased capillary density in the infarct borderzone by 21%, compared with saline-treated animals in the sh.sham virus-treated mice (Figure 7A through 7C). In contrast, capillary densities in Aplnr-silenced groups were reduced by 9%, compared with the sham-silenced groups. Again, apelin treatment did not alter myocardial capillary density in Aplnr-silenced animals (Figure 7C). No effect on arteriolar density was observed between the experimental groups (Online Figure V).

**Aplnr+ Cells Release Proangiogenic Cytokines**

Although transgenic EGFP+ BM-derived cells were abundant in the peri-infarct zone after MI in the control groups with and without apelin treatment, immunohistological analysis using CD31 to identify the vasculature showed negligible numbers of EGFP+ incorporated in the neocapillaries (data not shown). We further hypothesized that recruited cKit+/Flk1+ cells may have a paracrine proangiogenic effect, rather than provide cardioprotection by active transdifferentiation into neovasculature. Therefore, the cytokine profile of heart samples were assed 6 days after MI. Treatment with apelin resulted in an upregulation of VEGFA protein expression compared with saline-treated animals (Figure 8A). In line with these observations, VEGFA in Aplnr-silenced recipients of Aplnr-silenced BM was significantly downregulated compared with sham-silenced animals (Figure 8A), indicating that recruitment of BM-derived cells by Aplnr/apelin interaction was
responsible for the observed increase in local VEGFA levels. In addition, angiopoietin-2 (Ang-2) levels relative to Ang-1 levels (Ang-2/Ang-1 ratio) in apelin-treated animals were increased compared with the sh.sham-treated animals (Figure 8B through 8D). Myocardial Ang-2/Ang-1 ratio was markedly decreased in Aplnr-silenced animals (Figure 8B through 8D), suggestive of an antiangiogenic milieu. Aplnr-silenced animals were not responsive to apelin treatment and did not show a change in protein levels of any of these angiotrophic cytokines.

To assess if cKit+/Flk1+/Aplnr+ cells were indeed capable of producing these growth factors, sham-operated and MI animals were euthanized 5 days after surgery, and these cells were isolated from the heart using a flow cytometric cell sorter. Analysis of the mRNA expression showed that after MI these cells enhanced the expression of VEGFA and Ang-2, as compared with sham-operated animals (Figure 8F and 8G). Ang-1 expression in these cells was diminished after MI as compared with sham-operated animals (Figure 8E). Moreover, mRNA expression levels of other known angiogenic and cardioprotective factors were also enhanced after MI (Online Figure VI). Interestingly, when cells isolated after MI were added to human umbilical vein endothelial cells (HUVECs) in an in vitro tube formation assay, an improvement in tube formation was observed (Online Figure VII). Taken together, these data point toward a role for Aplnr/apelin recruited BM-derived cells in providing a proangiogenic environment in affected heart areas after MI.

Discussion

In the current study, we have demonstrated that apelin-recruited Aplnr+ cells improve cardiac neovascularization and improve remodeling and cardiac function after MI. Apelin was specifically upregulated in the myocardium and circulation during the first days after MI but not after HLI, resulting in the recruitment of cKit+/Flk1+/Aplnr+ cells to the circulation and the ischemic heart. Apelin induced recruit-
ment of this specific subpopulation resulted in an increase in proangiogenic factors at 6 days after MI and subsequently the capillary density, resulting in diminished cardiac scar formation with sustained cardiac function. Silencing of Aplnr in the BM abolished the recruitment of this population after MI, leading to a failure to mount a neovasculogenesis response, resulting in marked loss of cardiac function and enhanced scarring. These data demonstrate the important role of apelin and Aplnr+ cells in the orchestration of the myocardium-specific ischemic repair response.

Given the marked expression of Aplnr and its ligand apelin in angioblasts during mammalian embryonic development and the expression of apelin after hypoxia, we hypothesized that Aplnr/apelin signaling plays a central role in ischemia-driven neovascularization. Aplnr is highly expressed in the circulating cKit+/Flk1+ subpopulation. The ligand apelin was significantly upregulated particularly early after MI.36 Interestingly, this was not observed after HLI, suggesting that Aplnr/apelin signaling plays a specific role in a myocardium repair response.

Also cKit+/Flk1+/Aplnr+ cell numbers were increased in the circulation in response to myocardial ischemia but not in response to hind limb ischemia. In contrast, Sca1+/Flk1+ cells were recruited after HLI but failed to be recruited after MI. These data suggest a specific cell response for MI versus HLI. After a single intramyocardial injection, apelin induced an accelerated mobilization and homing of the cKit+/Flk1+/Aplnr+ cells not only into the myocardium but also into striated muscles after a local injection of apelin into the ischemic hind limb. In contrast, cells with a lower Aplnr expression, like the Sca1+/Flk1+ and Lin+ cells, failed to respond to apelin. These cells, however, showed a response to an injection of known chemotactic factors VEGFA or SDFα, whereas this was not the case for cKit+/Flk1+ cells, suggesting separate but additive recruitment pathways. These data further underline the remarkably potent but specific

Figure 7. Apelin treatment and subsequent recruitment of Aplnr+ cells enhance myocardial neovascularization after MI. A and B, Representative lectin staining of myocardial sections 14 days after sham or MI induction. Apelin increased capillary density in sham-silenced mice after MI (A). Aplnr-silenced animals showed diminished capillary density per field of view, whereas apelin treatment failed to increase the capillary density in these animals (B). C, Quantification of the capillary density. Sham-operated animals from the 2 BM transplantations are shown as reference. (n=7 per group), *P<0.05 versus sh.sham saline and control. #P<0.05 versus corresponding sh.sham.
chemoattractive function of apelin, even in tissue in which its expression is normally not enhanced in response to ischemia.

Recruitment of cKit+/Flk1+/Aplnr+ cells by apelin promotes local neoangiogenesis resulting in preserved cardiac function and diminished adverse remodeling with reduced scar formation after MI. The importance of cell recruitment was earlier suggested in animals with impaired cKit function.7 In these animals, recruitment of cells after MI was diminished and resulted in pronounced scar formation with poor residual cardiac function.

Interestingly, recruited cells did not differentiate into endothelial cells. This suggests that these cKit+/Flk1+/Aplnr+ cells may express proangiogenic factors and locally stimulate resident ECs to initiate angiogenesis, consistent with earlier findings.37,38 In Aplnr-silenced hybrid mice, cKit+/Flk1+ cell recruitment was severely impaired after MI, resulting in diminished neovascularization and pronounced scar formation with poor residual cardiac function after MI. The recruitment of Sca+/Flk+ and Lin+ cells was not affected after Aplnr silencing, suggesting that apelin signaling does not play a crucial role in the mobilization of these cells. Importantly, apelin treatment in animals with Aplnr-silenced expression in BM cells failed to enhance the recruitment of the cKit+/Flk1+ cell population; nor did it change capillary density, suggesting that the beneficial effect is mainly mediated by recruitment of BM derived cells and not a local effect on resident cells.

Increased recruitment of cKit+/Flk1+/Aplnr+ cells by apelin injection was associated with an increase in local VEGFA expression and Ang-2/Ang-1 ratio, whereas loss of Aplnr in the BM was associated with a decreased VEGFA expression and Ang-2/Ang-1 ratio. Ang-2 has been shown to block the quiescent signal of Ang-1 and to enhance the sensitivity of endothelial cells to VEGFA.39,40 Isolated cKit+/Flk1+/Aplnr+ cells proved to have an enhanced expression of VEGFA and Ang-2 and a reduced expression of Ang-1 after MI, indicating that they are at least in part responsible for the production of these cytokines. In line with our observations, another study showed that a mutation in the cKit gene, resulting in a dysfunctional protein, was associated with the reduced recruitment of cKit+ cells into the ischemic myocardium.7 This reduced recruitment resulted in a local decrease in VEGFA and Ang-2 production, suggesting a role of these cells in the release of the angiotropic factors.

Figure 8. Aplnr+ cells promote the release of angiotropic factors in the myocardium. A, VEGFA protein expression in the myocardium was increased in apelin-treated sham-silenced mice. Aplnr-silenced animals showed reduced VEGFA protein expression, as compared with sham virus-treated animals. Apelin treatment did not result in an increase of VEGFA protein in these animals. (n=5 per group). B and C, Myocardial expression of Ang-1 and Ang-2. D, Apelin treatment in sham-silenced mice increased Ang-2/Ang-1 ratio, whereas Aplnr-silenced animals showed inverse effects (n=5 per group). *P<0.05 versus sham saline. E through G, mRNA expression in isolated cKit+/Flk1+/Aplnr+ cells after MI. cKit+/Flk1+/Aplnr+ cells enhanced their mRNA expression of VEGFA (G) and Ang-2 (F) after MI, as compared with cKit+/Flk1+/Aplnr+ isolated from sham-operated hearts. The mRNA expression of Ang-1 (E) was decreased in these cells after MI as compared with cKit+/Flk1+/Aplnr+ isolated from sham-operated hearts (n=3 for each group). *P<0.05 versus sham.
We also observed an enhanced mRNA expression of other known angiogenic and cardioprotective factors in these isolated cells after MI, indicating other protective mechanisms. In vitro experiments to study if cKit+/Flk1+/Aplnr+ cells could directly affect angiogenesis again indicated a stimulating effect of these cells. When HUVECs were cultured in combination with these cells in an in vitro tube formation assay, we observed an increase in the number of tubes and junction formed. These observations proved that cKit+/Flk1+/Aplnr+ can directly stimulate local angiogenesis.

Our data indicate a central role of Aplnr and apelin interaction in myocardial repair response to ischemia, by attracting a specific subset of cells toward the myocardium. Indeed, apelin expression is reduced in diabetes and hypercholesterolemia,22,23 2 known risk factors that have been associated with low circulating angiogenic cell numbers with impaired function, resulting in an attenuated neoangiogenesis response.44,45 Moreover, physical exercise has been shown to promote the neoangiogenesis response by recruitment of circulating cells46 and has also been associated with increased apelin levels.47

In summary, the current study provides evidence for a central role of Aplnr/apelin interaction in the recruitment of cKit+/Flk1+/Aplnr+ cells toward the myocardium in the early phase after MI, where these cells enhance the local release of proangiogenic cytokines to promote neovascularization and limit myocardial damage and cardiac dysfunction. It is even possible that some growth factors produced by recruited cKit+/Flk1+/Aplnr+ cells mobilize and recruit other cell subset that repair the myocardium. To evaluate this interesting concept of possible interactions and dependence between subsets of cells in the MI condition, further research is needed. Other mediators, such as VEGFA, GM-CSF, SDF-1, and Ang-1 have been proven to enhance the number of circulating cells and neovascularization after ischemia,10–13 but to our knowledge, none of these pathways is specific for the myocardial repair response. The apelin/Aplnr pathway may therefore represent a unique repair mechanism that may represent a new therapeutic approach to limit myocardial damage and dysfunction after MI.

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**Disclosures**

None.

**References**

Novelty and Significance

What Is Known?
- Transplantation or mobilization of stem cells can be used to repair the damage caused by a myocardial infarction (MI).
- Neovascularization is one of the key processes to repair the heart after an infarction.
- Apelin signaling is important for a wide range of vascular actions, including angiogenesis, cardiac contractility, and blood pressure regulation.

What New Information Does This Article Contribute?
- Apelin functions as a specific chemoattractant for a potent sub population of bone marrow–derived cells that are recruited early after MI via apelin/apelin receptor (APJ) binding.
- The attracted circulating Aplnr+ cells improve local neovascularization and subsequently diminish scar formation and benefit cardiac function after MI.

Myocardial infarction causes irreversible damage to cardiac tissue, which is difficult to repair. Therefore, new therapies to regenerate cardiac tissue are needed. Results of recent attempts to repair the damage after MI are encouraging, but the approach needs further improvement. To optimize cell therapy, we need a better understanding of the environmental cues that are required for stem cell trafficking to define the genetic and cellular mechanisms involved in myocardial repair. In the present study, we found that in mice the ligand apelin mobilizes and recruits Aplnr+ bone marrow–derived cells to the heart after MI. These recruited cells secrete growth factors that locally stimulate neovascularization and subsequently improve cardiac function and diminish infarct size. These findings suggest that apelin functions as a new and potent chemoattractant for bone marrow–derived Aplnr+ cells early during myocardial repair. On the basis of these observations, we conclude that the apelin/Aplnr pathway may represent a unique repair mechanism, which could be a target for a new therapeutic approach to stem cell therapy for myocardial repair and regeneration.
Apelin Enhances Cardiac Neovascularization After Myocardial Infarction by Recruiting Aplnr+ Circulating Cells
Dennie Tempel, Martine de Boer, Elza D. van Deel, Remco A. Haasdijk, Dirk J. Duncker, Caroline Cheng, Stefan Schulte-Merker and Henricus J. Duckers

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Supplemental Material

Material and methods

Lentivirus production
Lentiviral vectors encoding short hairpin RNA targeting mouse Aplnr (sh.Aplnr), the control plasmid (sh.sham), encoding a scrambled non-targeting short hairpin RNA, and a positive control vector, which expresses EGFP (EGFP) were used (RNAi Consortium, Sigma Aldrich, The Netherlands). Human embryonic kidney 293 FT cells (Invitrogen, The Netherlands) were co-transfected with the lentiviral shuttle vector and compatible packaging plasmid mixture (Mission Lentiviral Packaging Mix, Sigma) using Fugene® 6 (Roche, The Netherlands), in accordance with the manufacturer's instructions. Viral titers were quantified by a limiting dilution method using puromycin selection in HEK 293 FT cells. 1.0x10^7 c57BL/6J Unfractionated bone marrow cells were transduced using different titers of EGFP in complete DMEM supplemented with 10% FCS and 10 µg/ml diethylaminoethyl (DEAE)-dextran at 37°C. After 24 hours, cells were washed and fixed in 4% paraformaldehyde for flow cytometric analysis to determine transfection efficiency.

Measurement of infarct size
24h and 2 weeks after infarct induction 1 ml of 1% Evan's blue dye was injected into the jugular vein to delineate the area at risk. The heart was quickly excised, frozen for a few minutes at −20°C, and then immediately sliced with a scalpel into 1-mm-thick sections perpendicular to the long axis of the heart. Thereafter each slice of LV was weighed and photographed on both sides. Evan's blue stained area and Evan's blue negative area (IA) were digitally measured using SigmaScan (SPSS, USA). The infarct size was measured and expressed as a percentage of infarct area (IA) over total area.

qPCR and protein analysis
mRNA levels were determined from freshly isolated myocardial and gastrocnemic muscles, or BM aspirates. RNA was extracted using the RNeasy kit (Qiagen, The Netherlands). Quantity and quality were verified by optical dosimetry (Nanodrop) and Agilent 2100 Bioanalyzer electrophoresis (Agilent Technologies, UK). Isolated RNA was reverse transcribed into cDNA (Iscript, Biorad, the Netherlands) and analysed by real-time fluorescence assessment of SYBR Green signal in the iCycler iQ Detection system (Bio-Rad, The Netherlands). MRNA expression levels were analyzed and corrected for the housekeeping gene HPRT. Reported values were normalized to sham (Figure 1 and Supplemental figure 6) or control values (Figure 4), which were arbitrarily, assigned the average value of 1.
Apelin protein levels were assessed from freshly isolated left ventricles, gastrocnemic muscles and blood plasma. 40 mg of homogenized tissue, was boiled in 0.1 mol/l acetic acid for 10 min, and centrifuged at 15,000 rpm for
10 min. The supernatant was used to quantify total protein concentration using a Bradford Assay. Equal amounts of total protein (300 µg/ml) were used in the apelin-12 EIA kit (Phoenix pharmaceutical, US), according to the manufacturers’ protocol. VEGFA, Ang-1 and Ang-2 protein levels were assessed by commercially available Elisa kits (Antibodies-online, Germany) according to the manufacturers’ protocol.

Primer sets used for qPCR analysis

<table>
<thead>
<tr>
<th>Primer set</th>
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<th>Sense</th>
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<td>Murine Apelin</td>
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<td>Murine Ang2</td>
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<td>Murine IGF-1</td>
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<tr>
<td>Murine TB4</td>
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**Tube formation assay**

*In vitro* formation of tube structures was studied on BioCoat Matrigel tissue culture plates (BD Biosciences, The Netherlands). HUVECs were plated at 25000 cells/well in 96-well plates precoated with a solution of Matrigel basement membrane matrix. 1000 cKit+/Flk1+/Aplnr+ cells isolated from hearts 5 days after MI were added to study the effect of these cells on tube formation. After 18 hours of incubation at 37°C, the tubes were visualized by Calcein-Am uptake (BD biosciences, The Netherlands) the tube organization was examined using an inverted fluorescence microscopy, and the photographs were subsequently analyzed using the commercial image analysis system (AngioSys, UK).
Online figures

Online Table I: Characteristics of BM repopulated mice 14 days after MI.

<table>
<thead>
<tr>
<th>Animal</th>
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<th>BW (g)</th>
<th>TL (cm)</th>
<th>LV weight (mg)</th>
<th>Lung fluid weight (mg)</th>
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<tr>
<td>Control</td>
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<td>19.3 ± 0.2</td>
<td>1.74 ± 0.01</td>
<td>86 ± 3</td>
<td>134 ± 5</td>
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<td>19.9 ± 0.3</td>
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<td>87 ± 2</td>
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<tr>
<td>Apelin</td>
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<td>7</td>
<td>19.7 ± 0.3</td>
<td>1.74 ± 0.01</td>
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<tr>
<td>Control</td>
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<td>19.9 ± 0.3</td>
<td>1.73 ± 0.01</td>
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<td>Sh.Aplnr</td>
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<td>19.3 ± 0.4</td>
<td>1.73 ± 0.01</td>
<td>89 ± 2#</td>
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</table>

Data are provided as mean ± SEM. * P<0.05 vs Sh.sham control. # P<0.05 vs apelin Sh.sham. BW = Body weight, TL = Tibia length and LV = Left ventricle.

Online figure I: cKit+/Flk1+ circulating cell recruitment to specific myocardial areas after myocardial ischemia.
(A) Quantification of cKit+/Flk1+ cells in different areas of the myocardium 3 days after MI. cKit+/Flk1+ cells are preferentially recruited to the borderzone (n=6 per group). * P<0.05 compared with naïve hearts.

Online figure II: Apelin, but not VEGFA or SDFα, mobilizes cKit+/Flk1+
(A) Quantification of circulating cKit+/Flk1+ cells following an intravenous injection. Apelin mobilized cKit+/Flk1+ cells into the circulation. In contrast, VEGFA and SDFα did not show an effect on the mobilization of these cells. (n=6 per group). * P<0.05 compared with saline-injected animals and non-injected animals. (B) Quantification of circulating Sca+/Flk1+ cells following an intravenous injection. VEGFA and SDFα mobilized Sca+/Flk1+ cells into the circulation, whereas apelin failed to mobilize this specific subset of cells. (n=6 per group). * P<0.05 compared with saline-injected animals and non-injected animals.

Online figure III: Bone marrow transplant efficiency
(A) Quantification of eGFP-positive cells in the circulation 4 weeks after transplantation. (B) Quantification of eGFP-positive cells in the BM 4 weeks after transplantation. (C) Quantification of different cell types in the circulation 4 weeks after transplantation.
Online figure IV: Apelin treatment and Aplnr knockdown in the BM do not affect initial infarct size.
(A, B) Representative pictures of heart slices from evans blue perfused sham (A) and Aplnr silenced animals (B) 24 hours after MI. (C) Quantification of the infarct area (n=6 per group).

Online figure V: Apelin treatment and subsequent recruitment of Aplnr+ cells have no effect on the arteriolar density of the myocardium after MI.
(A, B) Representative arteriole staining of myocardial sections from sham (A) and Aplnr silenced animals (B) 14 days after MI. Blue staining identifying arterioles. (C) Quantification of the arteriolar density (n=7 per group).

Online figure VI: cKit+/Flk1+/Aplnr+ cells recruited after MI express angiogenic and cardioprotective growth factors.
(A-D) mRNA expression of growth factors in cKit+/Flk1+/Aplnr+ cells isolated from the heart 5 days after MI. cKit+/Flk1+/Aplnr+ cells isolated after MI showed a higher expression of HGF (A), SDF-1 (B), TB4 (C) and IGF-1 (D), as compared with cKit+/Flk1+/Aplnr+ cells isolated from the hearts of sham-operated animals (n=3 for each group). * P<0.05 versus sham.

Online figure VII: cKit+/Flk1+/Aplnr+ cells isolated after MI improve endothelial tube formation in vitro.
(A) cKit+/Flk1+/Aplnr+ cells isolated after MI were co cultured with HUVECs to induce tube formation. Tubes were visualized by Calcein Am dye. cKit+/Flk1+/Aplnr+ cells increased the angiogenic capacity of HUVECs, as shown by an increase in (B) number of tubes, and (C) number of junctions (n=3 for each group). * P<0.05 versus control.
Online figure II

Online figure III
Online figure IV
Online figure V