Published in final edited form as:

Stem Cell Rev. 2018 October ; 14(5): 702–714. doi:10.1007/s12015-018-9833-x.

Cathelicidin Related Antimicrobial Peptide (CRAMP) Enhances Bone Marrow Cell Retention and Attenuates Cardiac Dysfunction in a Mouse Model of Myocardial Infarction

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Abstract

Background—Acute myocardial infarction (MI) and the ensuing ischemic heart disease are approaching epidemic state. Unfortunately, no definitive therapies are available and human regenerative therapies have conflicting results. Limited stem cell retention following intracoronary administration has reduced the clinical efficacy of this novel therapy. Cathelicidin related antimicrobial peptides (CRAMPs) enhance chemotactic responsiveness of BMSPCs to low SDF-1 gradients, suggesting a potential role in BMSPCs engraftment. Here, we assessed the therapeutic

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DISCLOSURES AND CONFLICT OF INTEREST

None

Author contribution.

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efficacy of CRAMPs in the context of BMSPCs recruitment and retention via intracardiac delivery of CRAMP-treated BMSPCs or CRAMP-releasing hydrogels (HG) post-AMI.

Methods—For cell transplantation experiments, mice were randomized into 3 groups: MI followed by injection of PBS, BMMNCs alone, and BMMNCs pre-incubated with CRAMP. During the in vivo HG studies, BM GFP chimera mice were randomized into 4 groups: MI followed by injection of HG alone, HG + SDF-1, HG + CRAMP, HG + SDF-1 + CRAMP. Changes in cardiac function at 5 weeks after MI were assessed using echocardiography. Angiogenesis was assessed using isolectin staining for capillary density.

Results—Mice treated with BMMNCs pre-incubated with CRAMP had smaller scars, enhanced cardiac recovery and less adverse remodeling. Histologically, this group had higher capillary density. Similarly, sustained CRAMP release from hydrogels enhanced the therapeutic effect of SDF-1, leading to enhanced functional recovery, smaller scar size and higher capillary density.

Conclusion—Cathelicidins enhance BMMNC retention and recruitment after intramyocardial administration post-AMI resulting in improvements in heart physiology and recovery. Therapies employing these strategies may represent an attractive method for improving outcomes of regenerative therapies in human studies.

Keywords

Bone Marrow Derived Mononuclear Cells (BMMNCs); Cathelicidin Related Antimicrobial Peptide (CRAMP); LL-37; myocardial infarction; regeneration; stem cells homing

INTRODUCTION

Acute myocardial infarction (AMI) leads to irreversible cardiac cell damage or death secondary to inadequate blood supply to the heart muscle. Ischemic heart disease (IHD) is the leading cause of morbidity and mortality in the United States and worldwide (1). The prognosis of AMI and IHD remains poor despite significant advances in medical and revascularization therapies (2). Recently, regenerative studies utilizing bone marrow and cardiac stem cells have generated great interest in the field. However, the therapy has faced limited success, partly due to the limited engraftment of transplanted cells which reduced their efficacy (3). Studies examining the role of SDF-1 and cytokines to enhance the engraftment of transplanted stem cells in the myocardium have achieved limited success. To improve myocardial regenerative therapies, enhanced cell retention and survival are desperately needed. To address this need, multiple approaches have been investigated to precondition cells for ischemia, modify cells genetically to make them more resistant to ischemic conditions, and biomaterials devised to limit inflammatory responses from attacking transplanted cells.

The antimicrobial peptides (AMP) are a family of polypeptides found in lysosomes of macrophages, polymorphonuclear leukocytes, and keratinocytes of humans and many different species of mammals (4). In mice, AMP include cathelicidin-related antimicrobial peptides (CRAMP) and defensins. Human cathelicidins identified as hCAP-18/LL-37 serve an essential role in the innate immune defense against invasive bacterial infection without affecting the viability of normal eukaryotic cells. These selective effects of AMPs in

destroying bacteria, but not normal eukaryotic cells, depend on differences in electrostatic and hydrophobic properties of cell membranes between prokaryotic and eukaryotic cells (4). The α -chemokine stromal derived factor 1 (SDF-1) is one of the major chemotactic factors responsible for bone marrow-derived stem/progenitor cell (BMSPC) egress and is significantly upregulated in the infarcted myocardium during the events of AMI (5). However, the local inflammatory response following AMI yields a proteolytic environment that degrades SDF-1, suggesting alternative mechanisms for BMSPC mobilization post-AMI (6,7). Strategies aimed at the local sustained delivery of recombinant SDF-1 (rSDF-1) through its integration in crosslinked hyaluronic acid (HA) hydrogels resulted in enhanced bone marrow cell engraftment in the heart (8). Furthermore, the effect of HA impregnated with an engineered SDF-1 mimetic led to significant reduction in cardiac remodeling and fibrosis at 4 weeks after AMI (9).

It has been reported that LL-37 enhances the chemotactic responsiveness of BMSPC to a low SDF-1 gradient (10). This effect is dependent on incorporation of the SDF-1 receptor, CXCR4, into lipid rafts of cell membrane. Mice transplanted with BMSPCs primed by LL-37 *ex vivo* showed accelerated recovery of platelet and neutrophil counts when compared to mice transplanted with unprimed BM cells (10). We recently showed that myocardial ischemia and tissue hypoxia lead to increased expression of CRAMP in cardiac fibroblasts (11).

In this study, we hypothesized that CRAMP could be applied to facilitate the engraftment of mobilized BMSPCs to the ischemic myocardium and ultimately attenuate maladaptive remodeling and enhance function after AMI. To test this hypothesis, we utilized clinically relevant strategies, including incubation of BMSPCs with CRAMP prior to their transplantation or by releasing CRAMP from biodegradable hydrogels transplanted in the peri-infarct region following AMI. This is the first study to utilize CRAMP in the setting of AMI to enhance the engraftment and homing of BMSCs towards development of a novel therapy.

MATERIALS AND METHODS

Animal studies

All procedures were conducted under the approval of the University of Kentucky IACUC in accordance with the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. [NIH] 85-23, rev. 1996). Mice were fed *ad libitum* with a normal chow diet (R36, Lactamin, Sweden) and randomly assigned to experimental groups.

Isolation of bone marrow nucleated cells

Bone marrow mononucleated cells (BMMNCs) were isolated by flushing the femurs and tibias of pathogen-free C57BL/6 mice using 1 ml of PBS per animal. Whole BM cells were suspended in BD Pharm Lyse buffer (BD Biosciences, San Jose, CA, USA) to remove RBCs to yield bone marrow mononuclear cells (BMMNCs), washed twice and resuspended in RPMI medium (Thermo Fisher Scientific, South Logan, UT, USA) containing 0.5% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA). For cell transplantation studies, cells

(1×10^6 cells/100 μ L) were incubated with either CRAMP (Anaspec, Fremont, CA, USA) at 2.5 ng/ml or placebo for 3 hours prior to transplantation. Cells were then washed once and resuspended at 3×10^6 cells/25 μ L.

Chemotaxis assays

Cell migration assays were performed using the chemotactic (Boyden) chambers (Neuroprobe, Gaithersburg, MD, USA). BM- and PB-derived cells were lysed as described earlier. Cells were then suspended in RPMI with 0.1% FBS with and without CRAMP (2.5 ng/ml) for 3h before the migration assays. The lower chambers were loaded with control medium (RPMI with 0.1% FBS) or RPMI + 0.1% FBS + SDF-1 at 12.5 ng/ml or 300 ng/ml (R&D systems, Minneapolis, MN, USA). Cell suspension (1×10^6 cells/100 μ L) was loaded into the upper chambers on a 5 μ m pore membrane; the chambers were incubated (37°C, 95% humidity, and 5% CO₂) for 3 h; and subsequently, cells in the lower chambers were harvested and stained against the following stem cell markers: anti-mouse Abs against CD45 PE (BD Pharmingen 553081), c-Kit APC (BD Pharmingen 558162) and aLy-6A/E PECy7 (Sca-1) (BD Pharmingen 558162). All anti-mouse lineage markers (Lin) were FITC conjugated and purchased from BD Pharmingen: anti-CD45R/B220 (553088); anti-T-cell receptor β (TCR β ; 553170); anti-TCR $\gamma\delta$ (553177); anti-CD11b (553310); anti-Ter-119 (557915); anti-Ly6G and Ly6C (553127). To examine the effect of CRAMP incubation on the expression of CXCR4 receptor, BMMNCs incubated with CRAMP (2.5 ng/ml) (Anaspec) for 1, 3 and 6 hours were washed twice in PBS then stained against c-Kit APC (BD Pharmingen), Sca1 PECy7 (BD Pharmingen), lineage cocktail FITC, and CXCR4 PE (BD Pharmingen 551966). The expression of CXCR4 was quantified in Sca1⁺/cKit⁺/Lineage⁻ cells using flow cytometry. Experiments were performed in triplicate. The mean difference and 95% standard error of the mean are reported.

Flow Cytometry

Heart tissue was harvested at 5 days and placed in ice cold PBS instantly. Heart tissue was minced then digested using a collagenase B (Roche, Indianapolis, IN) and dispase II (Roche, Indianapolis, IN) solution for 30 minutes at 37°C with mixing every 5 minutes. The enzymatic reaction was stopped by dilution with Flow Buffer (PBS + 5 % normal goat serum + 0.1 % sodium azide) and the heart cell suspensions were passed through 40 μ m strainers. Cells were centrifuged at 400 $\times$ g for 5 min at 4 °C, then suspended in Flow Buffer. Cells were incubated directly for 30 minutes with eFluor 660 conjugated GFP Antibody Clone 5F12.4 (eBioscience, Thermo Fisher Scientific, Waltham MA) and APC-CY7-conjugated CD45 (Biolegend, San Diego CA). After incubation, cells were washed twice using flow buffer and analyzed using flow cytometry. All flow experiments were conducted using LSR II (Becton Dickinson) in the University of Kentucky Flow Cytometry Core. Laser calibration and compensation were carried out utilizing unstained and single fluorescent control beads (eBioscience). We utilized FlowJo v7 (FlowJo, FlowJo Ashland OR) software to generate dot plots and analyze the data.

Global cardiac ischemia

Mice were anesthetized with ketamine/xylazine (90/10 mg/kg i.p.), and hearts were rapidly excised. Retrograde perfusion with an oxygenated low-potassium Tyrode's buffer

maintained at 37°C was initiated by aortic cannulation. After clearing blood and equilibration, flow was halted for 15 minutes, after which hearts were then perfused with oxygenated low-potassium Tyrode's buffer for 30 minutes. The left ventricle was then isolated and frozen immediately in liquid nitrogen.

Western blotting

Left ventricular sections of frozen murine hearts after myocardial infarction (~200mg) were homogenized in 600 µl RIPA buffer (150mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) + complete[®] protease inhibitors (Roche) via bead beater (MillMix 20). The homogenate was then centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was isolated, sonicated, and processed for the Bradford protein assay (Bio-Rad). Forty micrograms of lysate protein of each sample was boiled for 4 min in SDS-sample buffer containing 5% β-mercaptoethanol, and separated on a 12% SDS polyacrylamide gel. After electrophoresis, the gel was transferred to PVDF membrane and the blot was blocked in PBS with 1% casein, and 0.1% Tween 20, and incubated with anti-CRAMP mouse antibody (Santa Cruz; 1:200 dilution) and anti-actin rabbit antibody (Sigma-Aldrich; 1:1000) in blocking buffer overnight at 4 °C. The next day the blot was washed six times in PBS with 0.1% Tween 20, and incubated with anti-mouse IRDye[®] 680-conjugated IgG (1:5000) and anti-rabbit IRDye[®] 800-conjugated IgG (1:5000) in blocking buffer for 1 h at room temperature. The blot was then washed six times in PBS 0.1% Tween 20 and quantified via LI-COR[®] Odyssey Infrared Imaging System.

In vitro ischemia/reperfusion experiments

Murine cardiac fibroblasts were isolated from C57BL/6 mice (Jackson Labs), aged 5–6 weeks and used for the *in vitro* studies. Heart tissue was harvested and placed in ice cold PBS (VWR International) instantly. Heart tissue was minced then digested using a collagenase B (Roche, Indianapolis, IN) and dispase II (Roche, Indianapolis, IN) solution for 30 minutes at 37°C. The enzymatic activity was then stopped using PBS with 10% FBS and cells were washed twice using PBS with 10% FBS. Cells were plated in 6 well plates at concentration 0.3×10^6 cells/well in media consisting of DMEM, 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, L-Glutamine, and Glucose. Following cell adhesion (~1 week), cultured cells were established in a gas-tight modular chamber (STEMCELL Technologies Inc., Seattle, WA) containing 1% O₂, 5% CO₂, 94% N₂, or in normal oxygen tension with 20% O₂, 5% CO₂ as control for 72 hours at 37°C followed by reperfusion with 21% O₂, 5% CO₂ for 2 hours. Cells were washed using PBS and lysed using RNA lysis buffer (ThermoFisher Scientific).

Real-Time Polymerase Chain Reaction

We utilized PureLink RNA Mini Kit and reverse transcribed with PowerUp SYBER Green Master Mix Reverse Transcription Reagents to collect the total mRNA from heart and peripheral blood (ThermoFisher Scientific). We carried quantitative mRNA expression of markers identifying hypoxia inducible factor-1α (HIF-1α) and CRAMP genes. Relative quantitation of mRNA expression was performed with the comparative Ct method. The relative quantitative value of target, normalized to an endogenous control (β2-microglobulin gene) and relative to a calibrator, was expressed as 2^{-Ct} (-fold difference), where $Ct =$

(Ct of target genes [Oct-4, Nanog, Rex-1, Rif1, Dppa1, Gata4, Nkx2.5]) – (Ct of endogenous control gene [β 2-microglobulin]), and $Ct = (Ct \text{ of samples for target gene}) - (Ct \text{ of calibrator for the target gene})$. To avoid the possibility of amplifying contaminating DNA (i) all primers for real-time RT-PCR were designed to span an intron for specific cDNA amplification; (ii) reactions were performed with appropriate negative controls (template-free controls); (iii) a uniform amplification of the products was rechecked by analyzing the melting curves of the amplified products (dissociation graphs); and (iv) the melting temperature (T_m) was 57–60°C, and the probe T_m was at least 10°C higher than primer T_m . Three independent experiments were performed for each set of genes.

Murine model of myocardial infarction

Mice were anesthetized with 2% isoflurane with an inhaled delivery system. The heart was exposed, pushed out of the thorax with a direct visual control and the left anterior descending coronary artery (LAD) was sutured and ligated at a site approximately 3 mm from its origin using a 6-0 silk suture as previously described (13). The ligation was deemed successful when the anterior wall of the LV turned pale and ST-elevation was noted by ECG. After ligation, BMMNCs or PBS were injected around the infarct border zones (7 μ L per injections, 3 injections, 1×10^6 cells per injection site). For HG injection studies, hydrogels were prepared as described, containing SDF-1 (150 ng), CRAMP (15 μ g), both SDF-1 and CRAMP, or no factors (HG alone); 25 μ L was injected into the border zone in three different areas in the anterior, lateral, and posterior walls surrounding the infarct border. After injections, the heart was immediately placed back into the intrathoracic space, followed by manual evacuation of pneumothorax and closure of muscle and the skin by means of the previously placed purse-string suture. Mice were injected daily with BrdU (Sigma B9285) (80 mg/kg, i.p.) beginning the day of operation until sacrifice. Mortality was observed mainly in the first week and was not different between the treatment groups. Animals were sacrificed at 35 days after the operation via CO₂ asphyxiation followed by cervical dislocation.

Echocardiography

Echocardiograms were obtained using a Vevo 2100 system (VisualSonics, Toronto, CA) equipped with 15-7 MHz linear broadband transducer and a 12-5 MHz phased array transducer. *In vivo* cardiac function was assessed at baseline before cardiac surgery, at 48 hours post-MI and immediately prior to sacrifice 35 days post-MI by echocardiography. Using a rectal temperature probe, body temperature was maintained carefully at 37°C throughout the study using a heating pad. Modified parasternal long axis and short axis views were obtained to assess the left ventricular function and volume in M-mode, 2D, and Doppler echocardiography modes. Systolic and diastolic parameters were obtained using M-Mode tracings at the mid papillary level. LV volumes were estimated using the Teichholz formula at end-systole and end-diastole. Echocardiography was performed with mice under 1–3% isoflurane anesthesia. All echocardiography analyses were performed by a blinded investigator.

Histology

After final echocardiography and peripheral blood collection, hearts were harvested in diastole with saturated KCl and CdCl (100 mM) injected through the apex into the LV cavity. The LV apex was then cannulated and the heart perfused with PBS followed by 10% buffered formalin at 75 mmHg with severed inferior vena cava serving as the outlet. Hearts were then cut into 2 mm cross sectional slices and processed for paraffin embedding. Slices were cut into 4 μ m sections for histology and immunofluorescent staining. LV area, LV cavity area, and infarct area were measured in Masson's Trichrome stained sections. Images were acquired digitally and areas were measured using NIH ImageJ (v1.37). Cell turn-over was evaluated by staining against BrdU (Roche, Indianapolis, IN) and α -sarcomeric actin (Sigma, St Louis, MO) or myosin heavy chain (MHC) (Abcam, Cambridge, MA) and counting BrdU^{pos} α -SA^{pos}/MHC^{pos} cells in both ischemic and remote regions. GFP bone marrow cell retention was assessed by anti-GFP staining in infarct border regions (Abcam). Capillary density was measured by staining against FITC- isolectin B4 (FL1201, Vector Labs, Burlingame, CA) in cardiac sections. 10–15 adjacent areas in the peri-infarct and remote zones per section were analyzed (1–2 sections/animal) at 40x magnification using Nikon Confocal Microscope A1 in the University of Kentucky Confocal Microscopy facility.

Materials synthesis

Sodium hyaluronic acid (HA, 90kDa, Lifecore Biomedical) was exchanged against Dowex 100 resin and neutralized by tetrabutylammonium hydroxide to obtain the tetrabutylammonium salt of HA (HA-TBA). β -Cyclodextrin (CD, TCI America) was aminated through the conventional tosylated intermediate and anhydrous reflux against hexane diamine. All other chemicals were purchased from Sigma-Aldrich and used as received. HA modified by 1-adamantane acetic acid (Ad-HA) and aminated cyclodextrin (CD-HA) were prepared by previously reported methods by corresponding anhydrous esterification and amidation reactions with HA-TBA ¹H NMR spectra were acquired (360MHz, Bruker) in deuterated water from which Ad-HA and CD-HA modifications were determined to be 24.9% and 22.0%, respectively (12). To permit quantification of CRAMP release *in vitro*, the fluorescent analogue was prepared by standard solid phase peptide synthesis (PS3 Peptide Synthesizer, Protein Technologies) using glycinol 2-chlorotrityl resin and Fmoc protected amino acids (Novabiochem). The amine terminus was reacted with the acid of 5(6)-carboxyfluorescein by substitution in place of N-acetylation.

Hydrogel formation and in vitro release assays

Hydrogels were prepared from solutions of Ad-HA and CD-HA in PBS at a concentration of 5.0 wt%. For hydrogel formation, Ad-HA and CD-HA solutions were combined in volumetric ratios such that adamantane and cyclodextrin were present in a 1:1 ratio and mixed to ensure homogenous hydrogel formation. For inclusion of CRAMP (10 μ g/10 μ L), SDF-1(100ng/10 μ L) or their combination, molecules were included during hydrogel formation.

Hydrogels (n=4 per group) were prepared as described and 15 μ L was loaded into the depression (3 mm diameter, 5 mm height) of acrylamide molds. Hydrogels were covered by 0.75mL reagent diluent (R&D, DY995) and allowed to degrade at 37°C. The complete

buffer volume was sampled periodically, with replacement, and hydrogels degraded at the study terminus by 1.0 mg/mL hyaluronidase. Release of HA, CRAMP, and SDF-1 were respectively quantified by uronic acid assay, carboxyfluorescein fluorescence relative to standard curves, and ELISA (R&D, DY460).

Generation of GFP BM chimeras

Female recipient C57BL/6 mice (Jackson Labs), aged 5–6 weeks were lethally irradiated to consume the BM stem/progenitor cells. Irradiation was performed in two doses: the first does was 700 rad followed in three hours by a second dose of 500 rad. Male donor GFP transgenic mice (Jackson Labs #006567) were anesthized using 4% isofluarane and euthanized using carbon dioxide overdose followed by cervical dislocation. BM was harvested from hip bones, tibias, and femurs. The irradiated mice were injected via lateral tail vein with 5×10^6 fresh GFP bone marrow cells in 0.3 mL of medium. Chimerism was confirmed using flow cytometric analysis of peripheral blood cells for GFP positive cells at 6 weeks post-transplantation.

Statistical analyses

Data are expressed as mean $\pm$ standard error of the means. Differences were analyzed using the unpaired Student *t*-test or analysis of variance (one way or multiple comparisons) as appropriate. Post hoc multiple comparison procedures were performed using 2-sided Dunnett or Dunn tests as appropriate with PBS or HG alone treated mice as the control category. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the Prism 6 package (GraphPad, La Jolla, CA, USA). All authors had full access to and took full responsibility for the integrity of the data. All authors have read and agreed with the article as written.

RESULTS

Treatment with the priming factor CRAMP enhanced the migration of SKL murine stem cells to a gradient of SDF-1 *in vitro*

Previous studies demonstrated the role of CRAMP in enhancing BM cell mobilization towards low, albeit physiological, levels of SDF-1 (10). We conducted a transwell migration assay to study the migration of murine BM stem cells, with or without CRAMP incubation (at a concentration of 2.5 ng/mL), toward DMEM medium supplemented with 0.1% fetal bovine serum alone (Control), physiological dose of SDF-1 (12.5 ng/mL) or supra-physiological dose (300 ng/ml). SKL cells migrated in significantly higher numbers toward physiological levels of SDF-1 after incubation with the priming factor CRAMP (Figure 1A). However, the migration towards supra-physiological levels of SDF-1 (300 ng/ml) did not change with the addition of CRAMP, suggesting possible saturation of CXCR4 receptors. Furthermore, our preliminary studies indicated that cell surface expression of CXCR4 is enhanced by CRAMP incubation (at a concentration of 2.5 ng/ml) peaking at 3 hours of incubation *in vitro* (Figure 1B and Supplemental Figure 1).

Ischemic myocardium releases CRAMP

Multiple studies including our previous work suggest that the myocardium releases homing factors for circulating mobilized BM cells (11,15). In this study, we performed experiments to examine the *in vivo* peptide expression of CRAMP following AMI induced by LAD ligation. We observed significant elevation in the peptide level of CRAMP in cardiac tissues starting on day 5 after AMI. This enhanced expression continued until day 10 following ischemic injury (Figure 2A). Interestingly, these time points coincide with the mobilization of SKL cells and their peak circulating numbers as we have shown previously (16). When cardiac fibroblasts were cultured in ischemia/reperfusion conditions, the expression of CRAMP was increased and correlated with increased expression of HIF1 α (Figure 2B).

CRAMP enhances the retention and therapeutic effect of transplanted BMCs

In vitro studies discussed above demonstrate the potential therapeutic effects of CRAMP to enhance the response of BMCs to physiological levels of SDF-1, which is expressed in the myocardium after ischemic injury (15). 3×10^6 GFP BMMNCs were incubated with CRAMP at 1.25 ng/ml for 3 hours prior to heart transplantation. WT mice underwent LAD ligation followed immediately by injection of 25 μ L of PBS alone or PBS suspension of BMMNCs incubated with CRAMP or placebo.

Pre-incubation of BMMNCs with CRAMP resulted in higher cell retention as assessed using flow cytometry of cardiac tissue at 5 days following MI. Additionally, GFP+ cells demonstrated higher proliferative capacity in the form of higher percentage of cells in the G2M phase of cell cycle and higher percentage of BrdU+ cells (Supplemental Figure 1). However, this strategy does not provide long-term cell survival since we observed very rare GFP cells in the myocardium at 5 weeks in both BMMNCs alone or BMMNCs incubated with CRAMP (Supplemental Figure 2).

Mice were assessed for cardiac functional recovery using echocardiography at 48 hours and 35 days after AMI. At 48 hours, there were no significant differences of various echocardiographic parameters of cardiac function between groups (Figure 3). However, at 5 weeks following AMI, BMMNCs primed using CRAMP demonstrated better therapeutic efficacy when compared to BMMNCs alone and PBS controls with significant improvement of left ventricular ejection fraction (LVEF) (23.3 vs. 22.3 vs. 30.9% for PBS, BMMNCs alone and BMMNCs + CRAMP respectively, $P < 0.05$); LV end systolic volume (LVESV) (91.3 vs. 92 vs. 60.2 μ L for PBS treated mice, BMMNCs alone and BMMNCs incubated with CRAMP, respectively, $P < 0.05$); and end-diastolic volume (122.5 vs. 117.2 vs. 81.8 μ L for PBS, BMMNCs alone and BMMNCs incubated with CRAMP treated mice respectively, $P < 0.05$).

Given the improvement noted in the echocardiographic assessments, we explored the underlying mechanism responsible for these findings. We initially assessed the amount of scar in different groups at 5 weeks after AMI. The scar size as assessed using mason-trichrome staining (45.6 vs. 25.1 vs. 16.5% for PBS treated mice, BMMNCs alone and BMMNCs incubated with CRAMP respectively, $P < 0.01$) (Figure 4A and B).

The reduction in scar and functional improvements can be explained by multiple underlying mechanisms, potentially including angiogenesis and myocardial regeneration. To examine the potential role of angiogenesis, we stained the peri-infarct remote areas for capillaries using isolectin staining (Figure 5). Mice treated with BMMNCs cells or BMMNCs incubated with CRAMP resulted in significantly higher capillary density in the peri-infarct border (51 vs. 83.1 vs. 99.5/mm² for PBS, BMMNCs alone and BMMNCs incubated with CRAMP treated mice respectively, $P<0.05$). In the remote zone, treatment with BMMNCs, with and without CRAMP pre-incubation, resulted in significant increase in capillary density compared to PBS treated mice (148 vs. 184.9 vs. 266/mm² for PBS, BMMNCs alone and BMMNCs incubated with CRAMP treated mice respectively, $P<0.05$).

Hydrogel-based sustained delivery of CRAMP and SDF-1 enhances myocardial recovery after AMI

Incubation with CRAMP enhanced the therapeutic efficacy of BMMNCs, likely due to enhanced cell retention. However, this approach necessitates cell harvest from the bone marrow, which is an invasive procedure and may not be feasible in MI patients short after their ischemic injury. Therefore, we adopted a clinically relevant approach to provide localized delivery of homing signals, including both CRAMP and SDF-1, at the peri-infarct border. To achieve this goal, we utilized a previously reported injectable hydrogel (HG) (12) to provide sustained local release of contained SDF-1 and CRAMP (Figure 6). Peptide release proceeded simultaneously with hyaluronic acid hydrogel erosion (Figure 6A). SDF-1 release was sustained over the course of 5 weeks *in vitro* with a near complete release (~95% cumulative release) at the study endpoint of 35 days (Figure 6B). Furthermore, HG delivery sustained CRAMP release in a near-linear fashion over the first 7 days (~80% cumulative release) with continued slow delivery afterwards (Figure 6C). Biomolecule release was identical under co-delivery conditions.

Following LAD ligation, mice were injected with 25 μ L HG alone or containing CRAMP (10 μ g/10 μ L), SDF-1 (100ng/10 μ L) or their combination in 3 separate injections in the peri-infarct zone. Mice did not demonstrate significant cardiac function differences at 48 hours following AMI (Figure 7). At 5 weeks after treatment, mice treated with HG incubated with SDF-1 + CRAMP demonstrated significant improvement in LVEF (23.3 vs. 25.8 vs. 29.5 vs. 26.5 vs. 40.4% for PBS, HG alone, HG + CRAMP, HG + SDF-1 and HG + SDF-1 + CRAMP respectively, $P<0.01$) (Figure 7A); as well as parameters of LV remodeling such as LVESV (54.9 vs. 36.3 vs. 26.8 vs. 37.1 vs. 29.2 μ L for PBS, HG alone, HG + CRAMP, HG + SDF-1 and HG + SDF-1 + CRAMP respectively, $P<0.01$) (Figure 7B); and LVEDV (78.5 vs. 49.2 vs. 38.6 vs. 49.8 vs. 47.2 μ L for PBS, HG alone, HG + CRAMP, HG + SDF-1 and HG + SDF-1 + CRAMP respectively, $P<0.01$) (Figure 7C).

Improvements in cardiac function correlated with a trend towards reduction in scar size assessed at 5 weeks in all HG treated groups compared to PBS treated mice (41.9 vs. 34.2 vs. 28.7 vs. 27.6 vs. 25.4% for PBS, HG alone, HG + CRAMP, HG + SDF-1 and HG + SDF-1 + CRAMP, respectively, $P = \text{NS}$) (Figure 8A and B). Capillary density was significantly increased in all HG treated groups compared to PBS treated mice both at the infarct border (51 vs. 125.1 vs. 125.5 vs. 162.6 vs. 161.7/mm², $P<0.01$) and remote zone

(148 vs. 243 vs. 192.8 vs. 228 vs. 239.9/mm² for PBS, HG alone, HG + SDF-1, HG + CRAMP and HG + SDF-1 + CRAMP respectively, $P<0.01$) with the highest density seen in the HG + CRAMP and HG + CRAMP + SDF-1 in the peri-infarct zone (Figure 9). When compared to HG alone, capillary density was significantly higher with the addition of CRAMP and CRAMP + SDF-1 in the infarct border but not in the remote zone. Of note, HG + SDF-1 did not show significant difference compared to HG alone or PBS. Additionally, adding SDF-1 or CRAMP to HG does not affect the capillary density in the remote zone.

To determine whether supplementing HG with CRAMP and/or SDF-1 resulted in increased cardiomyocyte turn-over, we examined the number of BrdU+ cells that express the cardiac markers, alpha-sarcomeric actin and myosin heavy chain, by immunofluorescent confocal microscopy at 5 weeks post-MI. We observed significantly higher numbers of BrdU+ cardiomyocytes in the infarct border (infarct border: 2.7 ± 0.4 vs. 3.3 ± 0.5 vs. 4.8 ± 0.4 vs. 5.9 ± 0.5 cells/high power field [HPF], $P<0.01$) and remote zone: 2.2 ± 0.4 vs. 4 ± 0.6 vs. 4.4 ± 0.5 vs. 5.8 ± 0.9 cells/HPF, $P<0.01$) (Supplemental Figure 4).

DISCUSSION

There is a great need for effective therapies to treat ischemic heart disease as an alternative to heart transplantation, which faces significant limitations. However, attempts at achieving myocardial regeneration after ischemic tissue loss have achieved limited success. Stem cell based regenerative therapies in cardiac applications are hampered by low engraftment of transplanted cells. In this study, we demonstrate novel approaches to enhance the engraftment of transplanted BM stem cells as well as their homing. Cathelicidin related antimicrobial peptide, a naturally occurring peptide, enhanced the engraftment and therapeutic benefit of BMMNCs transplanted following AMI. Furthermore, direct application of homing factors, including both CRAMP and SDF-1 with an injectable hydrogel, enhanced the recovery of cardiac function and reduced remodeling and scar size after AMI. This is the first study to examine the enhancement of stem cell engraftment for myocardial regenerative therapies using the naturally occurring cathelicidins as priming factors.

Human and animal studies suggest limited engraftment of stem cells after their transplantation in acute and chronic ischemic heart disease (17). In acute ischemic injury, SDF-1 and homing chemokines are expressed in the heart (15,18,19). However, upregulated metalloproteinases and proteases lead to their degradation (6,7). Indeed, studies on mice lacking the expression of CXCR4 (SDF-1 receptor) demonstrated similar homing of transplanted BM stem cells compared to WT mice (20). In this study, we adopt a novel approach to address this limitation. CRAMP was shown to improve the response of BM hematopoietic stem cells to physiological concentration of SDF-1, attributed to enhanced incorporation of CXCR4 receptor in the lipid rafts of cell membrane (10). In our hands, BM-derived SKL cells incubated with CRAMP migrate more readily towards low gradients of SDF-1. *In vivo*, mice treated with BMMNCs preincubated with CRAMP showed significantly higher therapeutic benefit when compared to BMMNCs alone or placebo treatment. This benefit was most obvious in our functional analyses such as global cardiac function, cardiac adverse remodeling, and infarct size reduction. This functional recovery

was paralleled with smaller scar size as assessed by histological examination. Interestingly, our histological examination for transplanted BMMNCs at 5 weeks revealed small numbers of GFP+ cells in cardiac tissue, mostly in mice treated with BMMNCs incubated with CRAMP and none in animals treated with BMMNCs alone (Supplemental Figure 2). Nonetheless, angiogenesis, as manifested by capillary density, was significantly higher in the BMMNCs incubated with CRAMP group suggesting that while BMMNCs are short lived in the myocardium, they lead to enhanced angiogenesis and resulting functional improvements. Multiple studies have shown the beneficial effects of BMMNCs to be derived primarily from their secretory and paracrine function (25,26).

Multipotent stem cell populations are mobilized from the bone marrow into peripheral blood in response to AMI in both murine models and in patients as demonstrated by us and others (21–23). However, the rate of their homing to the hearts is a limiting factor to their beneficial effects (24). In the heart, BM stem cells play a role in enhancing cardiomyocyte renewal, cardiac stem cell (CSC) proliferation, and reduce apoptosis (25,26). Indeed, in our studies, we observed increased angiogenesis and smaller scar size in mice treated with HG impeded with CRAMP and SDF-1 compared to HG alone and control treated mice. HG by itself has been associated with enhanced angiogenesis and improved cardiac recovery which could be explained by the mechanical support and enhanced cardiac free wall integrity. However, the addition of CRAMP resulted in a significantly higher rate of capillary formation. This translated into improved cardiac functional recovery and reduction in scar size in these groups. Indeed, CRAMP has been associated with improved angiogenesis in other tissue injury models. Pfosser et al primed human endothelial progenitor cells (EPCs) with human cathelicidin (LL37) prior to their transplantation in ischemic limbs in a murine hind limb ischemia model. EPCs incubated with LL37 demonstrated improved therapeutic efficacy with enhanced collateral vessel formation and limb perfusion compared to EPCs alone. This effect appears to be achieved through the stimulation of the $\text{i}\kappa\text{B}$ and $\text{NF}\kappa\text{B}$ signaling pathways (27).

Most importantly, the findings presented herein have significant translational relevance. CRAMP administration can enhance the homing and engraftment of BM stem cells to cardiac tissue and increase their therapeutic utility. While incubating cells with CRAMP before transplantation improves their response to SDF-1 and homing, we believe the effect is rather transient. Utilizing biomaterials such as hydrogels carries multiple benefits. First, hydrogels provide mechanical support to wall integrity and reduce adverse remodeling (28). Second, the sustained release of homing factor over a 4–5-week period can enhance homing of mobilized cells for extended period compared to the current single cell transplantation models which could also explain the enhanced angiogenesis response in the remote zone. The use of biosynthetic materials has gained traction in clinical studies (29–31), and advances in biomaterial engineering promise more readily tunable hydrogel systems suited specifically for clinical application in myocardial therapies. Indeed, in our studies, the use of HG alone resulted in improved cardiac recovery, reduced adverse remodeling and smaller scars when compared to controls. These effects were enhanced through concurrent delivery of CRAMP to produce an effective off-the-shelf regenerative therapy, eliminating the need for prior cell sourcing. Our findings open the door to large animal and human studies utilizing this approach to improve outcomes in myocardial regenerative studies.

In conclusion, our findings demonstrate that cathelicidin-related antimicrobial peptides improve engraftment of BMMNCs to the heart following AMI. Augmenting BM cell engraftment correlated with the formation of new blood vessels and functional recovery. These novel findings on the cellular level were associated with functional cardiac recovery, reduced adverse remodeling, and decrease in scar size. Taken together, these data indicate that priming BM cells with naturally occurring cathelicidins can be beneficial in the early phase following cardiac ischemic injury resulting in significant cardiac structural and functional improvements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Dr. Abdel-Latif is supported by the University of Kentucky Clinical and Translational Science Pilot Award (UL1TR000117), the UK COBRE Early Career Program (P20 GM103527) and the NIH Grant R56 HL124266.

Dr. Nagareddy is supported by the NIH Pathway to Independence Award (1K99HL122505-01)

Dr. Ye is supported by the T32 grant (HL091812)

Dr. Ratajczak is supported by NIH grants 2R01 DK074720 and R01HL112788

Christopher B. Rodell is supported by an American Heart Association Predoctoral Fellowship.

Jason A. Burdick is supported by an American Heart Association Established Investigator Award.

Non-standard Abbreviations

AMI	Acute Myocardial Infarction
BM	Bone Marrow
BMMNCs	Bone Marrow mononuclear cells
CRAMP	Cathelicidin-related antimicrobial peptides
EF	Ejection Fraction
ESV	End Systolic Volume
LAD	Left Anterior Descending Artery
LV	Left Ventricle
PB	Peripheral Blood

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Figure 1. Treatment with the priming factor CRAMP enhanced the migration of SKL murine stem cells to low but physiological levels of SDF-1 in vitro

Migration of SKL (Sca1+, c-kit+, Lin-) murine stem cells (**Panel A**), with and without CRAMP incubation, toward SDF-1 (* $P < 0.05$ as compared with controls, $N = 3$). Incubation with CRAMP significantly enhanced the migration of SKL cells towards low, yet physiological, levels of SDF-1. However, incubation with CRAMP did not affect the migration of SKL cells towards high levels of SDF-1. **Panel B** demonstrates the effect of CRAMP incubation on CXCR4 surface expression. CXCR4 median fluorescence intensity and geometric means peaked after 3 hours of incubation with CRAMP. CRAMP; cathelicidin related antimicrobial peptide, SDF-1; stromal derived factor 1.

Figure 2. Ischemic cardiac tissue releases CRAMP

Tissue levels of CRAMP increased steadily after myocardial infarction, peaking around 10 days after injury (**Panel A**). Similarly, in a langendorff experiment, CRAMP expression in cardiac tissue increased in a similar manner to the expression of hypoxia induced factor alpha (HIF-1α). In tissue culture experiments, cardiac fibroblasts expressed CRAMP and HIF-1α genes in an ischemia/reperfusion model (72 hours of hypoxia followed by 2 hours reperfusion) (**Panel B**). CRAMP; cathelicidin related antimicrobial peptide, HIF-1α; hypoxia induced factor 1α, I/R; ischemia/reperfusion. Values are presented as mean ± SEM (* $P < 0.05$ as compared to sham/control, N=3).

Figure 3. Mice treated with CRAMP–preincubated BMMNCs demonstrated enhanced functional recovery of LV parameters following AMI

Echocardiographic analysis of left ventricular function and remodeling parameters.

Treatment with CRAMP-preincubated BMMNCs enhanced cardiac recovery after acute myocardial infarction induced by left coronary artery ligation, after 5 weeks. Left ventricular ejection fraction (**Panel A**), left ventricular end systolic volume (**Panel B**) and left ventricular end-diastolic volume (**Panel C**), were assessed before myocardial infarction, 48 hours, and 5 weeks after myocardial infarction. Values are presented as mean ± SEM (* $P < 0.05$ as compared to controls, $N = 12-15$ animals/group). BMMNCs; bone marrow mononuclear cells, CRAMP; cathelicidin related antimicrobial peptide, LVEF, Left Ventricular Ejection Fraction; LVESV, Left Ventricular End-Systolic Volume.

Figure 4. Treatment with CRAMP-preincubated BMMNCs reduced scar size following AMI Mice treated with CRAMP-preincubated BMMNCs showed significant reduction in cardiac scar size as assessed with mason-trichrome staining (**Panels A and B**). **Panel A** - representative short axis images of mason-trichrome stained sections at 5 weeks after AMI showing reduced scar size in the CRAMP-preincubated BMMNCs-treated arm, arrows indicate the boundaries of the scar. **Panel B**-Quantitative analysis of the scar size presented as a percentage of the left ventricular area. Values are mean $\pm$ SEM (* P <0.05 compared to control and values, N=7–15 animals/group). BMMNCs; bone marrow mononuclear cells, CRAMP; cathelicidin related antimicrobial peptide.

Figure 5. Mice treated with CRAMP-preincubated BMMNCs demonstrated increased capillary density

Capillaries in the peri-infarct risk area and remote zone of the LV were labeled with FITC-conjugated isolectin B4 and counted. **Panel A** - representative fluorescent confocal images of FITC-isolectin stained infarct border (risk zone) cross sections. **Panel B** - Quantitative analysis of capillary density demonstrating increased peri-infarct region capillary density in CRAMP-preincubated BMMNCs-treated mice after AMI. Values are mean $\pm$ SEM (* P <0.05 vs. PBS control, N=10–12 animals/group). Scale bars represent 50 μ m.

Figure 6. Hydrogels demonstrate sustained degradation and molecule release

Hydrogels (5.0wt%) were loaded with stromal derived factor 1 (SDF-1; 150ng/15uL), fluorescently labeled cathelicidin related antimicrobial peptide (CRAMP; 15μg/15uL), or both (SDF-1 + CRAMP). Cumulative release profiles of hyaluronic acid (HA, **Panel A**), SDF-1 (**Panel B**) and CRAMP (**Panel C**). For both CRAMP and SDF-1, no significant difference in release concentration was determined between individual vs combined release samples at any timepoint (N=4).

Figure 7. Mice treated with HG+SDF-1+CRAMP demonstrated enhanced functional recovery of LV parameters following AMI

Echocardiographic analysis of left ventricular function and remodeling parameters.

Treatment with HG+SDF-1+CRAMP enhanced cardiac recovery after acute myocardial infarction induced by left coronary artery ligation. Left ventricular ejection fraction (**Panel A**), left ventricular end systolic volume (**Panel B**) and left ventricular end diastolic volume (**Panel C**), assessed at 48 hours after and 5 weeks after myocardial infarction. Values are presented as mean $\pm$ SEM (** $P < 0.01$ as compared to controls, $N = 12-15$ animals/group). BMMNCs; bone marrow mononuclear cells, CRAMP; cathelicidin related antimicrobial peptide, HG; hydrogel, LVEF; Left Ventricular Ejection Fraction, LVEDV; Left Ventricular End-diastolic Volume, LVESV; Left Ventricular End-Systolic Volume, SDF-1; stromal derived factor 1.

Figure 8. Treatment with CRAMP–preincubated BMMNCs reduced scar size following AMI Mice treated with HG+SDF-1+CRAMP showed reduction in cardiac scar size as assessed with mason-trichrome staining (**Panels A and B**). **Panel A** - representative short axis images of mason-trichrome stained sections at 5 weeks after AMI showing reduced scar size in the HG-treated arms, arrows are indicating the boundaries of the scar. **Panel B**-Quantitative analysis of the scar size presented as percentage of left ventricular area. Values are mean $\pm$ SEM (N=5–15 animals/group). CRAMP; cathelicidin related antimicrobial peptide, HG; hydrogel, SDF-1; stromal derived factor 1.

Figure 9. Mice treated with HG demonstrated increased capillary density

Capillaries in the peri-infarct risk area and remote zone of the LV were labeled with FITC-conjugated isolectin B4 and counted. **Panel A** - representative fluorescent confocal images of FITC-isolectin stained infarct border (risk zone) cross sections. **Panel B** - Quantitative analysis of capillary density demonstrating increased peri-infarct region capillary density in all HG-treated mice after AMI. However, the changes in capillary density in the remote zone was not significant. Values are mean $\pm$ SEM (** $P < 0.01$ vs. vehicle control, $N = 3-5$ animals/group). Scale bars represent 50 μ m. CRAMP; cathelicidin related antimicrobial peptide, HG; hydrogel, SDF-1; stromal derived factor 1.