Hydrogels With Integrin-Binding Angiopoietin-1–Derived Peptide, QHREDGS, for Treatment of Acute Myocardial Infarction

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Background—Hydrogels are being actively investigated for direct delivery of cells or bioactive molecules to the heart after myocardial infarction (MI) to prevent cardiac functional loss. We postulate that immobilization of the prosurvival angiopoietin-1–derived peptide, QHREDGS, to a chitosan-collagen hydrogel could produce a clinically translatable thermoresponsive hydrogel to attenuate post-MI cardiac remodeling.

Methods and Results—In a rat MI model, QHREDGS-conjugated hydrogel (QHG213H), control gel, or PBS was injected into the peri-infarct/MI zone. By in vivo tracking and chitosan staining, the hydrogel was demonstrated to remain in situ for 2 weeks and was cleared in ≈3 weeks. By echocardiography and pressure–volume analysis, the QHG213H hydrogel significantly improved cardiac function compared with the controls. Scar thickness and scar area fraction were also significantly improved with QHG213H gel injection compared with the controls. There were significantly more cardiomyocytes, determined by cardiac troponin-T staining, in the MI zone of the QHG213H hydrogel group; and hydrogel injection did not induce a significant inflammatory response as assessed by polymerase chain reaction and an inflammatory cytokine assay. The interaction of cardiomyocytes and cardiac fibroblasts with QHREDGS was found to be mediated by β1-integrins.

Conclusions—We demonstrated for the first time that the QHG213H peptide–modified hydrogel can be injected in the beating heart where it remains localized for a clinically effective period. Moreover, the QHG213H hydrogel induced significant cardiac functional and morphological improvements after MI relative to the controls. (Circ Heart Fail. 2015;8:333-341. DOI: 10.1161/CIRCHEARTFAILURE.114.001881.)

Key Words: chitosan † collagen ‡ hydrogel ‡ myocardial infarction

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The acute phase after MI might be the most appropriate time to use hydrogel-based therapy because therein hydrogel injections can prevent cardiac remodeling, deliver cells to replace the damaged tissue, and recruit endogenous stem cells.3 Chitosan and collagen are natural, biodegradable, and bio-compatible polymers that have been explored for their potential use in the treatment of cardiac dysfunction.4,9 Typically, collagen and chitosan, alone or in combination, are cross-linked using exogenous, sometimes toxic, chemical cross-linkers to improve the hydrogel mechanical properties.4,10,11 However, we have previously shown that chitosan–collagen composites gel because of ionic interactions at physiological temperature and pH to form mechanically stable hydrogels that are appropriate for in vivo application.12 Furthermore, the collagen–chitosan interaction within the gels resembles the collagen–glycosaminoglycan interaction found in vivo in the extracellular matrix.13 Thus, chitosan–collagen may mediate physiological cell–matrix interactions.

The functional success of hydrogel-based cardiac and cell therapies can be improved by modifying biomaterials with bioactive molecules because bioactive molecules (cytokines,
growth factors, etc) have the potential to increase transplanted cell survival, reduce resident cell apoptosis, recruit desired regenerative cells, and promote stem/progenitor cell differentiation.\(^{14,15}\) One such bioactive molecules is the growth factor angiopoietin-1 (Ang1). In endothelial cells, Ang1 binds the Tie2 receptor\(^{16-18}\) but in cells such as neonatal rat cardiomyocytes that lack the Tie2 receptor Ang1 binds to integrins\(^{19}\) and activates prosurvival pathways.\(^{20}\) We identified the short sequence QHREDGS as the integrin-binding motif of Ang1, and the QHREDGS peptide was found to support cardiomyocyte attachment and survival similar to full-length Ang1.\(^{5,21}\) It is therefore possible that the QHREDGS peptide could retain/restore cardiac contractile function after MI by promoting cardiomyocyte survival. Importantly, the QHREDGS peptide is water-soluble, stable, fully synthetic with a precisely defined composition and does not require a specific orientation to function.

We therefore incorporated the prosurvival peptide QHREDGS into our chitosan–collagen hydrogel to design a novel hydrogel-based cardiac regenerative therapy. We used ≈85% deacetylated, chitosan-glutamate salt (UPG213), in the form of a linear polysaccharide. We covalently immobilize the QHREDGS peptide to this chitosan backbone using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC)/Sulfo-N-hydroxysulfosuccinimide chemistry. EDC is a zero-length cross-linker; therefore, no other chemical moieties aside from immobilization has been demonstrated, in previous studies, to both reduce the amount of bioactive molecules required and prolong signaling by stabilizing the receptor/ligand complex.\(^{22-26}\) In our previous study using the QHREDGS-modified chitosan–collagen hydrogel, we demonstrated increased cardiomyocyte survival in vitro culture and negligible hydrogel degradation therein, as well as in vivo biocompatibility in a subcutaneous model.\(^{12}\) This indicates that peptide release, which depends on hydrogel degradation, was not required for improved cardiomyocyte survival.

Based on these findings, we sought to investigate the effect of our QHREDGS-modified chitosan–collagen hydrogel in vivo after MI. We examined the in vivo biodistribution, localization, and lifespan of the hydrogel in the heart, and its effect on cardiac remodeling in a rat acute MI model at 6 weeks, when pathological remodeling is considered to be complete.\(^{27-29}\)

### Methods

All animal experimental procedures were approved by the Animal Care Committee of the University Health Network and the University of Toronto Committee on Animal Care, according to the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care.

For expanded Methods, see Data Supplement.

### Peptide-Modified Chitosan–Collagen Hydrogel (QHG213H)

QHG213H peptide (Biomatik) was conjugated to chitosan (UPG213, Novamatrix) using EDC chemistry as described previously.\(^{12}\) Briefly, 2.5-mg/mL 1:1 (mass:mass) chitosan–collagen hydrogel was prepared to produce control (no peptide) or QHG213H hydrogel (651±8 nmol peptide/mL gel) and kept on ice for ≤3 hours before use.

### In Vivo Studies

#### Animal model

Lewis rats (200–250 g) were obtained from Charles River Laboratories (Saint-Constant, QC, Canada), and MI was generated under general anesthesia by occluding the left anterior descending coronary artery as previously described.\(^{5,30,31}\) Three weeks after injection, rats were assessed by echocardiography and only those exhibiting 20% to 40% fractional shortening were included in the study.

#### Experimental Groups and Injection Timeline

Synges with PBS (MI-only), control gel (no peptide), or QHG213H (peptide-modified) hydrogel were prepared before surgeries as previously described.\(^{12}\) Treatments were randomized among the animals and the surgeon was blinded to the treatment given. Injections were performed using a 28-gauge needle (BD Biosciences) inserted into the peri-infarct left ventricle (LV) wall and directed toward the developing scar (MI zone). A total of 50 μL was injected into 3 locations immediately after left anterior descending ligation, before closure of the thorax.

#### Statistical Analysis

Statistical analysis was performed using SPSS Statistics 17.0 and GraphPad Prism 5.0. Differences between experimental groups were analyzed using 1- or 2-way ANOVA with Bonferroni post hoc tests unless otherwise specified. \(P<0.05\) was considered significant for all statistical tests. Results were plotted with GraphPad Prism 5.0.

### Results

#### In Vivo Hydrogel Degradation

DyLight800-conjugated hydrogel was injected into rat LVs. After 1 hour, animals were imaged and labeled hydrogel was seen to localize in the heart with unconjugated dye and gel washout evident in the chest (Figure 1A). After 24 hours, labeled hydrogel was visible in the heart but no longer in the chest. At 3, 5, 7, and 14 days after injection, labeled hydrogel was visible in the heart but the extent of staining decreased with time. By day 14, labeled hydrogel was only visible with increased exposure. To confirm heart localization and to quantify the amount of gel remaining, hearts were excised at 1 hour and 14 days and fluorescence intensity was measured. By day 14, ≈40% of the gel present at 1 hour remained in the heart (Figure 1B). Heart sections were also excised at 3 weeks and stained for chitosan, wherein ≈20% of animal hearts stained positive (Figure 1A).

#### Functional Data

All animals had similar echocardiograms before surgery. Left anterior descending ligation caused significant and progressive ventricular dysfunction, decreased fractional shortening, and increased LV internal diameter at systole and diastole at 3 and at 6 weeks after MI (Figure 2A–2C). Injection of the control gel significantly increased the cardiac shortening fraction and the LV internal diameter at systole compared with the MI-only group at 3 and 6 weeks, with additional improvements in these...
parameters with QHG213H gel injection (Figure 2A and 2B). Most of the decrease in fractional shortening for the MI-only and control gel groups occurred in the first 3 weeks after MI, as there was no significant reduction in fractional shortening between 3 and 6 weeks for either group, whereas the decrease was lesser and more gradual with QHG213H gel injection (Figure 2A and 2D). The LV internal diameter at diastole was not markedly affected by either treatment (Figure 2C).

At 6 weeks after MI, load-dependent measurements of ejection fraction, dP/dt max and dP/dt min were all significantly improved in the QHG213H gel group relative to either the control gel or the MI-only group (Figure 3A–3C). Control gel injection significantly improved these parameters relative to the MI-only group (Figure 3A–3C). The end-systolic LV volume was lowest in the QHG213H group, followed by the control gel group, which was significantly lower than the MI-only group (Figure 3D). No difference was observed in the end-diastolic LV volumes between groups (Figure 3E).

Load-independent measurements of end-systolic pressure–volume relationship (Figure 4A) and preload recruitable stroke work (Figure 4B) were also significantly improved with hydrogel injection relative to the MI-only group, and addition of the peptide further improved these parameters (Figure 4A and 4B).

**Gross Morphology and Histology**

Untreated MI-only hearts appeared larger than control and QHG213H gel–injected hearts, and the MI-only cross-sections showed the most remodeling (Figure 5A). The largest scar fractional area and the smallest scar thickness were in the MI-only group. The control gel group had significantly smaller scar areas and thicker scars than the MI-only group, parameters that were significantly improved in the QHG213H gel group (Figure 5B and 5C).

Differences in scar thickness and area were also evident by Masson’s trichrome staining, which showed more healthy (red) tissue in the MI zone of the QHG213H group relative to the controls (Figure 1A in the Data Supplement). Heart sections were stained for smooth muscle cell (marker of mature vasculature), CD31 and FVIII (endothelial cell marker for all
vasculature, including immature neovasculature; Figure IB–ID in the Data Supplement). The mean vessel density and diameter of all vasculature (FVIII+; Figure II in the Data Supplement, top) and mature vasculature (smooth muscle cell+; Figure II in the Data Supplement, bottom) were quantified in the MI and border zones. Although no difference in vascular metrics was

**Figure 3.** Load-dependent cardiac function and left ventricular (LV) volumes at 6 weeks after myocardial infarction (MI). Steady-state pressure–volume analysis was used for load-dependent cardiac functional analysis. **A** to **C**, Injection of the control gel significantly improved cardiac function compared with MI-only, and injection of the QHG213H gel further improved function. **D**, End-systolic LV volume was significantly lower in the QHG213H gel group vs either control, and in the control gel group vs MI-only group. **E**, The end LV diastolic volume was unchanged between groups. Data were analyzed by 2-way repeated measures ANOVA and expressed as mean±SD, n=7 per group. *P<0.05, **P<0.01 vs MI-only; #P<0.05 vs control gel.

**Figure 2.** Cardiac function after myocardial infarction (MI) measured by echocardiography. **A**, QHG213H gel injection significantly enhanced the %fractional shortening (%FS) at 21 days (3 weeks) and 42 days (6 weeks) after MI compared with the control gel and MI-only groups. The control gel injection induced a significant improvement in %FS relative to MI-only group. **B**, Injection of gel with or without peptide significantly reduced left ventricular systolic dimension (LVDS) at 3 and 6 weeks relative to MI-only. The QHG213H gel significantly improved the LVDS relative to the control gel. **C**, No significant difference in LV diastolic dimension (LVDD) was evident between groups. **D**, Repeated measures ANOVA results for factor days after injection for data from **A** to **C**. Data were analyzed by 2-way repeated measures ANOVA and expressed as mean±SD, n=7 per group. *P<0.01, **P<0.001 vs MI-only; #P<0.01 vs control gel.
seen in the MI zone or in the border zone FVIII+ vessels, there were significantly larger border zone smooth muscle cell+ vessels in the QHG213H gel group compared with the controls.

Cardiomyocyte Survival Mechanism

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and cardiac troponin-T staining was used to determine whether the functional and morphological improvements seen with control and QHG213H gel injection could be attributed to improved cardiomyocyte survival. Fewer TUNEL+ (apoptotic) cells were visible within the border zone of the control and QHG213H gel groups compared with the MI-only group (Figure 6A and 6B). In the MI zone, there was no difference in the apoptotic cell fraction among the treatment groups but the QHG213H gel group had a higher proportion of cardiac troponin-T+ cells (cardiomyocytes) than the controls (Figure 6C and 6D).

To gain further insight into the cardiomyocyte survival mechanism, RNA isolated from MI and border zone tissue excised 24 hours after injection was subjected to quantitative polymerase chain reaction. We did not observe any significant differences between the treatment groups, but there were some large fold changes that may warrant further investigation. Specifically, there was a 4.50- and 2.29-fold increase in interleukin-6 and interleukin-1β (proinflammatory cytokines), respectively, in the QHG213H gel compared with the MI-only group. Also, there was a 7.69-fold decrease in caspase-9 (proapoptotic), a 2.32-fold decrease in phosphatidylinositol-4,5-bisphosphate 3-kinase (prosurvival), and a 2.17-fold increase in B-cell lymphoma 2 (antiapoptotic) gene expression in the QHG213H gel compared with the MI-only group (Figure III in the Data Supplement). For all the aforementioned genes, peptide addition to the gel resulted in a larger fold change than the control gel with the exception of B-cell lymphoma 2, which was downregulated by control gel treatment and upregulated by QHG213H gel treatment (Figure III in the Data Supplement). We also performed an inflammatory cytokine array on the border zone tissues extracted at 24 hours.

Figure 4. Load-independent pressure–volume analysis at 6 weeks after myocardial infarction (MI). Occlusion pressure–volume analysis was used for load-independent cardiac functional analysis. Significant differences were seen in both (A) end-systolic pressure–volume relationship and (B) preload recruitable stroke work between the QHG213H gel group and either control, as well as between the control gel group and the MI-only group. Data are expressed as mean±SD, n=7 per group. *P<0.05.

Figure 5. Gross heart morphology at 6 weeks after myocardial infarction (MI). A. Representative whole heart and heart sections (from just below suture to apex of heart, 5 sections per heart) illustrate differences in gross morphology between groups (scale=1 mm). Significant differences in (B) fractional MI scar coverage and (C) MI scar thickness between the QHG213H gel and the control gel and MI-only groups, as determined from (A) gross section images. Data are expressed as mean±SD, n=7 per group. *P<0.05, **P<0.01 vs MI-only; #P<0.05 vs control gel.
hours but did not detect any significant differences in protein expression, indicating a lack of significant inflammatory reaction (Figure IV in the Data Supplement).

Western blot analysis was performed to determine whether integrin-linked kinase and mitogen-activated protein kinases (MAPK) were involved in the cardiomyocyte survival mechanism as both proteins have been reported to be upregulated by QHREDGS.33 In the border zone, integrin-linked kinase protein levels and MAPK phosphorylation did not differ between groups but there was a significant increase in MAPK protein expression in the QHG213H gel group relative to the controls (Figure V in the Data Supplement).

To identify the receptor mediating the cardiomyocyte–QHREDGS and cardiac fibroblast–QHREDGS interaction, major cell types in the native myocardium,34 polyethylene glycol (PEG) hydrogels were generated with and without immobilized QHREDGS peptide. PEG was used because it is nonfouling and nonadhesive in short-term culture. The peptide concentration in the PEG hydrogel was 11.7±4.5 mg/mL (9.7±3.7 nmol/L).35 The addition of QHREDGS to PEG significantly increased the number of adherent cells relative to PEG alone (Figure 7A and 7B). Preincubation of either cell type with an anti–β1-integrin antibody reduced adhesion to the QHREDGS-PEG hydrogel to that of PEG alone.

Discussion
Hydrogel degradation is most often characterized in vitro or by histological staining of excised in vivo samples36,37 because tracking hydrogels in the heart is extremely difficult because of the dynamic nature of the beating heart, the depth of the heart within the animal, and the small volume of material injected therein (≈20–200 μL).36,38 The near-infrared dye

![Figure 6](https://circheartfailure.ahajournals.org/)

Figure 6. Apoptosis at 6 weeks after myocardial infarction (MI). Heart sections were stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; red), cardiac troponin-T (cTnT; green), and 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (blue). A, Representative images showed fewer TUNEL+ cells in the control gel and QHG213H gel groups than the MI-only group in the border zone (scale bars, 100 μm). B, Quantification of the fraction of TUNEL+ cells (number of TUNEL+ cells/number of DAPI+ cells) in the border zone. The control and QHG213H gel groups had a significantly smaller TUNEL+ cell fraction relative to the MI-only group. C, There was no difference in the TUNEL+ cell fraction between groups in the MI zone. There was a significant increase in cTnT expression in the QHG213H group relative to either control. Data are expressed as mean±SD, n=7 per group. *P<0.05, **P<0.01.

![Figure 7](https://circheartfailure.ahajournals.org/)

Figure 7. Cardiac cell attachment to polyethylene glycol (PEG) and PEG-QHREDGS gels. Rat neonatal rat cardiomyocytes (NMs; A) or cardiac fibroblasts (B) were seeded onto PEG-only (no peptide) or PEG-QHREDGS disks with or without preincubation with an anti–β1-integrin (CD29) antibody. After 50 minutes, the attached cells were counted. The data are normalized to the PEG-only gels. Both NCMs and cardiac fibroblasts have significantly more attached cells to PEG-QHREDGS surfaces over PEG alone, but the difference is abolished when preincubated with anti–β1-integrin antibody. Data are expressed as mean±SE, n=6 per group. **P<0.01.
DyLight800 and the Kodak In-Vivo FX Pro Imaging System were used in this study to provide online information on hydrogel localization and lifespan. Near-infrared dyes permit deep tissue imaging without affecting the surrounding tissue; a potential advantage in comparison with the long-term use of radiolabels or the use of contrast agents (eg, iron oxides or gadolinium), which might be taken up by macrophages, skewing the degradation profiles, although PET and MRI imaging have much higher resolution.

By imaging the injected labeled hydrogel for 14 days, we confirmed localization and retention of the hydrogel within the heart (Figure 1A). We were unable to perform quantification because the 2-dimensional imaging system could not provide volumetric information for the injected material and images were distorted by heart movement over the 40-second imaging period. Therefore, we quantified degradation from excised tissue samples using the assumptions: (1) the bulk of unbound dye was removed from the gel by 1 hour after injection and (2) the gel present at 1 hour after injection represented the total amount of successfully localized gel. We determined that ≈60% of the gel was degraded/removed from the heart by day 14 (Figure 1B).

Notably, there was no observable fluorescent signal in the processing organs (eg, kidneys or liver) 24 hours after injection. The collagen and chitosan-Dylight800 that leaked into the chest cavity before gelation (Figure 1, first panel) was cleared in 24 hours (Figure 1, second panel). These results are consistent with reports that polysaccharides (dextrans and pullulans of 5–850 kDa) are cleared in under 24 hours.28 Even the largest sugars had a tissue clearance rate of 10 μL/h and an excretion rate of 100 μL/h, which suggests that a polysaccharide-like chitosan (150–600 kDa) would be cleared in hours, thus further motivating its incorporation into a stable hydrogel. In addition, fluorescein isothiocyanate-labeled chitosan (≈100 kDa) injected directly into murine peritoneum was found to rapidly distribute to the urine and kidneys.40 Within 1 hour, ≈25% of the injected chitosan was detected in the urine.40 By 14 hours, chitosan was completely absorbed from the peritoneal cavity, >90% of the injected chitosan was detected in the urine and <6% remained in the kidneys.40

On gelation, the hydrogel would have undergone degradation then clearance. Because of the absence of exogenous crosslinking, hydrogel degradation and chitosan release are dependent on the digestion of the collagen fibers holding the hydrogel together. The degraded collagen and chitosan would then be quickly cleared and expelled from the body.

We injected into the peri-infarct and MI-border region to prevent the damaged area from expanding. In rat and dog studies, cardiomyocyte apoptosis was shown to continue through the first 4 months after MI, which correlated with deterioration of pump function.38,39 We performed TUNEL staining at 6 weeks to determine whether there was expansion of the scar and border zone, which would induce apoptosis in the expanding region. Injection of either the control or QHG213H gel attenuated apoptosis in the border zone (Figure 6). We observed no difference in the MI zone (scar) with the treatments; however, by 6 weeks the scar tissue was fully formed and nonapoptotic.

Importantly, the amount of QHG213H hydrogel that localized in the heart and its lifespan therein was sufficient to improve cardiac functional outcomes relative to the controls at 6 weeks after MI (Figures 2, 3 and 4). Specifically, QHG213H gel treatment resulted in a 62% improvement in ejection fraction and a 35% improvement in fractional shortening from the MI-only group (Figures 2 and 3), which translates into an improvement in ejection fraction and fractional shortening from severe to mild impairment, on a normal–mild–moderate–severe scale of impairment.42 At 6 weeks after MI, we observed a significant decrease in the systolic dimension and volumes but did not find any difference in the diastolic parameters. This may be because while systolic function decreases significantly because of cardiomyocyte necrosis after MI, a longer time frame (eg, 12 weeks) may be required to observe changes in diastolic parameters because of matrix remodeling and scar expansion.

One explanation for the functional results is that the QHG213H hydrogel stabilized the infarct wall and increased its thickness thus altering the surrounding tissue properties and reducing wall stress. This is unlikely because the storage modulus of the control and QHG213H gels were measured at ≈55 and ≈45 Pa, respectively, well below the stiffness of native rat myocardium (1–140 kPa, diastole to systole).42,43 Also, there was no difference in end LV diastolic volume between groups, which would be expected if the hydrogel was aiding LV mechanics and altering the geometry (Figure 3).

Alternatively, the QHG213H hydrogel may act at the cellular level to promote survival. We investigated the number of cardiomyocytes and apoptotic cells in the various areas of the infarct at 6 weeks. There was significantly more MI zone cardiomyocytes in the QHG213H gel group compared with controls. There was no difference in apoptotic cells in this same region (Figure 6). It is possible that the increased cardiomyocyte numbers in the QHG213H injected group were because of the increased size of the mature vessels in the QHG213H gel group border zone (Figure 2 II in the Data Supplement). By delivering more nutrients and oxygen to the infarcted tissue, these vessels may have promoted cell survival. Interestingly, apoptosis was significantly reduced in the control and QHG213H gel group border zone compared with MI-only (Figure 6). The chitosan–collagen gel may therefore have a long-term prosurvival effect on the progressing front of apoptosis after MI.

We observed no difference in total vascularization (neo- and mature) but a significant increase in large (smooth muscle cell+ ) vessels within the border zone with QHG213H gel treatment. This may be because Ang1 angiogenic effects are mediated through the receptor Tie2, whereas the peptide QHREDGS is the Ang1 integrin-binding site30 and may not elicit identical angiogenic responses. Other Ang1-derived peptides (eg, vasculotide) have been demonstrated to bind Tie2 and are angiogenic.44 Moreover, the Ang1–cardiomyocyte interaction is integrin dependent and Tie2 independent46; whereas QHREDGS has been demonstrated to interact with integrins on endothelial cells (αvβ3 and αvβ5) and induced pluripotent stem cells (β3-type) to promote cell adhesion and survival.33,42 Thus, the QHG213H gel treatment may have promoted the survival of existing vessels after MI.

We also investigated the acute phase (24 hours) after MI wherein critical apoptotic and immune/inflammatory
responses predominate. Both our polymerase chain reaction (Figure III in the Data Supplement) and inflammatory cytokine array data (Figure IV in the Data Supplement) indicated no significant difference in the cytokines measured. This suggests that the hydrogel treatments did not elicit an inflammatory response that differs significantly from PBS injections after MI within the first 24 hours after an MI.

We have recently shown that the integrin subunits α<sub>c</sub>, β<sub>1</sub>, and β<sub>3</sub>, and the downstream effectors integrin-linked kinase and MAPK are involved in QHREDGS-mediated prosurvival pathways in stem cells and endothelial cells. Although we did not detect upregulation of either of these genes at 24 hours (Figure III in the Data Supplement) or of integrin-linked kinase protein or MAPK phosphorylation between groups, we did observe a significant increase in MAPK protein expression in the QHG213H gel group compared with the controls (Figure V in the Data Supplement). Timing of the assessment is critical as phosphorylation and translation can occur within minutes to hours, whereas transcription occurs within hours to days. Once again, an extensive and individualized time course for each event would be required to fully delineate the mechanism by which the QHG213H gel promotes cardiomyocyte survival after MI.

By conjugating QHREDGS to a PEG hydrogel, we found that both the cardiomyocytes and the cardiac fibroblasts interact with QHREDGS through β<sub>1</sub>-integrins (Figure 7). We have previously demonstrated that QHREDGS interacts with endothelial cells through α<sub>c</sub>β<sub>1</sub> and α<sub>c</sub>β<sub>3</sub> integrins to promote cell survival. It has also been reported that Ang1-mediated effects on monocyte adhesion and chemotaxis are both Tie2 and integrin independent. Hence, QHREDGS may bind and promote the survival of cardiomyocytes, cardiac fibroblasts, and endothelial cells through integrin-binding but may not affect monocyte/macrophage function, which would account for the absence of a significant inflammatory response.

Conclusions

This is the first study to evaluate the effect of the Ang1-derived peptide QHREDGS on remodeling after MI. Injection of the QHG213H hydrogel into the LV of rats with acute MI showed significant improvements in cardiac morphology and functionality at 6 weeks relative to the controls. Scar thickness improved by 53%, fractional scar area decreased by 34%, fractional shortening improved by 35%, and ejection fraction improved by 62% compared with the MI-only group. The injected hydrogel was demonstrated to remain in situ for 14 days and was ≈80% cleared by 3 weeks. Based on our results, cardiac cells bind via β<sub>1</sub>-integrins to the QHREDGS peptide. The novelty of our work lies in the expanded insight into the integrin-mediated effects of the Ang1-derived peptide, QHREDGS, on cardiac cells after MI in vivo. The developed hydrogel, collagen:chitosan mixture with covalently immobilized QHREDGS, could form the basis of a new cardiac therapy that is fully chemically defined based on biocompatible and biodegradable molecules.

Acknowledgments

We thank Dr Peter Liu for helpful discussions.

Sources of Funding

This work is funded by the Heart and Stroke Foundation GIA T5946, the Canadian Institutes of Health Research (CIHR) Operating Grant (MOP-126027), Natural Sciences and Engineering Research Council of Canada (NSERC)-CIHR Collaborative Health Research Grant (CHRPJ 385981-10), NSERC Discovery Grant (RGPIN 326982-10), NSERC Discovery Accelerator Supplement (RGPAS 396125-10), and National Institutes of Health grant 2R01 HL076485. L.A. Reis is funded by the NSERC PGS-D Fellowship.

Disclosures

None.

References

Ischemic cardiomyopathy is a major clinical problem worldwide and cytokine therapy for angiogenesis is an effective treatment for those patients with diffuse vascular abnormality. Here, we presented a potential therapy for ischemic cardiomyopathy that relied on the use of a hydrogel and the angiopoietin-1-derived peptide QHREDGS. The rationale for this combination of materials is that the hydrogel, designed for possible noninvasive intramyocardial application, can localize to the desired site in the heart. Relying on the cardioprotective properties of angiopoietin-1, the novel peptide sequence, QHREDGS, was selected because of its ability to promote survival of cardiomyocytes in an integrin-dependent manner. Our research data suggested that application of the peptide-modified hydrogel attenuated pathological ventricular remodeling and improved cardiac function, which has a potential for clinical application.
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_Circ Heart Fail._ 2015;8:333-341; originally published online January 28, 2015;
doi: 10.1161/CIRCHEARTFAILURE.114.001881
_Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/8/2/333

Data Supplement (unedited) at:
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Supplementary Material
1. Supplementary Material

Supplementary Methods:

1.1. Peptide-modified polyethylene glycol (PEG-QHREDGS)

QHREDGS peptide was conjugated to polyethylene glycol (PEG) as previously described \(^1\), with some modification. Briefly, 8.0mg acrylate-PEG-NHS was incubated with or without 29mM (24mg/mL) QHREDGS solution in a final volume of 200µL. To the lyophilized powder, 80mg polyethylene glycol diacrylate and 0.8mg 2-Hydroxy-2-methyl-propiophenone was added; 150µL of this solution was added to a circular silicon mold (11mm diameter, 1mm thick) placed atop a glass microscope slide and a polyvinyl coverslip was placed on top of the mold. After 1h at 4°C the cover-slip was gently peeled off the hydrogel, the mold was removed, the hydrogel was sterilized, then placed in sterile PBS for 1h at 37°C.

NCMs or cardiac fibroblasts, isolated as previously described \(^2,3\), were washed and resuspended in equal volumes of PBS or 50µg/mL anti-β\(_1\)-integrin antibody (α-CD29; BD Biosciences, Mississauga, ON) and incubated at 37°C for 20min. The NCMs or cardiac fibroblasts were spun for 5min at 200g, resuspended in CM media, seeded onto the PEG-hydrogels (22,000 cells per hydrogel) in a volume of 10µL and incubated at 37°C for 50min. After 50min, the hydrogels were washed, fixed, stained and imaged as previously described \(^1\).

1.2. In vivo studies

1.2.1. Assessment of cardiac function (6 Week time point)

N=7 animals per group were used for a long term 6 week study. Cardiac function was evaluated by echocardiography (Sequoia C256 System, Siemens Medical; 15 MHz linear array transducer) before MI (pre-ligation baseline), 3 and 6 weeks post-MI. M-mode images were
obtained in the parasternal short-axis view at the level of the papillary muscles. The measurements were performed by a single, blinded examiner. LV internal diastolic dimension (LVIDd) and internal systolic dimension (LVIDs) were measured. Three consecutive cardiac cycles were recorded and averaged. Percent fractional shortening (%FS) was calculated as follows: 

\[
\%FS = \frac{\text{LVIDd} - \text{LVIDs}}{\text{LVIDd}} \times 100
\]

The 6 week time point was selected as it is assumed the heart has completed remodeling by this time. PBS, Control gel (no peptide), or QHG213H (peptide-modified) hydrogel was injected into 3 locations in the LV wall (50µL total) surrounding the infarct using a 28-gauge needle. End (6 weeks after treatment) cardiac function was assessed with a pressure-volume catheter, as described. Briefly, the rats were anesthetized and intubated with mixed oxygen and room air by a rodent ventilator (Harvard apparatus, Canada). A pressure-volume catheter-tipped pressure transducer (2F, Millar Instruments, USA) was inserted into LV cavity via right carotid artery. Volumes were measured by conductance, and end-systolic and -diastolic values were recorded. Ejection fraction, dP/dt Max, and dP/dt Min were measured, and end systolic pressure-volume relationship (ESPVR) and preload recruitable stroke work (PRSW) were calculated.

After pressure-volume analysis, hearts were perfusion fixed with 10% formaldehyde, excised, and sectioned into five 2-mm thick slices as previously described.

### 1.2.2. 24h time point

N=6 animals per group were used in a short 24h study to elucidate the mechanism by which the injected hydrogel preserved cardiac function. Surgery and injections were performed in the same manner as described above and animals were monitored overnight. The animals were anaesthetized 24h after injection with isoflurane, intubated, ventilated, and maintained with 2% isoflurane. The heart was accessed in the same manner as performed for LAD surgery and
arrested using 10% KCl injected directly into the LV. Hearts were then immediately excised and
the right ventricle and atria were removed. The LV was then sectioned into a MI region, border
region, and remote region. These sections were placed in separate cryo-tubes and snap frozen in
liquid N₂. Frozen tissues were subsequently ground using standard mortar and pestle with liquid
N₂ and kept at -80°C prior to RNA extraction using TRIZOL reagent according to
manufacturer’s instructions (Life Technologies).

1.2.3. Hydrogel lifespan

To assess the in vivo lifespan of our hydrogel, chitosan was conjugated with the near-infrared
dye DyLight800 (Thermo Scientific, Waltham, MA) and injected into the heart of live animals.
Briefly, 1 vial of DyLight800 label (suitable for 1mg of protein) was dissolved in 57.7μL PBS
and mixed immediately with 92.3μL of chitosan at 20mg/mL in 0.9% normal saline (final
reaction volume of 150μL and chitosan concentration of 12.31mg/mL). The mixture was
protected from light, placed on a microtube shaker at 650rpm for 1h, and then stored at 4°C until
use. Upon use, the DyLight800-labelled chitosan was used to make hydrogels as per the standard
protocol 6, kept on ice, and then 50μL of the labelled gel was injected directly into 3 locations of
the healthy LV wall of N=7 Lewis rats. Rats (anesthetized under isoflurane) were then imaged
using a Kodak In-Vivo FX Pro Imager (Kodak Molecular Imaging Systems), with a 5sec
exposure X-ray image overlaid with a 40sec exposure fluorescent image (760nm excitation,
830nm emission). Full-sized images were processed to illustrate fluorescent decrease over time
by applying identical threshold and gamma adjustment to all images using Kodak MI 4.0
software (Kodak Molecular Imaging Systems). Inset images were processed to highlight
fluorescent localization by applying the optimal brightness/contrast and threshold to each
individual image using ImageJ (NIH). N=3 rats were sacrificed 1h after injection, and N=4 were

Supplementary Material 3
monitored up to 14 days post injection. Excised hearts were stored at -80°C until use, at which time they were homogenized and fluorescently imaged to quantify degradation.

### 1.3. Immunohistochemistry

Paraffin embedding and sectioning was done on samples prepared as described above by the Pathology Research Program (PRP) at the University Health Network. Additionally, they performed immunohistochemical staining for hematoxylin and eosin (H&E), Mason’s trichrome, smooth muscle actin (SMA), Factor VIII (FVIII), and CD31. Sections were also stained for the presence of chitosan using a published protocol based on Cibacron Brilliant Red-3BA (CBR-3BA, Sigma Aldrich) and Weigert’s Iron Hematoxylin.

Cardiac troponin-T (cTnT) and TUNEL staining was done on sections deparaffinised and rehydrated with subsequent baths of 100% xylene, 100% EtOH, 95% EtOH, 75% EtOH, and distilled water (3 baths each for 3min per change). Antigen retrieval was through microwaving, immersion in Tris-EDTA buffer for 5min at room temperature (RT), permeabilization with 0.1% Triton-X, 0.1% Sodium Citrate in Tris-buffered saline (TBS) solution (10min, RT), and blocked with Dako Serum Free Protein Block (Dako Canada, Inc., Burlington, ON X0909; 30min, RT). After each step 3x3min TBS washes were performed. Primary antibody (mouse monoclonal cTnT antibody, Thermo Scientific MS-295-P, diluted 1:200) was applied for 2h at RT, followed by 3x3min TBS wash, and then secondary antibody (Alexafluor 594 goat anti-mouse IgG (H+L), Invitrogen) at 1:400 dilution for 1h at RT. TUNEL label and enzyme (Roche Applied Science, Indianapolis, IN, 11767291910) were applied as per manufacturer’s instructions and DAPI (Sigma Aldrich) nuclear stain was applied at a 1:1000 dilution for 10min. Sample were mounted with Dako Fluorescence Mounting Medium (Dako Canada, Inc., S3023), and imaged.

### 1.4. Quantitative PCR
Whole heart or heart sections frozen at -80°C were ground using a mortar and pestle and liquid N$_2$. TRIZOL Reagent was used for RNA extraction, following the manufacturer’s protocol. cDNA synthesis and qPCR were performed as previously described$^8$. All oligonucleotide sequences used are listed in **Supplementary Table 1**. Data was standardized between samples to reflect gene expression changes of the progressing scar, quantifying gene expression in the border zone compared to the established MI zone. Fold changes in gene expression between groups were analyzed and genes showing a >2-fold or <0.5-fold change in the QHG213H gel group over the MI Only (PBS) injection group were considered significant.

**1.5. Western Blotting & Inflammatory Cytokine Array Analysis**

Whole heart or heart sections frozen at -80°C were ground using a mortar and pestle and liquid N$_2$. Protein was isolated using Pro-Prep Protein Extraction Solution (iNtRON Biotechnology, Inc., Seongnam, KR) according to the manufacturer’s instructions. Western blots were performed as previously described$^1$.

Protein isolated from border zone tissues and used for Western blots were also analyzed using a rat inflammatory cytokine array kit (RayBio, AAR-CYT-1-8) as per the manufacturer’s protocol. Assayed array membranes were exposed as described for western blots, and data was collected by performing densiometry using the Dot Blot Analyzer plug-in in ImageJ.

**1.6. Image analysis techniques**

Morphometric analysis was performed as described previously$^4,5$. TUNEL/DAPI cell counts were made using a macro developed in Olympus CellSens Dimension software and tested on control images. TUNEL/cTnT MI border zone quantification was based on 2 independent images on either side of the MI zone in each animal, and MI zone quantification performed by averaging at least 4 independent images within the scar. All counts and normalizations were performed for
each image and averaged to get a single value per sample. Quantifying differences in vasculature in the MI zone and border zone of hearts excised 3 and 6 weeks post-MI was done using SMA and FVIII stained sections as described previously \(^9\).

Automation of the image quantification process eliminates human errors and bias between images, ensuring consistent quantification among all images and more accurate results.
Supplementary References


Supplementary Figure Captions:

**Supplementary Figure 1: Histological staining 6 weeks post-MI**
(A) Mason’s trichrome, (B) smooth muscle actin (SMA), (C) CD31, and (D) Factor VIII (FVIII) stained sections of hearts excised 6 weeks post-MI. Black outlined sections in (A) are enlarged in (B-D). A large collagenous scar is seen in all groups (A), but there appears to be more SMA, CD31 and FVIII staining in the Control gel and QHG213H gel groups compared to MI Only (PBS) group, suggesting increased vascularization (B-D). Scale bars = 1 mm (A), 50 µm (B-D), N=7/group.

**Supplementary Figure 2: Vascularization at 6 weeks post-MI**
Vascularization within the MI and border zones was quantified at 6 weeks from heart cross sections. Vessel density and diameters were quantified from SMA stained sections to assess mature vasculature, and from FVIII stained sections for neovascularization. The QHG213H gel group had significantly larger mature SMA+ vessels in the border zone. Data expressed as Mean±SD; N=7/group.

**Supplementary Figure 3: qPCR of MI and border zone tissues from hearts excised 24h post-MI**
Hearts subjected to MI and immediate treatment were excised 24h later, sectioned into MI, border, and remote regions, snap frozen then ground and used for qPCR. Overall, 27 genes conventionally associated with apoptosis, necroptosis (Necrop), survival, anti- and pro-inflammatory responses, as well as cardiac specific genes were assessed. Data was standardized in samples to reflect changes in gene expression in the progressing border zone to the established MI zone. No significance was determined between groups. Data expressed as Mean±SD, N=5/group.

**Supplementary Figure 4: Cytokine expression in border zone tissues from hearts excised 24h post-MI**
Inflammatory cytokine expression analysis within border zone tissues revealed no significant differences in any of the 19 cytokines analyzed (select data shown). Data expressed as Mean±SD, N=5/group.

**Supplementary Figure 5: Western blot analysis of border zone tissue from hearts excised 24h post-MI**
Western blot analysis of border zone heart tissues showed no differences in the levels of ILK or phospho-MAPK (PMAPK) protein expression between groups. The MAPK protein expression was significantly increased in the QHG213H gel group compared to the Control gel and MI Only (PBS) groups. Data expressed as Mean±SD, * P<0.05, N=5/group.
## Supplementary Tables:

### Supplementary Table 1: Genes and primers used in qPCR

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<tr>
<th>Gene</th>
<th>Abbr.</th>
<th>Forward</th>
<th>Reverse</th>
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Supplementary Figures:

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Supplementary Figure 2: Vascularization at 6 weeks post-MI
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<table>
<thead>
<tr>
<th>Gene</th>
<th>QH/MI</th>
<th>QH/Cont</th>
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