Cardiac disease is a major national health concern with ≈15.4 million Americans having coronary heart disease and 5.1 million having heart failure, while incurring an annual cost of $300 billion. As interventions for acute cardiac events have evolved and become more effective, therapies aimed at mitigating the progression to heart failure have failed to keep pace. This void has led to the exploration of a variety of cell- and cytokine-driven vasculogenic and regenerative strategies. Preclinical studies of these approaches have yielded promising results, whereas early clinical trials have been encouraging but with more modest outcomes. As a result, it is necessary to build on these initial studies to enhance the translation to clinical therapeutics.

Neuregulin-1β (NRG) is a member of the epidermal growth factor family that plays a critical role in cardiomyocyte development and proliferation. Systemic administration of NRG demonstrated efficacy in cardiomyopathy animal models, leading to clinical trials using daily NRG infusions. This approach is hindered by requiring daily infusions and off-target exposure. Therefore, this study aimed to encapsulate NRG in a hydrogel to be directly delivered to the myocardium, accomplishing sustained localized NRG delivery.

**Background**—Neuregulin-1β (NRG) is a member of the epidermal growth factor family possessing a critical role in cardiomyocyte development and proliferation. Systemic administration of NRG demonstrated efficacy in cardiomyopathy animal models, leading to clinical trials using daily NRG infusions. This approach is hindered by requiring daily infusions and off-target exposure. Therefore, this study aimed to encapsulate NRG in a hydrogel to be directly delivered to the myocardium, accomplishing sustained localized NRG delivery.

**Methods and Results**—NRG was encapsulated in hydrogel, and release over 14 days was confirmed by ELISA in vitro. Sprague-Dawley rats were used for cardiomyocyte isolation. Cells were stimulated by PBS, NRG, hydrogel, or NRG-hydrogel (NRG-HG) and evaluated for proliferation. Cardiomyocytes demonstrated EdU (5-ethynyl-2'-deoxyuridine) and phosphorylated histone H3 positivity in the NRG-HG group only. For in vivo studies, 2-month-old mice (n=60) underwent left anterior descending coronary artery ligation and were randomized to the 4 treatment groups mentioned. Only NRG-HG–treated mice demonstrated phosphorylated histone H3 and Ki67 positivity along with decreased caspase-3 activity compared with all controls. NRG was detected in myocardium 6 days after injection without evidence of off-target exposure in NRG-HG animals. At 2 weeks, the NRG-HG group exhibited enhanced left ventricular ejection fraction, decreased left ventricular area, and augmented borderzone thickness.

**Conclusions**—Targeted and sustained delivery of NRG directly to the myocardial borderzone augments cardiomyocyte mitotic activity, decreases apoptosis, and greatly enhances left ventricular function in a model of ischemic cardiomyopathy. This novel approach to NRG administration avoids off-target exposure and represents a clinically translatable strategy in myocardial regenerative therapeutics. (Circ Heart Fail. 2014;7:619-626.)

**Key Words:** heart failure ■ ischemia ■ molecular biology ■ muscle cells ■ myocardial infarction

Cardiac disease is a major national health concern with ≈15.4 million Americans having coronary heart disease and 5.1 million having heart failure, while incurring an annual cost of $300 billion. As interventions for acute cardiac events have evolved and become more effective, therapies aimed at mitigating the progression to heart failure have failed to keep pace. This void has led to the exploration of a variety of cell- and cytokine-driven vasculogenic and regenerative strategies. Preclinical studies of these approaches have yielded promising results, whereas early clinical trials have been encouraging but with more modest outcomes. As a result, it is necessary to build on these initial studies to enhance the translation to clinical therapeutics.

**Clinical Perspective on p 626**

Neuregulin-1β (NRG) is a member of the epidermal growth factor family that plays a critical role in the development of neuronal, epithelial, and cardiac cells. It is a ligand to the ErbB4 tyrosine kinase receptor, activating an intracellular signaling cascade that leads to proliferation, differentiation, and migration. Importantly, the ErbB4 receptor is present in adult cardiomyocytes. The significance of NRG’s potential cardioprotective effects was highlighted in the clinical trials.
for trastuzumab, in which cardiac dysfunction was noted a coveted adverse effect. Trastuzumab is a monoclonal antibody that inhibits the ErbB receptor and disrupts the NRG–ErbB signaling pathway. More recently, it has been found that trastuzumab impairs human resident cardiac stem cells. These findings support the notion that activation of the NRG–ErbB pathway critically enhances cardioprotection.

From a therapeutic perspective, NRG has shown great promise in preserving cardiac function and inducing regeneration after myocardial injury. This has been demonstrated in small and large animal models; however, multiple systemic intravenous infusions of NRG were required. This model of multiple parenteral infusions was translated to early clinical trial in which a modest benefit in acute and chronic hemodynamics was observed in patients with stable heart failure receiving daily NRG infusions for 11 days. In addition, a phase II double-blind study demonstrated improvements in left ventricular (LV) ejection fraction and ventricular geometry using daily infusions of NRG for 10 days. The clear drawback of systemic NRG administration, however, is the significant potential for adverse effects and the consequent dosing limitations.

To improve on this strategy, our group hypothesized that targeted and sustained intramyocardial delivery of NRG would yield NRG’s known cardioprotective and regenerative effects, while avoiding systemic exposure and obviating the need for multiple infusions. We sought to accomplish this by using an established bioengineered hydrogel system to encapsulate NRG for sustained and localized intramyocardial delivery in a murine model of ischemic cardiomyopathy.

Methods

Macromer Synthesis

Sodium hyaluronate (74 kDa, Lifecore) was modified with hydroxyethyl methacrylate (HEMA) to incorporate a terminal methacrylate group for free-radical–initiated cross-linking and ester bonds to introduce hydrolytic degradation. Briefly, HEMA was reacted with succinic anhydride via a ring opening polymerization in the presence of N-methylimidazole to obtain HEMA-COOH, which was coupled to a tetrabutylammonium salt of hyaluronic acid in the presence of 4-dimethylaminopyridine. The resulting hyaluronic acid macromer with HEMA group modification (HEMA-HA) was purified via dialysis, lyophilized, and the percentage of HA dissociation modified with an HEMA group was determined to be ≈15% using 1H nuclear magnetic resonance.

Hydrogel Gelation and NRG Encapsulation With Release

To form hydrogels rapidly on injection into myocardium, a 2-component redox initiator system consisting of ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine was used. Ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine were added to a final concentration of 10 mmol/L in a 4% (wt/vol) HEMA-HA solution and kept on ice. The kinetics of gel formation were characterized with rheometry at 37°C by monitoring the storage (G′) and loss (G″) modulus with time, while applying oscillatory strain (20 mm Hz). Confocal microscopy

Animal Model

Myocardial infarction was induced in 60 mice using an established and highly reproducible model. Briefly, the mice were anesthetized in a 2-L induction chamber (VetEquip, CA) and 3% isoflurane was administered. A 20-gauge angiocatheter was used for endotracheal intubation and connected to mechanical ventilation (Hallowell EMC, MA) where 2% isoflurane was maintained throughout. A thoracotomy was performed through the left fourth intercostal space, the heart was exposed, and an 8-0 polypropylene suture was placed around the left anterior descending artery 2 mm below the left atrium producing an anterolateral myocardial infarction. The animals were randomized into 4 stimulation groups: PBS alone, NRG alone, hydrogel (HG) alone, or NRG-HG. This time point is labeled as t=0 days. The media was changed daily. The hydrogels were kept suspended over the cells in a 3-μm pore size transwell cell culture insert. By doing so, the daily medium changes did not wash away the hydrogel, which could continue to release NRG into the fresh medium. In the NRG alone group, the NRG was not replaced after the first medium change.

Cardiomyocyte Immunohistochemistry and Confocal Microscopy

Cell proliferation was tested using immunohistochemistry at t=6 days. Wells tested using the Click-iT EdU Imaging Kit (Life Technologies, Carlsbad, CA) required incubation of the EdU reagent at t=4 days at a 1 μmol/L concentration. On t=6 days, all wells of the plate were washed with PBS and fixed with 4% paraformaldehyde. The cells were permeabilized using 0.5% Tween-20. Wells tested for EdU were incubated with the EdU antibody and washed with rinse buffer. Cells were then stained with DAPI (Life Technologies) and imaged using a Zeiss LSM 710 confocal microscope (Oberkochen, Germany).

Animal Care and Biosafety

Male C57BL/6 mice weighing 25 to 30 g at 10 weeks of age were obtained from Charles River Labs (Wilmington, MA). Food and water were provided ad lib. All experiments conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (eighth edition, 2011). The protocol was approved by the Institutional Animal Use and Care Committee of the University of Pennsylvania (protocol No. 803190).

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In Vitro Cardiomyocyte Isolation

Cardiomyocytes were isolated from neonatal Sprague-Dawley rat pups using a trypsin-based dissociation method as previously described, with minor modifications. The cell samples were centrifuged to wash away the trypsin solution and suspended in growth media composed of 4:1 ratio of DMEM and Medium-199, 10% horse serum, 5% fetal bovine serum, 1% HEPES, and 1% solution of penicillin, streptomycin, and glutamine.

The cells were preplated onto multiple 10 mm2 petri dishes for 1 hour to allow fibroblasts to attach to the dishes. Cardiomyocytes still suspended in the media were retained and counted. Cells were seeded onto a glass bottom 24-well plate coated with vitronectin. The seeding density for each well was 3.33×10^5 cells in 1 mL of growth media. Growth media was changed 24 hours after seeding. Cells were kept in culture for 5 days so that background proliferation would subside and were randomized into 4 stimulation groups: PBS alone, NRG alone, hydrogel (HG) alone, or NRG-HG. This time point is labeled as t=0 days. The media was changed daily. The hydrogels were kept suspended over the cells in a 3-μm pore size transwell cell culture insert. By doing so, the daily medium changes did not wash away the hydrogel, which could continue to release NRG into the fresh medium. In the NRG alone group, the NRG was not replaced after the first medium change.

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were randomized into 4 groups (n=13/group) and received 3 peri-
infarct intramyocardial injections of either PBS (40 μL), NRG alone
(2.5 μg in 40 μL PBS), HG alone (40 μL), or NRG-HG (2.5 μg in
40 μL hydrogel). These borderzone injections were performed with
the needle at a 20° angle to the myocardium to achieve an injection
depth of 1 mm avoiding intraventricular delivery. The thoracotomy
was closed, and tissue adhesive (VetBond, MN) was applied over the
incision. All mice were implanted with subcutaneous microchips
(BioMedic Data Systems, ID) for tracking and recovered from anes-
thesia. Buprenorphine (0.5 mg/kg) was administered for pain control.

Echocardiographic Assessment
LV geometry and function (n=32) were evaluated by echocardiog-
raphy (Philips, Amsterdam) at 2 weeks after myocardial infarction and
therapy. LV parasternal short-axis 2-dimensional images were
obtained at the papillary muscles. All analyses were performed by a
single investigator who was blinded to the treatment groups.

Immunohistochemistry and Histological Analysis
At 6 days postoperatively, a subset (n=5/group) was anesthetized as
previously described, and hearts were explanted. They were flushed
with PBS, injected retrograde with tissue Tek optimal cutting tem-
perature compound (Netherlands), frozen at −80°C, and sectioned
onto slides using a Leica CM3050S cryostat (Leica, Germany) at 10-
μm thickness. Next, the samples were fixed with 4% paraformalde-
yde and blocked with 10% fetal bovine serum. Sections were stained
for PH3, Ki67, activated caspase-3, and NRG separately. All sections
were stained for cardiac troponin. All primary antibodies (Abcam)
were diluted at 1:200 in PBS and incubated for 2 hours at 37°C.
Importantly, the NRG primary antibody (ab80237) was specific to
the EGF-binding domain of human NRG to minimize cross-reactivity
with mouse neuregulin. The sections were washed in PBS, and the
appropriate secondary antibody was applied. The slides were washed
in PBS and counterstained for nuclei with DAPI (4’,6-diamidino-
2-phenylindole; Vector Labs). The sections were imaged with a Leica
DM5000B fluorescent microscope.

For histological analysis at 2 weeks, hearts were explanted, flushed
with PBS, and injected retrograde with optimal cutting temperature
compound through the aorta and pulmonary artery. Hearts were sub-
merged in optimal cutting temperature compound, frozen, and stored
in a −80°C freezer. Eight 10-μm-thick sections were prepared from
each heart at the papillary level and stained with Masson trichrome.
Standardized digital photographs were taken with a Nikon D5100 SLR
camera (Nikon, Japan). Photographs were uploaded to ImageJ (ver-
sion 1.46b), and the borderzone was assessed with digital planimetry.

Peripheral Blood NRG Quantification
At 6 days after myocardial infarction and therapy, a subset (n=5/
group) was anesthetized, and 500 μL of peripheral blood was ac-
quired. Blood was immediately placed in a biopsy tube with EDTA
to prevent clotting and flash frozen. ELISA (ab100614, Abcam) was
performed on all samples to quantify levels of human NRG. The sam-
plies were read on a microplate reader (TECAN, Austria) at 450 nm.

Statistical Analysis
All analyzed variables approximated a normal distribution, and val-
ues for continuous variables were reported as means±SD. For all ex-
periments, a Levene test was first performed to establish homogenous
variance across all groups. One-way ANOVA was then performed
followed by a Tukey honest significant difference test to compare 2
groups at a time (α=0.05).

Results

NRG Encapsulation and Sustained Release Kinetics
NRG release was sustained for 14 days in vitro. An initial 2-day
burst phase was observed where 60% of the NRG was released
from the hydrogel. This was followed by a steady and sus-
tained distribution of the remaining 40% of NRG for 12 days
(Figure 1A). This release pattern was consistent with previous
encapsulation of other similarly sized peptides. The uronic acid
assay demonstrated 100% degradation of the hydrogel during a
14-day period as well, paralleling the release of NRG (Figure 1B).

Sustained Release NRG Induces Cardiomyocyte
Mitotic Activity in Vitro
Isolated cardiomyocytes were stimulated with PBS, NRG
alone, HG alone, or NRG-HG at t=0 days. Importantly,
transwell plates that allowed passage of NRG released from
hydrogel were used, and the media were changed daily. This
allowed comparison of a 1-time administration of NRG ver-
sus sustained delivery. At t=6 days, the cardiomyocytes were
visualized with confocal microscopy to evaluate for EdU incor-
poration, marking DNA synthesis. The PBS, NRG alone,
and HG alone groups demonstrated no EdU incorporation
compared with 10% of the cardiomyocytes in the NRG-HG
group that did incorporate EdU (Figure 2A). Providing fur-
ther evidence of cardiomyocyte cell cycle activation, immu-
nohistochemistry was performed to determine the presence
of PH3. There was no PH3 observed in the PBS, NRG alone,
and HG alone groups, whereas the NRG-HG group exhibited PH3
positivity (Figure 2B) in 2% of the cardiomyocytes.

Sustained Release NRG Induces Cardiomyocyte
Mitotic Activity in Vivo
At 6 days postoperatively, hearts were explanted for immuno-
histochemistry (n=5/group) and determination of the presence

Figure 1. Sustained neuregulin (NRG) secretion. Release kinet-
ics of NRG from hydrogel (HG) are demonstrated for 14 days.
The typical burst phase of 60% release during 2 days is demon-
strated followed by sustained release for 14 days (A). The degra-
dation of the HG is displayed by quantifying uronic acid levels for
14 days (B).
of PH3 and Ki67. Heart sections in the 3 control groups did not demonstrate evidence of PH3 or Ki67. In all of the NRG-HG–treated animals, heart sections were positive for PH3 and Ki67 (Figure 3A and 3B).

Sustained Release NRG Provides Cardioprotection In Vivo

In the same subset at 6 days postsurgery, heart sections (n=5/group) were evaluated by immunohistochemistry for the presence of activated caspase-3, a mediator of apoptosis and parameter of cardioprotection.28 This was quantified by number of positive cells per 10× high-powered field. The PBS (5.6±0.8 cells; P=0.005) and HG (6.2±0.97 cells; P=0.006) groups demonstrated significantly higher levels of activated caspase-3 compared with the NRG-HG group (1.5±0.29 cells; Figure 4). The NRG group demonstrated a greater number of positive cells (3.5±0.29 cells) compared with NRG-HG, but was not statistically significant.

Encapsulated NRG in Hydrogel Provides Sustained Local Release in Vivo Without Peripheral Exposure

In the same subset at 6 days postsurgery, peripheral blood (n=5/group) was acquired along with explanted hearts. Immunohistochemistry of explanted hearts demonstrated the
presence of NRG in the myocardium in the NRG-HG group only (Figure 5A). The peripheral blood was assessed for the presence of NRG. There was no difference in peripheral blood NRG levels throughout all groups: native (3.6±0.4 ng/mL; P>0.5), NRG (3.4±0.3 ng/mL; P>0.5), HG (3.1±0.2 ng/mL; P>0.5), NRG-HG (3.5±0.2 ng/mL; Figure 5B).

Echocardiographic Assessment
At 14 days postoperatively, the animals were anesthetized and were evaluated by echocardiography (n=8/group). LV chamber area at the level of the papillary muscles was analyzed. The PBS (0.29±0.02 cm²; P=0.05), NRG alone (0.33±0.04 cm²; P=0.02), and HG alone (0.26±0.05 cm²; P=0.03) animals all demonstrated significantly greater LV chamber areas compared with NRG-HG–treated animals (0.18±0.02 cm²; Figure 6A). In addition, LV ejection fraction was determined at 14 days. The PBS (20.2±3.2%; P<0.01), NRG alone (20.1±4.7%; P<0.01), and HG alone (17.2±3.7%; P<0.01) groups all exhibited significantly reduced LV ejection fraction compared with NRG-HG–treated animals (37.5±3.6%; Figure 6B).

Histological Evaluation
Directly after echocardiography evaluation at 14 days, hearts were explanted and frozen sections were acquired for histological analysis (n=8/group). Masson trichrome staining was performed to evaluate borderzone thickness. The PBS (0.27±0.04 mm; P<0.01), NRG alone (0.30±0.05 mm; P<0.01), and HG alone (0.26±0.05 mm; P<0.01) groups revealed a significantly reduced borderzone thickness compared with animals receiving NRG-HG (0.65±0.07 mm; Figure 7).

Discussion
This study demonstrated that localized and sustained myocardial delivery of NRG stimulates cardiomyocyte mitotic activity, provides cardioprotection, reduces LV dilation, and enhances ventricular function in a murine model of ischemic cardiomyopathy. The novel and integral component of this study was the encapsulation of NRG in the hydrogel delivery system. Although previous preclinical and clinical studies have revealed a functional benefit of repeated systemic dosing of NRG, there remains a legitimate concern regarding NRG’s potential oncological consequences and its effect on the nervous system.29 The locally administered encapsulated NRG addresses this problem, allows for greater flexibility in dosing, and obviates the requirement for daily infusions. A crucial component of this study was confirming the presence of the administered NRG in the myocardium 6 days after injection.
only when delivered in a hydrogel. In addition, analysis of the peripheral blood at this time point exhibited no difference in NRG levels throughout all groups, including native mice. The low levels of NRG detected were probably attributable to minimal cross-reactivity between the detection antibody and native mouse neuregulin. These findings strongly support the strategy of sustained and local NRG delivery to the myocardium with minimal off-target exposure.

During the past decade, a multitude of bioengineered systems have been developed as adjuncts to cytokine, peptide, and cell therapies for heart failure. These include, but are not limited to, cell sheets, fibrin patches, algin ate scaffolds, and hydrogels. The hyaluronic acid hydrogel was used in this study because of its previous effectiveness in encapsulating similarly sized peptides and its demonstrated safety in humans. There is strong evidence to suggest that administration of hydrogel alone contributes to cardiac repair and improvement in function; however, this was not observed in this study. The most logical explanation is that a very small volume of 40 μL was injected into the myocardium. This volume was optimal for encapsulating the determined dose of 2.5 μg of NRG and treating the borderzone. After encapsulation of NRG, it was critical to determine that NRG could be released from the gel for an extended period of time without hindering its function. This was accomplished by incubating the NRG-containing hydrogel in PBS and evaluating the supernatant by ELISA for 14 days. The typical burst phase was observed followed by a steady release of NRG. After this analysis, in vitro experiments on isolated cardiomyocytes demonstrated that the released NRG retained its ability to activate the ErbB signaling axis. Importantly, these experiments were carefully performed such that the cells were cultured on a transwell plate where the hydrogel was suspended above the cells. This allowed for daily medium changes while keeping the hydrogel in place and continually releasing NRG into the new medium. In the NRG alone group, the NRG was not replaced after the first medium change. This allowed comparison between cardiomyocytes undergoing sustained NRG stimulation and a 1-time stimulation. The data demonstrated that activation of the cardiomyocyte cell cycle occurred only with sustained NRG administration. The NRG dosing was based on a previous study that characterized differentiated cardiomyocyte cell cycle re-entry after multiple days of NRG stimulation, which was consistent with the findings of this study. The visualization of EdU incorporation and PH3 positivity in NRG-HG–stimulated cells strongly supports that the integrity of the NRG–ErbB signaling axis was maintained.

After demonstration that NRG retains activity after hydrogel release, findings of reduced LV dilation and significantly improved LV ejection fraction coupled with increased borderzone thickness on histology 2 weeks after therapy illustrate enhancement of ventricular function and structure resulting from NRG-HG therapy. The in vitro and in vivo data exhibiting cardiomyocyte mitotic activity support the notion that NRG-HG–induced cardiomyocyte regeneration contributed to enhanced ventricular function. The decreased levels of activated caspase-3 after NRG-HG treatment also suggest an anti-apoptotic effect. It is logical that NRG administration would yield a protective effect in conjunction with a regenerative stimulus as the downstream pathway of the ErbB receptors includes PI3K (phosphoinositide 3-kinase) and Akt, which are well-established mediators of cellular proliferation and

![Figure 6. Echocardiography. Graphs demonstrating significantly decreased left ventricular (LV) chamber area at the papillary level (A) and significantly enhanced LV ejection fraction (EF; B) in the neuregulin (NRG)-hydrogel (HG) group compared with all controls. Measurements were determined by short-axis view on ECHO (*P<0.05 for each group vs NRG-HG).](http://circulation.ahajournals.org/content/129/4/396/F6.large.jpg)

![Figure 7. Increased borderzone thickness. Photographs of Masson trichrome–stained heart sections 14 days after treatment (A). Neuregulin (NRG)-hydrogel (HG)–treated animals demonstrated significantly augmented borderzone thickness (*P<0.01 for each group vs NRG-HG; B).](http://circulation.ahajournals.org/content/129/4/396/F7.large.jpg)
protection. It is possible that additional mechanisms of action such as inotropy are involved. NRG has been determined to modulate nitric oxide synthesis and affect cardiomyocyte calcium cycling, however, multiple studies have actually concluded that NRG has a slightly negative inotropic effect. In addition, the role of cardiac stem cells and NRG’s interaction with them were not studied here. Previous work has somewhat elucidated this mechanism in which fate mapping experiments showed that NRG-induced cardiomyocyte regeneration occurred with already differentiated cardiomyocytes.

There were limitations to the study. Although the experiments confirmed the presence of NRG in the myocardium 6 days after injection, further information concerning the kinetics of encapsulated NRG administration would be helpful. This could be accomplished in future experiments by conjugating a fluorophore to NRG and using serial optical imaging to track its location over time. Regarding limitations involving delivery, intramyocardial injection poses a manageable hurdle when translating to clinical application. In addition to being an adjunct to cardiac surgery, hydrogels that are more suitable for catheter-based administration are being explored. As a result, the translational potential of this therapy is greatly broadened.

Overall, this study demonstrated that targeted and sustained delivery of NRG to borderzone myocardium stimulates cardiomyocyte mitotic activity and cardioprotection and significantly enhances ventricular function and structure. It is an extremely translatable therapy in that it addresses a major national health concern, NRG alone has reached clinical trial and future directions.

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**Disclosures**

None.

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