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Infarct stabilization and cardiac repair with a VEGF-conjugated, injectable hydrogel

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ABSTRACT

Injectable scaffolds made of biodegradable biomaterials can stabilize a myocardial infarct and promote cardiac repair. Here, we describe the synthesis of a new, temperature-sensitive, aliphatic polyester hydrogel (HG) conjugated with vascular endothelial growth factor (VEGF) and evaluate its effects on cardiac recovery after a myocardial infarction (MI). Seven days after coronary ligation in rats, PBS, HG, or HG mixed or conjugated with VEGF (HG + VEGF or HG-VEGF, respectively) was injected around the infarct ($n = 8–11$/group). Function was evaluated by echocardiography at multiple time points. Pressure–volume measurements were taken and infarct morphometry and blood vessel density were assessed at 35 days after injection. HG-VEGF provided localized, sustained VEGF function. Compared with outcomes in the PBS group, fractional shortening, ventricular volumes, preload recruitable stroke work, and end-systolic elastance were all preserved ($p < 0.05$) in the HG and HG + VEGF groups, and further preserved in the HG-VEGF group. Conjugated VEGF also produced the highest blood vessel density ($p < 0.05$). The infarct thinned and dilated after PBS injection, but was smaller and thicker in hearts treated with HG ($p < 0.05$). Our temperature-sensitive HG attenuated adverse cardiac remodeling and improved ventricular function when injected after an MI. VEGF delivery enhanced these effects when the VEGF was conjugated to the HG.

1. Introduction

After a myocardial infarction (MI) [1], biodegradable biomaterials can be used, alone or in conjunction with cardiac cell or cytokine therapy, to stabilize and preserve elasticity in the infarct area [2]. For instance, previous studies found that alginates [3] or fibrin glue [4] were well-tolerated by the heart and prevented adverse remodeling by stabilizing the infarct and the matrix structure of the border zone. Some materials also provide a substrate for the attachment of injected or recruited cells.

Injectable materials that incorporate a gelation mechanism; for example, aqueous alginates (that gel in the presence of Ca_2^+ by ionic cross-linking [5]) or macromonomers (that can be cross-linked by UV light [6]), improve the efficiency of the repair because they assume a solid form *in vivo* and so are more readily retained at the injection site. Of these materials, temperature-sensitive and biodegradable gels are preferred for cardiac repair because they undergo a mild solidification process under physiological conditions that allows them to maintain cell viability and molecular bioactivity while avoiding damage to the surrounding tissue. Hydrogel (HG) is one such temperature-sensitive and injectable material that has been used successfully to inhibit ventricular remodeling and improve cardiac function after an experimental MI [7]. The HG's functional effects can be enhanced by co-injecting progenitor cells or growth factors [8,9]. One study recorded increased angiogenesis in the infarct area after treatment with a mixture of HG and plasmids carrying genes for the well-characterized angiogenic cytokine vascular endothelial growth factor (VEGF) [10].

We expected that the beneficial effects of HG-mediated VEGF delivery would be improved by controlling the release of VEGF from the biomaterial. Specifically, conjugating VEGF to the HG could achieve sustained local release, extending the duration of protein activity and the resultant angiogenesis. This approach to cytokine delivery may be more effective than either local bolus delivery or systemic administration [11] (since both are limited by transient cytokine levels at the site of delivery) and could improve protein stability, solubility, and biocompatibility [12]. To test this theory, we synthesized and evaluated a temperature-sensitive, aliphatic polyester HG [Poly (δ-valerolactone)-*block*-poly (ethylene glycol)-*block*-poly (δ-valerolactone) (PVL-*b*-PEG-*b*-PVL)] that permits the conjugation of cytokines. This polymer dissolves in water at room

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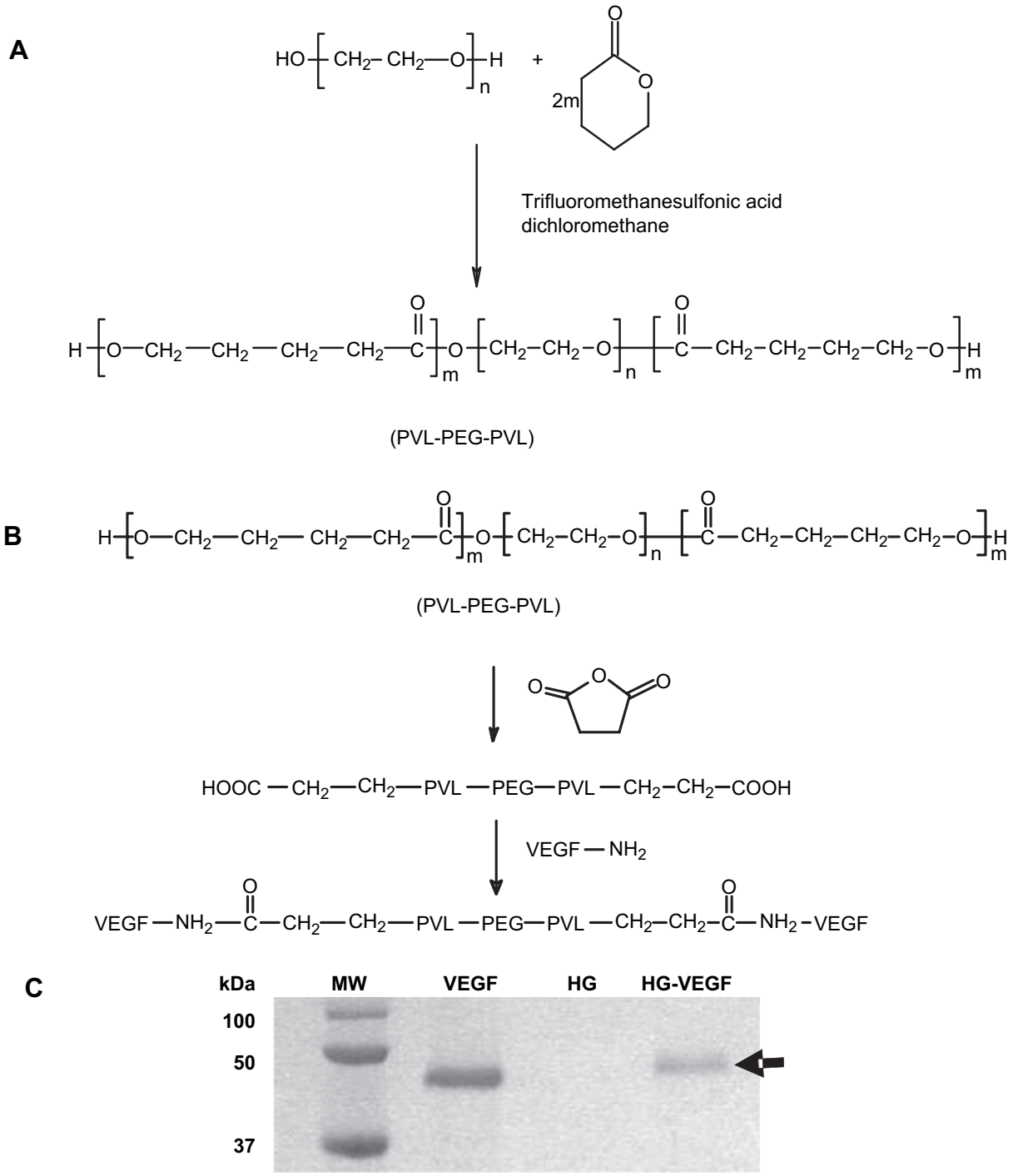

Fig. 1. Hydrogel synthesis. (A,B) Diagrams illustrating the synthesis of (A) HG and (B) HG-VEGF. (C) Western blot was used to assess VEGF protein expression in VEGF, HG, and HG-VEGF samples. Arrow indicates the presence of VEGF expression in VEGF and HG-VEGF. MW = molecular weight.

temperature, but gels at 37 °C. In a rat MI model, we measured blood vessel density and cardiac repair after injection of the new HG alone, HG mixed with VEGF, or HG conjugated with VEGF.

2. Materials and methods

2.1. PVL-b-PEG-b-PVL synthesis

The triblock copolymer was synthesized using a metal-free cationic method. Briefly, 0.5 g polyethylene glycol (PEG) (0.33 mmol) and 1.2 g of δ-valerolactone (VL, 12 mmol) were dissolved in 5 mL dichloromethane. Trifluorimethanesulfonic acid [catalyst, 61 μL (0.67 mmol)] was added to the mixture at 0 °C. The reaction was maintained for 3 h, and terminated by adding 0.2 g $NaHCO_3$ and then filtering the mixture. The copolymer was collected after precipitation in hexane. The molecular weight of the poly-VL (PVL) block was calculated from ^1H nuclear magnetic resonance, with the known molecular weight of the PEG macroinitiator used as a reference and $CHCl_3$ as the internal standard.

2.2. N-Hydroxysuccinimide (NHS) terminated PVL-b-PEG-b-PVL synthesis

The carboxyl-terminated block copolymer was synthesized by reacting the hydroxyl-terminated triblock copolymer with succinic anhydride as previously described [13]. Briefly, 0.5 g dicarboxy-terminated block copolymer (0.128 mmol), 0.0396 g *N,N*-dicyclohexylcarbodiimide (1.5× excess, 0.192 mmol), 0.0221 g NHS (1.5× excess, 0.192 mmol), and 5 mL dichloromethane were mixed. The reaction was

maintained for 24 h at room temperature, and then the reaction mixture was filtered. NHS-PVL-*b*-PEG-*b*-PVL-NHS was precipitated in cold diethyl ether.

2.3. VEGF conjugate synthesis

VEGF conjugates were synthesized as we previously described [14]. Briefly, 10 mg NHS-PVL-*b*-PEG-*b*-PVL-NHS was added to a solution of 100 ng VEGF in 0.5 mL phosphate buffered saline (PBS; pH = 7.4, equiv. 200 ng/mL). The reaction was maintained for 24 h at room temperature. To remove the uncoupled VEGF, the reaction mixture was dialyzed against water using Spectra/Por 2 dialysis membrane tubing with a molecular weight cut-off of 12–14 kDa for 48 h. The reaction products (VEGF conjugates) were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the VEGF proteins were stained with Coomassie Brilliant Blue.

2.4. Hydrogel and VEGF-conjugated hydrogel preparation

To prepare the temperature-sensitive HG, 0.2 g PVL-*b*-PEG-*b*-PVL was added to 1.0 mL PBS. The mixture was heated to 60 °C and stirred until the polymer was completely dissolved, and then cooled to 10 °C. A clear polymer solution formed. The gelling temperature was determined by increasing the temperature by 5 °C per min until a gel formed [15]. VEGF-conjugated HG (HG-VEGF) was prepared by adding VEGF conjugates to PVL-*b*-PEG-*b*-PVL solution at 10 °C.

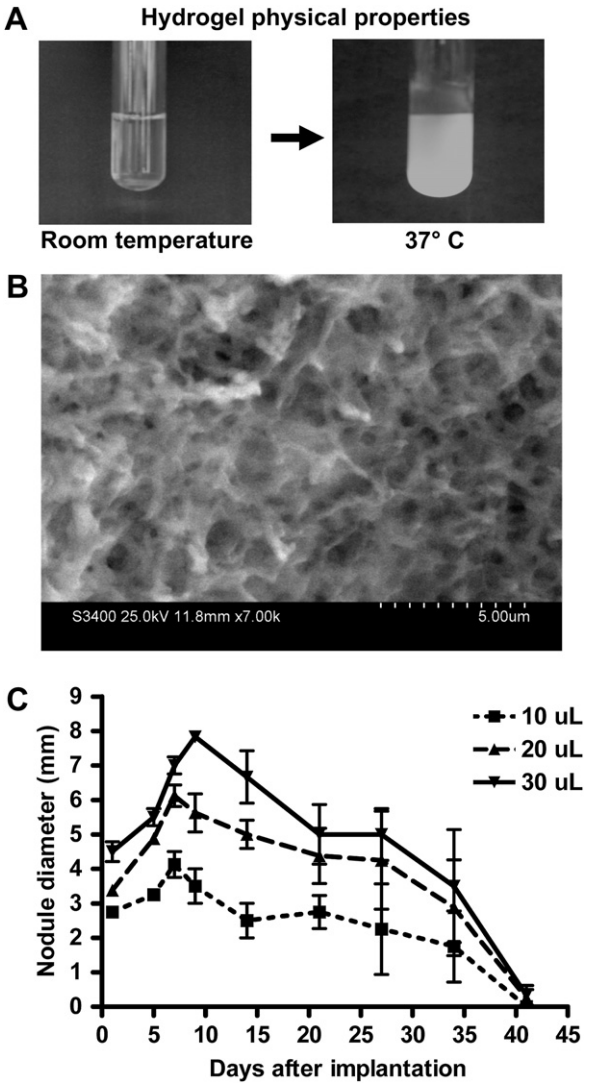

Fig. 2. Hydrogel: Physical properties and degradation. (A) HG exists as a clear solution (dissolves in water) at ambient temperature, but gels at 37 °C. (B) Representative scanning electron micrograph illustrating that HG has a honeycomb-like structure with pore size ~1 μm (C) HG degradation *in vivo*. Different doses of the gel solution (10, 20, 30 μL) were injected subcutaneously, and then nodule diameters were measured over 42 days after implantation. At all 3 doses, the gel the nodule size initially increased as the gel absorbed water, and then decreased beyond 7 days after implantation. By 42 days, the nodules were completely degraded.

2.5. In vitro and in vivo degradation assays

In vitro: Pellets of HG solution were dropped onto a cell culture dish (10, 20, or 30 μL per dish) and gelled at 37 °C. The pellets were soaked in culture media and incubated at 37 °C for 35 days. The diameter of each pellet was measured initially, and then at 7 day intervals (with several time points during the first week) to assess HG degradation.

In vivo: HG solution was injected subcutaneously (10, 20, or 30 μL per injection) in rats. The diameters of the resultant nodules were measured at regular intervals over a period of 42 days.

2.6. Myocardial infarction

We used Sprague Dawley rats (body weight = 200–250 g). All experiments were performed in accordance with the principles of laboratory animal care formulated by the guide for the care and use of laboratory animals by the Institute of Laboratory Animal Resources (Commission on Life Sciences, National Research Council). All animal procedures were approved by the University Health Network Animal Care Committee. Detailed surgical procedures for MI (coronary artery ligation) were as we previously described [16]. Cardiac function was evaluated using echocardiography at 7 days after MI; at this point, rats with no infarct or with a very large infarct were excluded from the study. The remaining animals were randomly assigned to 4 groups (n = 8–11/group).

2.7. Hydrogel injection

Under general anesthesia with ventilation, the heart was exposed through a thoracotomy. Next, 100 μL of PBS, HG, HG mixed with VEGF (40 ng/rat) (HG + VEGF), or HG-VEGF (40 ng VEGF/rat) was injected into 4 sites around the infarct with a 28-gauge insulin syringe (25 μL/injection), and the incision was closed. All animals received post-operative care.

2.8. Cardiac functional measurements

Function was evaluated using echocardiography immediately before MI, immediately before gel or PBS injection, and at 7, 14, 21, and 35 days after injection [16]. Cardiac function was also assessed at the end of the study (35 days after treatment) with a pressure–volume catheter as we previously described [17].

2.9. Morphometric and histological studies

After the pressure–volume analysis was complete, hearts were rapidly excised and fixed in 10% formaldehyde. Morphometry (measuring scar size and thickness) and immunohistochemical staining (measuring blood vessel density) were performed as we previously described [16,17].

2.10. Statistical analyses

All data were expressed as mean ± SD. Analyses were performed using GraphPad version 4.1 software with the critical α-level set at $p < 0.05$. One-way analyses of variance (ANOVA) compared the effects of treatment (PBS, HG, HG + VEGF, HG-VEGF) on pressure–volume cardiac function, infarct size and

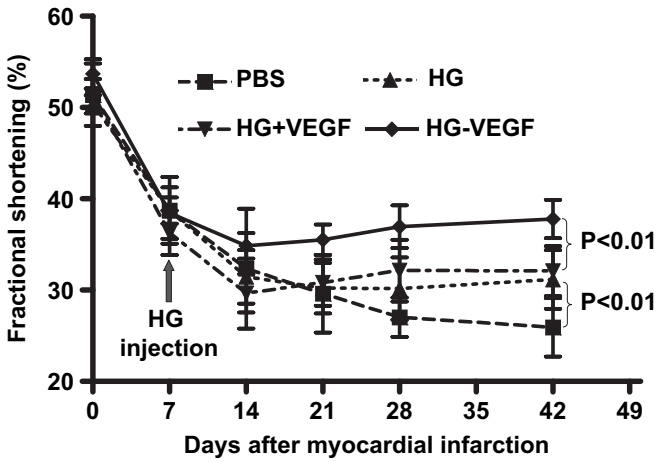

Fig. 3. Cardiac function by echocardiography. Percent fractional shortening in hearts implanted with PBS, HG, HG + VEGF, or HG-VEGF over 42 days after MI (35 days after injection). Cardiac function was significantly improved (vs. PBS) after the injection of HG or HG + VEGF, and further improved after HG-VEGF.

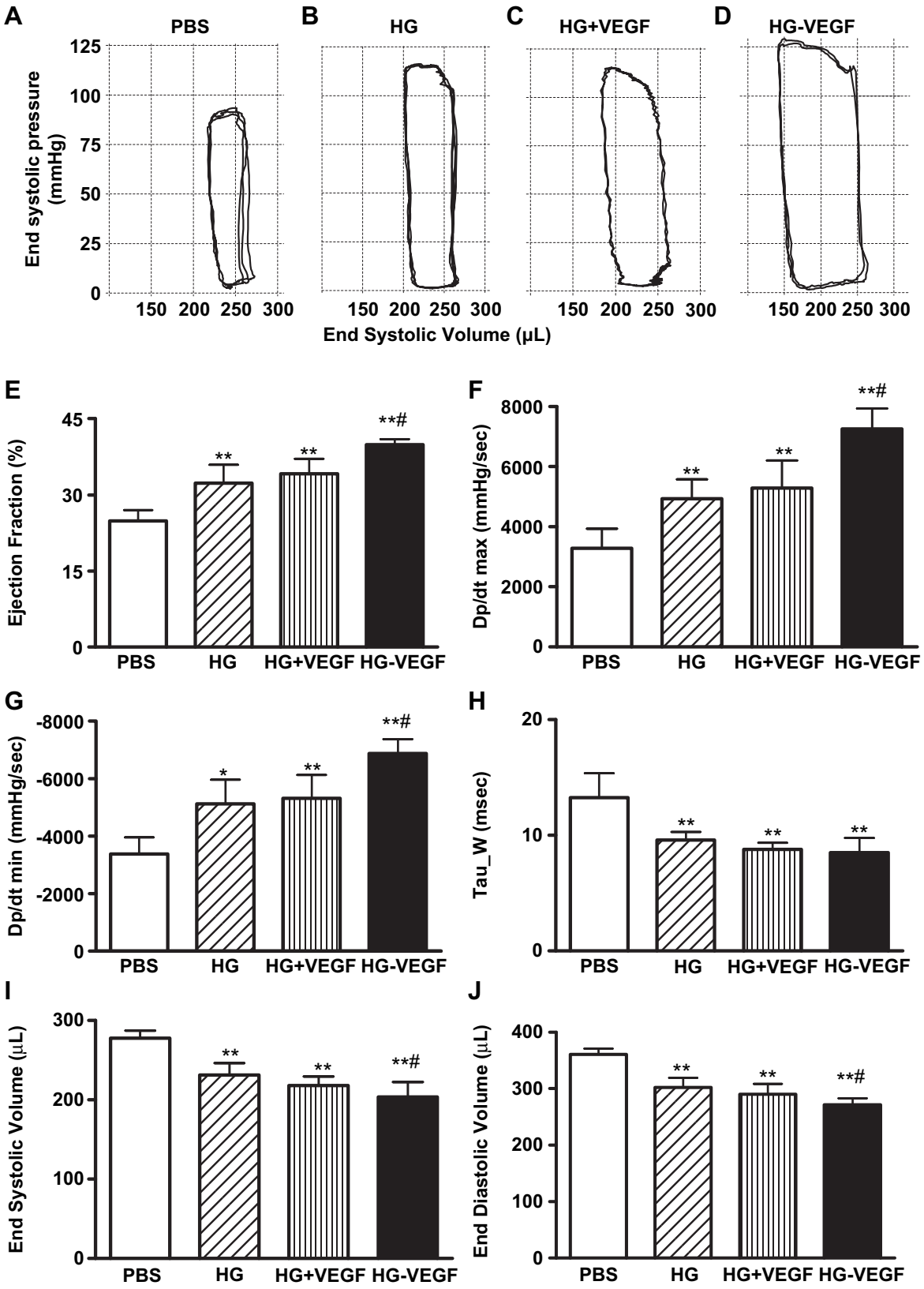

Fig. 4. Load-dependent cardiac function and **LV** volumes by pressure–volume (P–V) catheter. Left ventricular (LV) P–V relationships were measured at 35 days after post-MI injection of PBS, HG, HG + VEGF, or HG-VEGF. (A–D) Representative baseline P–V loops for each group. (E–J) Compared with the PBS group, load-dependent measures ejection fraction (E), Dp/dt max (F), Dp/dt min (G), and Tau_W (H) were significantly improved, and LV end-systolic volumes (I) and end-diastolic volumes (J) were smaller ($*p < 0.05$, $**p < 0.01$) in the HG and HG + VEGF groups (no differences between HG and HG + VEGF groups). HG-VEGF enhanced ($^{\#}p < 0.05$) the protective effects of HG and HG + VEGF for all measures except Tau_W (H).

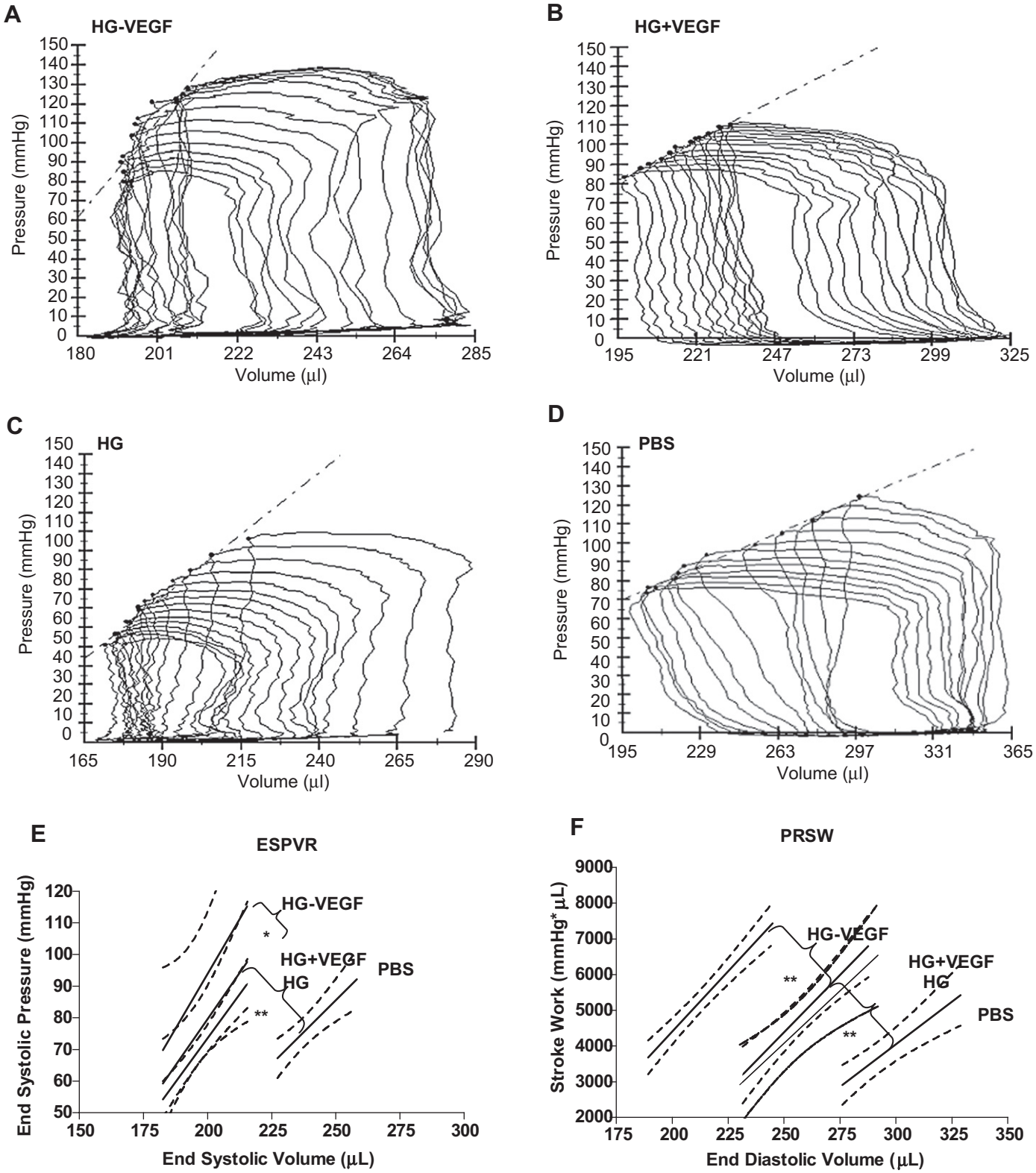

Fig. 5. Load-independent cardiac function by pressure–volume (P–V) catheter. (A–D) Representative series of P–V loops obtained during vena cava occlusion at 35 days after post-MI injection of HG-VEGF (A), HG + VEGF (B), HG (C), or PBS (D). (E,F) Load-independent measures end-systolic pressure–volume relationship (ESPVR, E) and preload recruitable stroke work (PRSW, F) were significantly improved (**$p < 0.01$) in the HG and HG + VEGF groups compared with the PBS group (no differences between HG and HG + VEGF groups). HG-VEGF enhanced (*$p < 0.05$, **$p < 0.01$) the protective effects of HG and HG + VEGF.

thickness, and vascular density. A two-way ANOVA compared the effects of treatment and time on fractional shortening (echocardiography). When the *F* ratio was significant, differences were specified using Tukey's multiple range tests.

3. Results

3.1. Synthesis and characterization of the VEGF polymer

The triblock copolymer (PEG-PVL) was synthesized by polymerizing PEG with VL using a metal-free cationic polymerization method (Fig. 1A). Triblock copolymers are water soluble at room temperature. HG-VEGF was synthesized by coupling the NHS functionalized polymer with VEGF protein in PBS solution (Fig. 1B). Successful conjugation was verified using a Western blot (Fig. 1C). We observed a clear VEGF expression band in the HG-VEGF sample. Unconjugated VEGF was not observed.

PEG-PVL is a clear solution in water at ambient temperature. The biomaterial gels at a concentration of 200 mg/mL, and forms an HG within 10 min at 37 °C (Fig. 2A). Scanning electron microscope analysis revealed a honeycomb structure with a pore size of approximately 1 μm, which may facilitate the diffusion of proteins or peptides (Fig. 2B).

3.2. Hydrogel degradation

In vitro: HG (10, 20, or 30 μL) pellets dropped onto a cell culture dish formed a gel at 37 °C. The pellet diameter did not change over 5 weeks (data not shown), indicating that the gel was stable *in vitro*.

In vivo: HG injected subcutaneously (10, 20, or 30 μL) formed nodules that initially increased in size as the nodules absorbed water at all 3 tested concentrations. The nodules decreased in size beyond 7 days after implantation, and were completely degraded after 42 days (Fig. 2C).

3.3. In vivo study

3.3.1. Animals

A total of 44 rats were initially included in the study. Three rats died during or immediately after undergoing the MI procedure, and 2 rats died during the second surgical procedure (gel or PBS injection). There were no further deaths. Of the remaining 39 animals, 10 received HG-VEGF, 10 received HG + VEGF, 11 received HG, and 8 received PBS control.

3.3.2. Cardiac function

3.3.2.1. Echocardiography. Coronary artery ligation produced significant left ventricle (LV) dilatation, evidenced by increased LV diameter and decreased LV function in all animals. There were no differences among the groups in any of the echocardiographic parameters at 7 days after MI (Fig. 3). Overall, HG-VEGF contributed more functional preservation than HG and HG + VEGF, though all HG treatments increased fractional shortening ($p < 0.01$) compared to PBS. Specifically, both HG and HG + VEGF preserved function ($p < 0.01$ vs. PBS) beyond 21 days after injection, while HG-VEGF increased fractional shortening earlier, by 14 days after injection ($p < 0.01$ vs. PBS, HG, HG + VEGF).

3.3.2.2. Pressure–volume catheter. Ventricular volumes and cardiac function were evaluated under load-dependent (Fig. 4) and load-independent (Fig. 5) conditions. Compared with PBS, HG and HG + VEGF improved ejection fraction (EF), Dp/dt max, Dp/dt min, and Tau (load-dependent indices), as well as end-systolic elastance (ESPVR) and preload recruitable stroke work (PRSW) (load-independent indices). HG-VEGF further improved EF, Dp/dt max, Dp/dt min, ESPVR, and PRSW ($p < 0.01$ vs. PBS, $p < 0.05$ vs. HG and HG + VEGF). End-systolic and end-diastolic volumes were smallest in the HG-VEGF group, followed by the HG + VEGF and HG groups. All HG treatments reduced end-systolic and end-diastolic volumes relative to PBS ($p < 0.01$ for all groups).

3.3.3. Infarct morphometry

Myocardial scar tissue was observed in all animals (Fig. 6A). Computerized planimetry (performed at 42 days after MI on explanted hearts fixed at physiologic pressures) showed that infarcts were larger and infarcted tissue was thinner in animals that received PBS rather than HG or HG + VEGF ($p < 0.05$). Scar size was smallest and

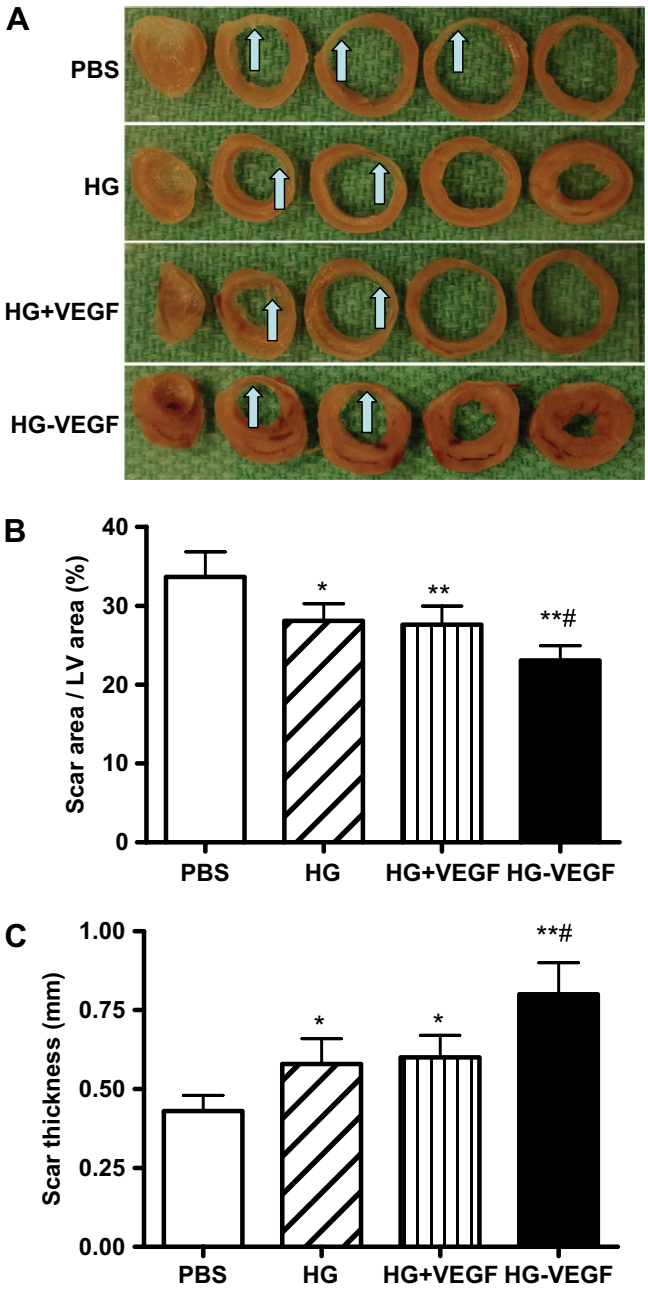

Fig. 6. Infarct morphometry by planimetry. (A) Representative heart slices obtained at 35 days after post-MI injection of PBS, HG, HG + VEGF, or HG-VEGF. Arrows indicate the location of the infarct in individual slices. (B, C) LV scar area (as a percentage of total LV area, B) was decreased and scar thickness (C) was increased after the injection of HG or HG + VEGF (*$p < 0.05$, **$p < 0.01$ vs. PBS for both measures). Scar area was smallest and scar thickness was greatest after the injection of HG-VEGF ($^{\#}p < 0.05$ vs. HG and HG + VEGF for both measures).

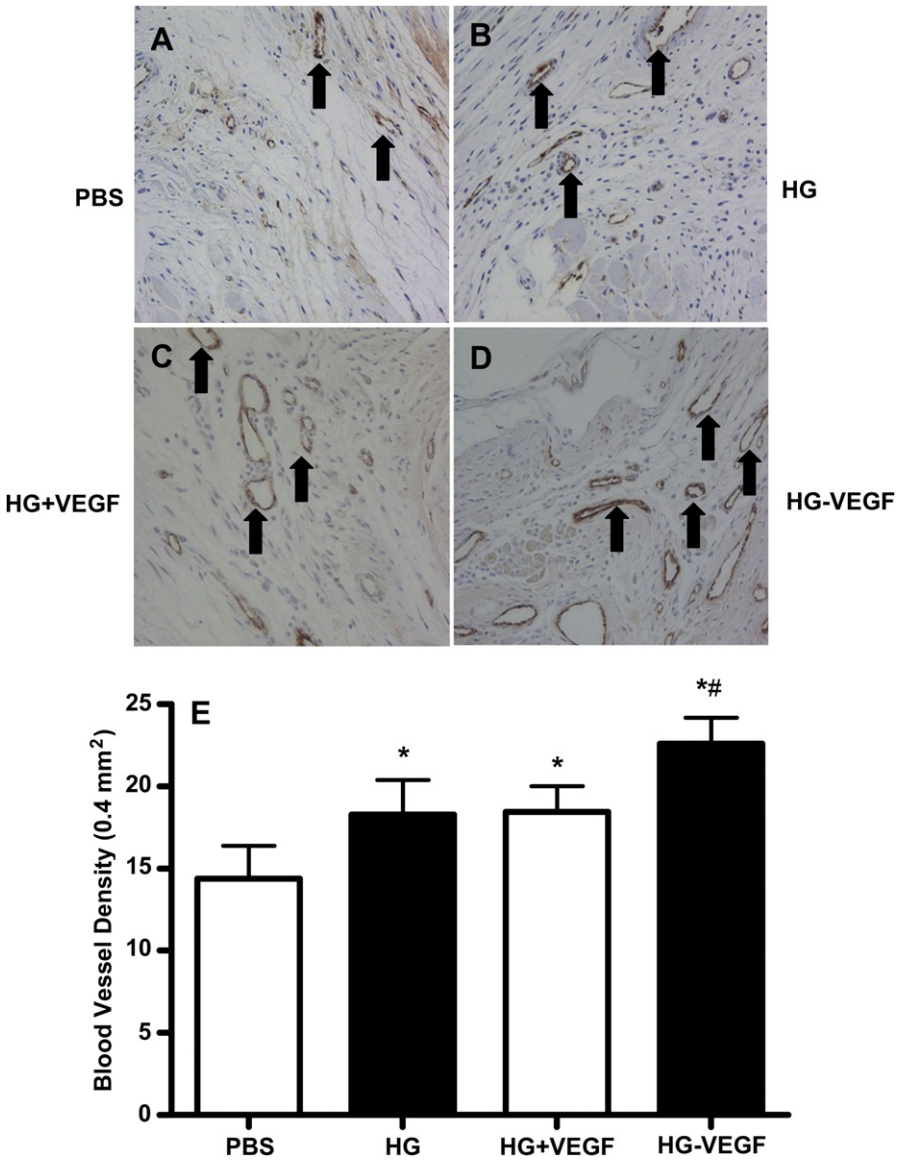

Fig. 7. Vascular density. (A–D) Representative micrographs illustrating vascular formations (arrows = Factor VIII + structures) in the peri-infarct area at 35 days after post-MI injection of PBS, HG, HG + VEGF, or HG-VEGF. (E) Capillary density was significantly greater after the injection of HG or HG + VEGF (*$p < 0.05$ vs. PBS), and highest after HG-VEGF (#$p < 0.05$ vs. HG and HG + VEGF).

scar thickness greatest in animals that received HG-VEGF ($p < 0.05$ vs. HG and HG + VEGF for both measures) (Fig. 6B,C). These findings suggest that conjugating VEGF with the biomaterial stabilized the infarct and prevented scar expansion and ventricular dilatation.

3.3.4. Blood vessel density

Blood vessels expressing Factor VIII were quantified immunohistochemically in the infarcted and peri-infarcted myocardium (Fig. 7). Vascular densities were increased in the hearts of animals that received HG or HG + VEGF ($p < 0.05$ vs. PBS), and further increased in those that received HG-VEGF ($p < 0.05$ vs. HG and HG + VEGF).

4. Discussion

This study reports the synthesis of a temperature-sensitive HG that is an injectable liquid at room temperature and becomes a biodegradable solid at physiological temperature (37 °C). Our HG also permits the conjugation of cytokines before myocardial implantation, which might extend the period of delivered cytokine activity. We demonstrated that intramyocardial injection of the HG significantly preserved scar thickness, attenuated adverse cardiac remodeling, and improved ventricular function (compared with PBS injection) for up to 35 days after an MI in rats. Further, compared with HG alone or HG mixed with VEGF, HG with conjugated VEGF boosted regional angiogenesis.

After an MI, cardiomyocyte loss triggers matrix degradation, fibrosis, and the progression to heart failure. Injectable biomaterials mitigate these effects by providing a simple method to physically stabilize the infarct (prevent ventricular remodeling) and preserve matrix structure [2–4]. Kelley et al. [18] first demonstrated that suturing a polypropylene mesh to the myocardium to restrain the infarcted wall preserves LV geometry and prevents cardiac functional decline. Our HG may have the same effect if applied soon after a coronary occlusion. An advantage of injectable biomaterials over external restraints is their ability to increase the recruitment of marrow progenitor cells and induce angiogenesis [2]. In this study, the temperature-sensitive HG (vs. PBS) increased scar thickness,

prevented LV dilation, improved cardiac function, and enhanced angiogenesis. It may also have prevented paradoxical systolic bulging by gelling immediately within the infarct.

The damaging effects of an MI can be further moderated by gene transfection, cytokine treatment, or implanted stem cells [19]. However, the infarcted myocardium is stiff and the marginal region is enriched with blood vessels, and so genes, cytokines, or cells injected directly into the scar often leak into the epicardial space or the circulation (for example, we found that cell retention was less than 50% immediately after implantation into a myocardial infarct [20]). Unsuccessful strategies to increase retention have included multiple small volume injections, sealing the injection holes, and encapsulating genes, proteins or cells. However, there has been some success with injectable biomaterials, which – beyond providing structural support for the infarcted heart – also act as agents of therapeutic cytokine delivery. For instance, biologically-derived materials such as fibrin, collagen, alginates, and self-assembling peptides [2,3,21] can provide a platform to increase the delivery and/or support of cells or cytokines – enhancing blood flow and preventing necrosis [22,23]. Here, we tested a PVL-*b*-PEG-*b*-PVL HG that provides additional advantages since it is relatively inert (unlikely to induce rejection), easy to manufacture (and therefore, more cost-efficient than the biologically-derived materials), and it is synthetic, with a very uniform, honeycomb-like structure that may facilitate the diffusion of proteins or peptides.

PVL-*b*-PEG-*b*-PVL gels at a physiological temperature (37 °C) and degrades within 6 weeks. Temperature-sensitive HGs are particularly attractive candidates for injectable therapeutics because they can easily incorporate biological elements while in liquid form (i.e., at room temperature) that are retained in the heart when the polymer gels rapidly at body temperature. In this way, the gel effectively "traps" therapeutic cells or cytokines and then prolongs their effects by releasing them gradually at the injection site as the biomaterial slowly degrades. Indeed, in this study, conjugating VEGF to the HG (vs. mixing VEGF and HG) increased regional angiogenesis *in vivo* – perhaps because the biological activity of VEGF was extended over the ~40 days of biomaterial degradation (post-MI healing phase). Boosting cell recruitment and angiogenesis at the site of myocardial repair is particularly important for older, debilitated patients because the regenerative capacity of endogenous stem cells declines with increasing age.

5. Conclusions

We synthesized a temperature-sensitive, biodegradable HG that preserved ventricular function after an MI by stabilizing the infarct and inducing angiogenesis. Many investigators are attempting to develop methods that will improve the delivery efficiency of VEGF and other cytokines at sites of ischemic injury. We found that conjugating VEGF to the HG material (rather than mixing VEGF and HG) before injection optimized the extent of myocardial and functional recovery – possibly by extending the angiogenic effects of the "trapped" VEGF throughout the period of HG degradation.

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Conflict of interest disclosures

None declared.

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