Arteriogenic Therapy by Intramyocardial Sustained Delivery of a Novel Growth Factor Combination Prevents Chronic Heart Failure

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Background—Therapeutic angiogenesis is a promising approach for the treatment of cardiovascular diseases, including myocardial infarction and chronic heart failure. We aimed to improve proangiogenic therapies by identifying novel arteriogenic growth factor combinations, developing injectable delivery systems for spatiotemporally controlled growth factor release, and evaluating functional consequences of targeted intramyocardial growth factor delivery in chronic heart failure.

Methods and Results—First, we observed that fibroblast growth factor and hepatocyte growth factor synergistically stimulate vascular cell migration and proliferation in vitro. Using 2 in vivo angiogenesis assays (n=5 mice per group), we found that the growth factor combination results in a more potent and durable angiogenic response than either growth factor used alone. Furthermore, we determined that the molecular mechanisms involve potentiation of Akt and mitogen-activated protein kinase signal transduction pathways, as well as upregulation of angiogenic growth factor receptors. Next, we developed crosslinked albumin-alginite microcapsules that sequentially release fibroblast growth factor-2 and hepatocyte growth factor. Finally, in a rat model of chronic heart failure induced by coronary ligation (n=14 to 15 rats per group), we found that intramyocardial slow release of fibroblast growth factor-2 with hepatocyte growth factor potently stimulates angiogenesis and arteriogenesis and prevents cardiac hypertrophy and fibrosis, as determined by immunohistochemistry, leading to improved cardiac perfusion after 3 months, as shown by magnetic resonance imaging. These multiple beneficial effects resulted in reduced adverse cardiac remodeling and improved left ventricular function, as revealed by echocardiography.

Conclusion—Our data showing the selective advantage of using fibroblast growth factor-2 together with hepatocyte growth factor suggest that this growth factor combination may constitute an efficient novel treatment for chronic heart failure. (Circulation. 2011;124:1059-1069.)

Key Words: angiogenesis ■ capsules ■ heart failure ■ remodeling

The presence of coronary collaterals is a favorable prognostic factor in coronary artery disease, associated with reduced myocardial infarct size and increased patient survival. However, the fact that an estimated 66% to 75% of coronary artery disease patients have insufficient coronary collaterals presents an incentive for therapeutic stimulation of arterial blood vessel growth, ie, collaterogenesis or arteriogenesis. In addition, ≈30% of myocardial infarction (MI) patients display inadequate myocardial perfusion (ie, the no-reflow phenomenon), suggesting that therapeutic stimulation of smaller-caliber blood vessel growth, ie, angiogenesis, also is a valid treatment approach for coronary artery disease. Chronic heart failure (CHF), an increasingly common consequence of MI and other cardiomyopathies, is characterized by extensive cardiac hypertrophy and fibrosis. It has been suggested that the switch between reversible physiological and irreversible pathological cardiac hypertrophy depends on angiogenesis. Thus, therapeutic stimulation of arteriogenesis and/or angiogenesis is an attractive common target in cardiovascular diseases.

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At the turn of the century, several clinical trials of therapeutic angiogenesis for cardiovascular diseases such as coronary artery...
disease were initiated. Today, 10 years later, there is still no approved treatment for patients who are poor candidates for surgical revascularization or have distal diffuse ischemic disease. Indeed, double-blind controlled clinical trials in coronary artery disease patients, based on administration of vascular endothelial growth factor or fibroblast growth factor-2 (FGF-2), have failed to demonstrate a durable effect.6,7 The main problems of current proangiogenic approaches include the limited duration of the therapy achieved with bolus delivery of naked proteins or gene delivery vectors, the high costs associated with protein therapy because of the general need for large doses, the transient effects of treatment likely due to the generation of unstable blood vessels that regress over time, and important safety issues related to the possibility of inadvertent stimulation of angiogenesis in distant, dormant micrometastases or in diabetic retinopathy.8,9 Responses to these challenges include the proposition that single growth factor therapy be replaced by growth factor combinations to stimulate the generation of mature and stable blood vessels, including large-caliber collateral arteries,10 or gaining control over growth factor release rates using delivery vectors that allow prolonged angiogenic stimulation.11–13 Of note, most experimental approaches have involved delivery of supraphysiological doses of angiogenic growth factors, often in the range of 10 to 100 μg/kg. However, it seems that it is not the total growth factor dose that determines the outcome of angiogenic therapy; rather, local microenvironmental concentration gradients are important for achieving functional revascularization responses.14

Here, we report a new therapeutic strategy specifically designed to stimulate angiogenesis and arteriogenesis in the failing heart based on targeted, intramyocardial, sequential slow release of a novel angiogenic growth factor combination, FGF-2 and hepatocyte growth factor (HGF), using albumin-alginate microcapsules.

Methods

A detailed description of all methods and reagents used for the experiments is provided in the online-only Data Supplement.

Cell Migration and Proliferation

Briefly, cells were pretreated with FGF-2 or HGF and then added to the upper wells of a modified Boyden 48-well chamber. The lower wells contained FGF-2 or HGF. Cells migrated across the membrane during 6 to 12 hours at 37°C. Results are reported as number of migrating cells per 1 mm² (n=6). For analyses of cell proliferation, heart microvascular endothelial cells (HMEs), human microvascular cardiac endothelial cells (HMEC-Cs), and rat aortic smooth muscle cells (SMCs) were pretreated with FGF-2 or HGF, added to 24-well plates, and stimulated with FGF-2 or HGF. Cell numbers were estimated with the WST-1 assay.

Corneal Angiogenesis Assay

The mouse corneal assay was performed as previously described in C57Bl/6 mice and as approved by the North Stockholm Animal Ethics Committee.15 Briefly, sucrose aluminum sulfate micropellets containing recombinant human HGF and/or FGF-2 were implanted into mice corneas (n=5 per group). The eyes were examined on days 5, 25, and 40 and ~1 year after pellet implantation.

Albumin-Alginate Microcapsules

Albumin-alginate microcapsules were prepared with a modified version of the previously described interfacial cross-linking method16 and as outlined in the Methods section in the online-only Data Supplement.

In Vitro Growth Factor Release

Briefly, lyophilized microcapsules were loaded with growth factors by imbibition,17 with 1 μg growth factor per 1 mg microcapsules (~35'000 particles). Growth factor release after incubation in extracellular fluid mimetic release buffer was quantified by ELISA. Data are presented as mean amount (nanograms) of growth factor released per day per 1 mg microcapsules (n=3).

Matrigel Plug Model

Growth factor–reduced Matrigel was used to evaluate angiogenic responses as outlined in the Methods section in the online-only Data Supplement and in accordance with National Institutes of Health guidelines, European Communities Council directives (86/609/EEC), and French National legislation (ethical approval No. 76-114). Briefly, 500 μL Matrigel was mixed with microcapsules loaded with growth factors as above. Controls contained Matrigel mixed with buffer or microcapsules without growth factors. The Matrigel mixture was subcutaneously injected in male Balb/c mice (n=5 per group). After 3 weeks, Matrigel plugs were harvested and snap-frozen.

Chronic Heart Failure Model

We induced MI in anesthetized, mechanically ventilated male Wistar rats by ligation of the proximal left coronary artery after a left thoracotomy (n=22 rats per group) as previously described18,19 and in accordance with National Institutes of Health guidelines, European Communities Council directives (86/609/EEC), and French National legislation (ethical approval No. 76–114). Immediately after ligation, microcapsules loaded with growth factors, as above, were injected in 3 spots (25 μL per spot) along the infarct border zone on the right anterior side of the left ventricular (LV) free wall adjacent to the septum. The total amount of growth factor administered per heart was 125 ng HGF or 500 ng FGF-2 alone or in combination. Controls were injected with the same numbers of microcapsules without growth factor. At the time of death, hearts were arrested in diastole by immersion in ice-cold saturated potassium chloride buffer.

Histochecmistry

Collagen density and MI size were determined in heart cryosections stained with Sirius Red as previously described18 and as outlined in the Methods section in the online-only Data Supplement. Collagen content was calculated as percentage of collagen area to total area of the image (n=14 to 15 animals per group). Infarct size was calculated as follows: total infarction perimeter/(epicardial LV perimeter + endocardial LV perimeter)×100. For immunohistochemical analyses, cryosections were stained according to standard protocols as detailed in the Methods section in the online-only Data Supplement. Micrographs were processed by an operator blinded to the treatment groups with Image Pro-Plus, AxioVision, or Adobe Photoshop image analysis software. Vessel density and vessel maturity were quantified as the number of CD31⁺ vessels and smooth muscle active–positive (SMA⁺) vessels per 1 mm², respectively. Matrigel plug vessel content was calculated as percentage of vascularized area to total section area. Endothelial proliferation was analyzed by Ki67 and CD31 double labeling and presented as number of Ki67⁺ endothelial cells (ECs) per 1 mm². Cardiomyocyte sizes were measured in wheat germ agglutinin–stained sections. The ratio of vessel to cardiomyocyte was calculated as the number of vessels to the number of cardiomyocytes per 1 mm².

Semiquantitative Fluorescence Microscopy

Angiogenic growth factor receptor protein expression levels were determined in Matrigel plugs implanted with microcapsules loaded with FGF-2 or HGF as above and detailed in the Methods section in the online-only Data Supplement. Briefly, sections were double labeled for either c-Met or FGF receptor-1 (FGF-R1) in conjunction...
Results

Fibroblast Growth Factor-2 and Hepatocyte Growth Factor Synergistically Stimulate Vascular Cell Migration and Proliferation and Induce Stable Blood Vessel Growth

We evaluated the effects of FGF-2 and/or HGF in vitro using murine HMEs, murine embryonic microvascular ECs, HMEC-Cs, and rat aortic SMCs. Whereas both FGF-2 and HGF used alone induced EC and SMC migration and proliferation (Figure 1), HGF pretreatment significantly potentiated EC motility response to FGF-2 (Figure 1A and 1B). Reciprocally, pretreatment with FGF-2 enhanced HGF-induced migration (Figure 1A and 1B and Figure 1 in the online-only Data Supplement). Similarly, in SMCs, FGF-2 strikingly increased motility responses to HGF, although HGF did not alter FGF-2–induced migration (Figure 1C). Furthermore, pretreatment of HMEC-Cs (Figure 1D) or HMECs (Figure 1E) with FGF-2 significantly increased HGF-induced cell proliferation. Conversely, pretreatment of HMEC-C (Figure 1G) or SMCs (Figure 1F) with HGF significantly increased cell proliferation responses to FGF-2. These data show that FGF-2 and HGF synergistically stimulate both EC and SMC migration and proliferation, suggesting that this growth factor combination may generate mature and thus stable blood vessels in vivo. To investigate this possibility, we applied the corneal angiogenesis assay. Whereas HGF used alone induced a potent angiogenic response within 1 week as previously reported,15 most new blood vessels had regressed within 1 month (data not shown). In contrast, the blood vessels induced by FGF-2 used alone or in combination with HGF remained largely intact throughout the first month after initial remodeling (Figure 1H). However, whereas after ∼1 year only a few vessels remained in FGF-2–implanted corneas, considerably more extensive vascular networks persisted in corneas implanted with HGF and FGF-2.

Sequential Slow Release of Growth Factors by Albumin-Alginate Microcapsules

Next, we aimed to obtain an injectable particulate growth factor delivery system to achieve spatiotemporally controlled release inside the myocardium. Alginate, a naturally occurring polysaccharide, is suitable for the delivery of positively charged proteins such as FGF-2 and HGF because it bears negatively charged carboxylic groups available for electrostatic interactions.22 Indeed, ionically cross-linked alginate hydrogels have been widely used for angiogenic growth factor delivery23 but generally display uncontrolled degradation, leading to unpredictable release kinetics.24 However, covalently cross-linking polysaccharides to proteins in a microcapsule membrane prevents hydrolysis-driven dissolution and delays protease-driven degradation, resulting in more stable particles with reproducible drug release rates.16,17 Thus, we developed microcapsules containing a thin, covalently cross-linked human serum albumin and propylene glycol alginate membrane surrounding a liquid center. Laser diffraction measurements and microscopic observations revealed that these albumin-alginate microcapsules had a mean diameter of 100 μm and were roughly spherical (Figure IIA in the

Magnetic Resonance Imaging

Cardiac perfusion was assessed by arterial spin-labeling magnetic resonance imaging with a 4.7-T small animal magnet (Biopac 47/40 advanced II, Bruker, Ettlingen, Germany). Briefly, the perfusion sequence was run in the short-axis plane, allowing determination of myocardial tissue perfusion. Global and slice-selective spin inversion recovery T1* (fitted time constant) maps were acquired.20 Perfusion images were analyzed with ParaVision 5.0 software (Bruker) by 2 independent observers, and regional perfusion in the treated area of the LV was calculated as described.41
online-only Data Supplement). Dehydration caused a partial and reversible collapse, resulting in the appearance of a very pleated surface in desiccated microcapsules as observed by scanning electron microscopy (Figure IIB in the online-only Data Supplement). The microcapsules were assayed in vitro for the release of angiogenic growth factors under conditions approximating the in vivo tissue environment. Whereas FGF-2 release from the microcapsules began immediately, the release of HGF and another angiogenic growth factor, platelet-derived growth factor (PDGF)-BB, was delayed for 1 week (Figures IIC and IIIA in the online-only Data Supplement). Furthermore, whereas PDGF-BB release lasted 4 weeks, that of FGF-2 and HGF lasted 6 weeks. We confirmed that the growth factors released from the microcapsules retained their full bioactivity using an in vitro assay (Figure IIIB in the online-only Data Supplement).

To investigate the stability of the microcapsules, we analyzed their morphology during in vitro incubation. Although FGF-2-loaded microcapsules started to disintegrate within 1 week, those loaded with HGF remained largely intact for 3 to 4 weeks (Figure IIIC in the online-only Data Supplement). Furthermore, to determine the growth factor localization, we performed confocal analyses of microcapsules loaded with fluorescently labeled FGF-2 or HGF (Figure IID and IIE in the online-only Data Supplement). The results show that although both growth factors bound to the microcapsule surface layer, confirming their interactions with the cross-linked protein-polysaccharide membrane, FGF-2 was also found in the liquid center of the microcapsule. These findings may in part explain why the microcapsules display different release profiles for FGF-2 and HGF.

**Sustained Delivery of Fibroblast Growth Factor-2 and Hepatocyte Growth Factor Enhances Angiogenic and Arteriogenic Responses**

To determine whether our slow-release system would influence the angiogenic effect of FGF-2 or HGF in vivo, we compared treatment with growth factor–loaded microcapsules and naked growth factors using the mouse Matrigel plug model. Growth factor delivery by microcapsules was found to be 3 to 6 times more potent to induce angiogenesis compared with bolus delivery of growth factors (Figure IID in the online-only Data Supplement).

The reciprocal stimulatory interactions observed between FGF-2 and HGF in vascular cells indicated that these growth factors may cooperatively regulate vessel growth. To further
investigate this possibility, microcapsules containing FGF-2 and/or HGF were injected in Matrigel plugs in mice. In each case, we used the lowest dose of growth factor resulting in a substantial angiogenic effect. Whereas each growth factor used alone induced a moderate angiogenic response, FGF-2 and HGF used in combination synergistically stimulated angiogenesis and arteriogenesis, as evidenced by the increased vascular density (Figure 2A and 2C), vascular maturity (Figure 2D), and vascularized area (Figure 2E) compared with single growth factor treatments. The most potent angiogenic growth factor combination described to date is the association of FGF-2 and PDGF-BB.12 To compare these growth factor combinations, microcapsules containing FGF-2 and/or PDGF-BB were injected in Matrigel plugs in mice. We found that whereas the vessel density induced by the combination of FGF-2 and PDGF-BB (Figure 2B, 2F, and 2I) was moderately greater than that induced by FGF-2 in combination with HGF, the number of mature vessels did not differ (Figure 2G and 2J). However, whereas FGF-2 together with PDGF-BB resulted in only 30% of the total plug area being vascularized, the combination of FGF-2 and HGF notably resulted in a Matrigel plug vessel content of \(\approx 80\%\) (Figure 2E and 2H). These results reveal our microcapsules to be particularly efficient for growth factor delivery in vivo. Furthermore, in agreement with the in vitro data, the combination of FGF-2 and HGF synergistically induced angiogenesis and arteriogenesis at a level comparable to or surpassing that of the most potent angiogenic growth factor combination currently described.13

**Intramyocardial Delivery of Fibroblast Growth Factor-2 and Hepatocyte Growth Factor Stimulates Angiogenesis and Arteriogenesis and Prevents Myocardial Infarction–Induced Cardiac Hypertrophy and Fibrosis**

To evaluate the effect of FGF-2 in combination with HGF in a setting of cardiovascular disease, we performed a randomized, blinded experiment in rats surviving coronary artery ligation \((n=102)\) or sham surgery \((n=11)\). The experimental MI model leads to the development of CHF within 3 months.18 We found that the albumin-alginate microcapsules...
displayed slow release of HGF or FGF-2 in vivo after intramyocardial injection in rats (Figure IV in the online-only Data Supplement). Thus, we used these microcapsules for cardiac delivery of angiogenic factors by local injection into the viable free wall of the myocardium bordering the LV infarct zone immediately after MI (Figure VA in the online-only Data Supplement). At 1 or 3 months after MI, the angiogenic and arteriogenic cardiac effects were evaluated by immunohistochemistry. At 1 month after MI, untreated controls displayed myocardial vessel rarefaction, including reduced levels of mature blood vessels, compared with healthy shams (1552 ± 71 vessels per 1 mm²; Figure VB and VC in the online-only Data Supplement). The reduction was limited mainly to SMA⁺ microvessels with lumen diameters inferior to 15 μm (Figure VIA and VIB in the online-only Data Supplement). The result of an inherent compensatory angiogenic response, evidenced by increased EC proliferation (Figure VD in the online-only Data Supplement), the total vessel (1848 ± 57 vessels per 1 mm²) and mature vessel densities were slightly improved in untreated control hearts at 3 months (Figure 3A through 3D). Monotherapy with FGF-2 resulted in a further increase in angiogenesis and arteriogenesis locally in the treated LV area, leading to slightly augmented vessel density (1809 ± 83 vessels per 1 mm²) and significantly increased mature vessel density at 1 month compared with controls. However, the effects were lost at 3 months (1839 ± 84 vessels per 1 mm²; Figure 3A through 3E). Monotherapy with HGF, on the other hand, showing limited arteriogenic effects at 1 month, tended to increase EC proliferation and vascular density at 3 months (2107 ± 80 vessels per 1 mm²; Figure 3A, 3C, and 3E). In contrast, the combination therapy induced a potent angiogenic (1919 ± 66 vessels per 1 mm²) and arteriogenic response, again strictly limited to the treated LV zone, with more than a doubling of the number of proliferating ECs (Figure VD in the online-only Data Supplement) and 3 times more mature blood vessels compared with untreated controls at 1 month after MI (Figure VC in the online-only Data Supplement). The increase in SMA⁺ vessels was due mainly to an increase in microvessels (Figure VIA and VIB in the online-only Data Supplement). Strikingly, by 3 months, the myocardial vessel density in the combination group had attained normal sham levels (2505 ± 106 vessels per 1 mm²; Figure 3A and 3C). Moreover, the mature vessel density in
the group treated by the combination even surpassed that of shams (Figure 3B and 3D). Furthermore, whereas the numbers of SMA+ microvessels had decreased, there was a significant increase in the number of small arterioles with lumen diameters between 15 and 50 µm (FigureVIC and VID in the online-only Data Supplement), suggestive of vascular remodeling.

Next, the extent of MI-induced cardiac hypertrophy and fibrosis was evaluated by histological analyses of cardiomyocyte sizes and collagen density, respectively. At 1 month, cardiomyocyte sizes were not significantly increased in MI controls compared with sham, although the mRNA level of the maladaptive cardiac hypertrophic marker β-myosin heavy chain was found to be increased, together with significantly higher expression levels of atrial natriuretic peptide and a tendency for increased brain natriuretic peptide (Figure VII in the online-only Data Supplement). In contrast, FGF-2 used alone or in combination with HGF tended to stimulate cardiac hypertrophy (data not shown), although it did not reach significance. This is in line with previous studies suggesting a direct stimulatory role of angiogenesis on cardiac hypertrophy.25 However, the cardiomyocyte hypertrophy was not associated with increased β-myosin heavy chain, suggesting, together with the increased vessel density observed, that the cardiomyocyte growth may have been adaptive. At 3 months, MI controls and FGF-2–treated rats showed equally enlarged cardiomyocytes and increased levels of collagen deposition associated with increased brain natriuretic peptide and a tendeny for increased brain natriuretic peptide (Figure VII in the online-only Data Supplement). Both regional and global cardiac contractility were reduced, as evidenced at 1 month by a decreased fractional shortening (FS; 16±1% for control versus 54±3% for sham; Figure 4E) and velocity of circumferential fiber shortening (Figure 4F). Monotherapy with HGF only slightly improved LV parameters by 3 months, as evidenced by a decreased LVESD and end-systolic volume (Table II in the online-only Data Supplement) and a tendency for increased FS and velocity of circumferential fiber shortening (Figure 4E and 4F) compared with untreated controls. Monotherapy with FGF-2, on the other hand, significantly reduced LV dilation (Figure 4B and 4D; LV end-systolic [LVESD] and end-diastolic [LVEDD] diameters in Tables I and II in the online-only Data Supplement). Both regional and global cardiac contractility were reduced, as evidenced at 1 month by a decreased fractional shortening (FS; 16±1% for control versus 54±3% for sham; Figure 4E) and velocity of circumferential fiber shortening (Figure 4F).

In contrast to these moderate effects of monotherapies, the combination of FGF-2 and HGF reduced LV dilation (Figure 4B and 4D; LVESD and LVEDD in Table I in the online-only Data Supplement) and LV dysfunction already at 1 month after MI, as shown by an increased FS (22±2%; Figure 4E) compared with untreated controls. This was associated with a tendency for increased velocity of circumferential fiber shortening (Figure 4F), LV anterior end-systolic wall thickness (Table I in the online-only Data Supplement), and wall thickening (Figure 4C). At 3 months, the combination treatment group displayed a significant further increase in LV wall thickness (anterior end-systolic and end-diastolic wall thickness, Table II in the online-only Data Supplement), and wall thickening was no longer significantly different from that of sham rats (Figure 4C). Moreover, the LV dilation was further reduced (Figure 4D; LVEDD and LVESD, Table II in the online-only Data Supplement) and associated with a marked recovery of LV function, evidenced by an increased FS (Figure 4E) and velocity of circumferential fiber shortening (Figure 4F) compared with both untreated controls and HGF-treated animals, indicating that the development of CHF was partially prevented.

Intramyocardial Delivery of Fibroblast Growth Factor-2 and Hepatocyte Growth Factor Improves Regional Cardiac Perfusion and Cardiac Function After Myocardial Infarction

To assess whether the blood vessels induced by the angiogenic therapies resulted in a functional improvement in cardiac perfusion, magnetic resonance imaging was used. Our results, obtained 3 months after MI, demonstrate that whereas untreated controls displayed significantly reduced cardiac perfusion compared with healthy sham-operated animals, only the combination treatment increased regional cardiac perfusion in the treated area of the LV (Figure 4A).

Next, to investigate whether the multiple beneficial myocardial alterations induced by the angiogenic therapies correlated with improved cardiac function, even though myocardial infarct sizes were similar in all groups (Figure IX in the online-only Data Supplement), echocardiographic analyses were carried out at 1 and 3 months after MI. As previously reported,18 untreated controls displayed severe cardiac dysfunction (Figure 4B through 4F and Tables I and II in the online-only Data Supplement), characterized by LV wall thickening at 1 month (Figure 4C), and progressive LV dilation associated with the development of CHF (Figure 4B and 4D; LV end-systolic [LVESD] and end-diastolic [LVEDD] diameters in Tables I and II in the online-only Data Supplement). Both regional and global cardiac contractility were reduced, as evidenced at 1 month by a decreased fractional shortening (FS; 16±1% for control versus 54±3% for sham; Figure 4E) and velocity of circumferential fiber shortening (Figure 4F). Monotherapy with HGF only slightly improved LV parameters by 3 months, as evidenced by a decreased LVESD and end-systolic volume (Table II in the online-only Data Supplement) and a tendency for increased FS and velocity of circumferential fiber shortening (Figure 4E and 4F) compared with untreated controls. Monotherapy with FGF-2, on the other hand, significantly reduced LV dilation (Figure 4B and 4D; LVEDD and LVESD in Tables I and II in the online-only Data Supplement) and, by 3 months after MI, increased both FS (Figure 4E) and velocity of circumferential fiber shortening (Figure 4F).

In contrast to these moderate effects of monotherapies, the combination of FGF-2 and HGF reduced LV dilation (Figure 4B and 4D; LVESD and LVEDD in Table I in the online-only Data Supplement) and LV dysfunction already at 1 month after MI, as shown by an increased FS (22±2%; Figure 4E) compared with untreated controls. This was associated with a tendency for increased velocity of circumferential fiber shortening (Figure 4F), LV anterior end-systolic wall thickness (Table I in the online-only Data Supplement), and wall thickening (Figure 4C). At 3 months, the combination treatment group displayed a significant further increase in LV wall thickness (anterior end-systolic and end-diastolic wall thickness, Table II in the online-only Data Supplement), and wall thickening was no longer significantly different from that of sham rats (Figure 4C). Moreover, the LV dilation was further reduced (Figure 4D; LVEDD and LVESD, Table II in the online-only Data Supplement) and associated with a marked recovery of LV function, evidenced by an increased FS (Figure 4E) and velocity of circumferential fiber shortening (Figure 4F) compared with both untreated controls and HGF-treated animals, indicating that the development of CHF was partially prevented.
different signaling pathways were analyzed in ECs. The levels of phospho-Akt (p-Akt) and phospho-42/44 mitogen-activated protein kinases (p-MAPKs) were assayed in HMEC-Cs after stimulation with FGF-2 and/or HGF. We found that FGF-2 induced maximal Akt activation within 10 to 15 minutes (1.7±0.5-fold induction over control) but p-Akt levels returned to baseline after 30 minutes (Figure 5A and 5C). In turn, HGF induced maximal Akt activation within 30 minutes (1.6±0.8-fold) persisting up to 1 hour, consistent with the potent antiapoptotic effects of HGF.26 Notably, the combination of FGF-2 with HGF induced a prolonged Akt activation lasting >2 hours, with a significantly increased maximal activation level (2.2±0.2-fold). Next, we found that FGF-2 induced maximal p^{42/44-MAPK} activation within 15 minutes (1.8±0.6 fold; Figure 5B and 5D). In addition, HGF activated maximal Akt activation within 30 minutes (1.6±0.5-fold). The increased levels of p-Akt persisted up to 1 hour, consistent with the potent antiapoptotic effects of HGF.26 Notably, the combination of FGF-2 with HGF induced a prolonged Akt activation lasting >2 hours, with a significantly increased maximal activation level (2.2±0.2-fold). Next, we found that FGF-2 induced maximal p^{42/44-MAPK} activation within 15 minutes (1.8±0.6 fold; Figure 5B and 5D). In addition, HGF activated maximal Akt activation within 30 minutes (2.4±0.8-fold) persisting for >1 hour. The combination of FGF-2 and HGF resulted in a strikingly potent MAPK activation within 10 to 15 minutes (5.8±1.0-fold). However, the signal duration was only slightly prolonged compared with single growth factor stimulation. To verify that synergistic activation of cell signaling pathways also may occur in vivo, we assayed the levels of p-Akt in cardiac samples obtained at 3 weeks after MI. We found that the combination treatment led to significantly increased Akt activation compared with MI controls (Figure XA and XB in the online-only Data Supplement).

Another mechanism by which dual growth factor treatment may produce synergistic effects involves the induction of growth factor receptor levels. For instance, the potent angiogenic synergy between FGF-2 and PDGF-BB is due in part to an upregulation in vascular cells of FGFR-1 by PDGF-BB and of PDGF receptors by FGF-2.12,27 To investigate whether similar mechanisms may operate in the combination of FGF-2 with HGF, real-time polymerase chain reaction analyses were performed in vascular cells. Indeed, we found that HGF stimulation significantly induced the expression of both FGFR-1 (Figure 5F) and the HGF receptor c-Met (Figure 5E) in murine HMEs. In a reciprocal manner, FGF-2 stimulation potently increased c-Met mRNA levels (Figure 5E) while displaying a weaker effect on FGFR-1 levels (Figure 5F). Similar results were obtained in human HMEC-Cs (Figure 5G and 5H). Moreover, we found that FGF-2 induced the expression of c-Met in rat SMCs (data not shown). Next, to
confirm these findings in vivo, FGFR-1 and c-Met protein levels were assayed by semiquantitative immunofluorescence analyses of mouse Matrigel plugs. We found that although vascular c-Met levels were comparable after 3 weeks of stimulation with either FGF-2 or HGF, the FGFR-1 levels were increased in blood vessels induced by HGF compared with FGF-2 (Figure 5I and 5J).

To extend these findings to the heart, we assayed by Western blot the levels of phosphorylated FGFR-1 or c-Met in the treated LV peri-infarct zone at 3 weeks after MI. We found that FGF-2 treatment used alone or together with HGF resulted in increased levels of activated FGFR-1 compared with MI controls (Figure XB and XC in the online-only Data Supplement). Similarly, HGF treatment used alone significantly increased phospho–FGFR-1 levels in the heart. Reciprocally, FGF-2 used alone increased the levels of phospho–c-Met to levels similar to those with HGF treatment used alone or together with FGF-2 (Figure XE and XF in the online-only Data Supplement). Taken together, this finding suggests that whereas FGF-2 may have induced HGF and/or c-Met expression, HGF may have induced FGFR-1 expression in the heart, in line with our in vitro findings in vascular cells.

**Discussion**

Current strategies of therapeutic angiogenesis or arteriogenesis in cardiovascular diseases, including CHF, have proved insufficient. Our study shows that therapeutic angiogenesis, based on targeted intramyocardial albumin-alginate microcapsule delivery of a synergistic combination of growth factors, results in potent stimulation of mature blood vessel growth, prevention of cardiac remodeling, and enhanced myocardial perfusion and cardiac function in a rat model of post-MI CHF.

HGF plays an important role in tissue regeneration. It displays considerable prosurvival effects in many cell types, including cardiomyocytes. In addition, HGF potently stimulates angiogenesis. During tissue ischemia, HGF and its receptor, cMet, are upregulated in vascular cells and cardiomyocytes. Most recently, encouraging results have been reported for a double-blind controlled HGF gene therapy trial for chronic limb ischemia, suggesting the utility of this particular growth factor for regenerative medicine. Interestingly, in the present study, we found that HGF monotherapy at a dose at least 1 to 2 orders of magnitude lower than most experimental studies, while efficiently preventing cardiac fibrosis and hypertrophy, had limited angiogenic effects. Thus, although the structural antiremodeling effects were indeed considerable, the fact that HGF monotherapy merely showed weak amelioration of cardiac function suggests that stimulation of angiogenesis and arteriogenesis plays a key role in prevention of cardiac dysfunction. However, whereas FGF-2 monotherapy failed to induce a stable increase in blood vessel density and further showed no effects on cardiac fibrosis or hypertrophy, it did successfully improve regional cardiac function, as evidenced by decreased LVESD and increased FS. This suggests that mechanisms other than stimula-
tion of angiogenesis were at play. Indeed, FGF-2 may have direct cardioprotective effects involving decreased cell-to-cell metabolic coupling in cardiomyocytes through inhibition of connexin-43 gap junctions. Thus, it seems probable that the improvement in both regional and global cardiac function observed with the combination of FGF-2 and HGF is multifactorial in origin and not due only to potent induction of arteriogenesis leading to a normalized ratio of cardiomyocytes to vessel and enhanced regional cardiac perfusion.

The cardiac fibrosis occurring during postinfarction remodeling causes increased ventricular wall stiffness and reduced cardiac relaxation rates, contributing to the cardiac dysfunction. HGF has been shown to reduce cardiac fibrosis, in part via suppression of transforming growth factor-β signaling. Similarly, in our study, we found that HGF used alone or in combination with FGF-2 resulted in an impressive reduction of cardiac collagen content. This antifibrotic effect of HGF may have played an important role in the observed cardiac functional improvement induced by the combination treatment.

With regard to the potential molecular mechanisms behind the observed angiogenic synergy, we found that the combination of FGF-2 and HGF significantly potentiated the activation of Akt and MAPKs compared with single growth factors. This may contribute to the synergistic stimulation of cellular migration and proliferation observed in vitro and to the potent angiogenic effects in vivo. Moreover, we found evidence, both in vitro and in vivo, that HGF induces FGF-R1 levels in ECs, which should lead to enhanced FGF-2–induced signaling. Similar results were found in human ECs in which FGF-2 also reciprocally increased c-Met levels.

Previous studies have indicated some further potential means of angiogenic interactions, including HGF induction in SMCs by FGF-2. Moreover, HGF induces vascular endothelial growth factor production in SMCs and has been suggested to act in synergy with vascular endothelial growth factor to induce EC migration. However, another study reported that HGF and vascular endothelial growth factor did not synergistically stimulate angiogenesis in vivo and further that vascular endothelial growth factor was not required for the angiogenic effects of HGF. Interestingly, the proangiogenic signal of angiopeptin-1 has been shown to depend on induction of HGF in ECs, suggesting that HGF is indeed a key mediator of vascular maturation similar to PDGF-BB.

Whereas angiogenesis generally requires prolonged stimulation of vascular cells, the in vivo half-life of most angiogenic growth factors is very limited, illustrating the need to incorporate sustained delivery systems for protein therapy approaches. In this study, we devised albumin-alginate microcapsules to gain spatiotemporal control over growth factor release. These microcapsules released active growth factors over a significant period of time, showing different release kinetics for FGF-2 versus HGF. This sequential release pattern, generating an initial strong angiogenic signal through FGF-2 followed by a potent arteriogenic signal via HGF, might be ideal to induce mature blood vessel formation. Furthermore, our sustained release system not only increased the efficacy of treatment, permitting the use of remarkably low doses of growth factors, but also resulted in the confinement of the angiogenic effect to a well-defined area of the LV. Both of these aspects should be instrumental in considerably reducing growth factor leakage to the circulation and thus the risk for serious side effects of treatment, including stimulation of angiogenesis at distant sites potentially harboring micrometastases.

**Conclusions**

Our data provide proof of principle that localized sustained delivery of low doses of a specific combination of angiogenic growth factors is sufficient to generate stable and functional blood vessels in the heart. Moreover, dual delivery of FGF-2 and HGF reduced MI-induced cardiac fibrosis and remodeling and partially prevented cardiac dysfunction. These findings may provide conceptual guidelines for future clinical trials of therapeutic angiogenesis for the treatment of cardiovascular diseases.

**Sources of Funding**

This work was supported in part by the 2009 Société General Asset Management Therapeutic Innovation Prize and by Inserm. Acquisition of the magnetic resonance imaging system was made possible through financial support from Servier Labaratories, France, but this work was conducted independently from the firm. S. Banquet was supported by a French Ministerial PhD student fellowship and a fellowship from the Fondation pour la Recherche Médicale. E. Gomez was supported by PhD student fellowships from the Société Française de Pharmacologie et de Thérapeutique and the Groupe de Réflexion sur la Recherche Cardiovasculaire.

**Disclosures**

None.

**References**


CLINICAL PERSPECTIVE

 Therapeutic stimulation of blood vessel growth, including microvascular growth (angiogenesis) and collateral growth (arteriogenesis), is an attractive common target in cardiovascular diseases. Such novel therapies may in particular benefit coronary artery disease patients with advanced-stage or small-vessel disease that responds poorly to standard pharmaceutical or surgical revascularization treatments or patients who develop chronic heart failure. However, despite significant preclinical efforts, there is still no available treatment to stimulate cardiac vessel growth, and clinical studies have so far been inconclusive. Indeed, current approaches for therapeutic angiogenesis or arteriogenesis result in the generation of unstable or nonfunctional blood vessels. Thus, there is an urgent need for new treatments and delivery methods to improve the efficacy of therapeutic blood vessel growth. In the present study, using a microparticulate slow-release system, we have evaluated targeted intramyocardial delivery of a growth factor combination of fibroblast growth factor-2 and hepatocyte growth factor in a rat model of myocardial infarction. We show that the combination treatment induces angiogenesis and arteriogenesis, resulting in enhanced cardiac perfusion at 3 months after myocardial infarction. Furthermore, the cardiac remodeling and dysfunction associated with the development of heart failure were partially prevented by fibroblast growth factor-2 and hepatocyte growth factor used in combination. Our data suggest that local intramyocardial delivery of fibroblast growth factor-2 together with hepatocyte growth factor in our slow-release formulation may constitute an efficient novel treatment to stimulate a durable angiogenic and arteriogenic response in the heart. Furthermore, on the basis of this study, we believe that this therapy might prevent the development of chronic heart failure in patients.
Arteriogenic Therapy by Intramyocardial Sustained Delivery of a Novel Growth Factor Combination Prevents Chronic Heart Failure

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SUPPLEMENTAL MATERIAL
Materials & Methods

Reagents and Animals

Murine Heart Microvascular Endothelial cells (HME) were a kind gift from Dr Marco Presta (Università di Brescia, Italy). Murine Embryonic Microvascular Endothelial cells (PmT-EC) were a kind gift from Drs. Karin Aase and Lars Holmgren (Karolinska Institutet, Sweden). Primary Human Microvascular Cardiac Endothelial cells (HMEC-C) and Rat Aortic Smooth Muscle Cells (SMC) were purchased from Lonza. All cell lines were maintained in DMEM (Gibco, Invitrogen, Paisley, UK) containing 10% FBS, except PmT-EC and HMEC-C, which were maintained in EGM-2 medium supplemented with MV bullet kit (Lonza). Primary cell cultures were used for experiments between passages 4-8. All cells were maintained at 5% CO₂ and 95% air at 37°C. Growth factors, rhFGF-2 (monomer, 157 a.a.), rmHGF (dimer, 463 and 232 a.a.), rhHGF (dimer, 697 a.a.), rhPDGF-BB (dimer, 109 a.a.), were obtained from RnD Systems Inc. (Minnesota, USA). Male Balb/c mice (20-22g) and male Wistar rats (200-220g) were purchased from Janvier (Le Genest St Isle, France). Male C57Bl/6 mice (20-22g) were obtained from the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet (Stockholm, Sweden). Animal experiments were performed in accordance with NIH guidelines, the European Communities Council Directives (86/609/EEC), and French National legislation (ethical approval no° 76-114) or were approved by the North Stockholm Animal Ethics Committee.

Cell migration and proliferation

Chemotaxis was assayed using a modified Boyden migration 48-well chamber (AP48; Neuro Probe Inc., Gaithersburg, USA). Briefly, membranes were coated with 0.15% gelatin for 1h at 37°C. Cells, serum starved for 24h in 1% FCS (without MV bullet kit supplements for HMEC-C and PmT-EC), were stimulated with growth factor for 24h in the case of pre-
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treatment using FGF-2 (25 ng/ml for HME, PmT and RAOSMC; 50 ng/ml for HMEC-C) or HGF (25 ng/ml for HMEC-C; 50 ng/ml for HME, PmT and RAOSMC). Next, cells were trypsinized and resuspended in fresh medium supplemented with 0.25% BSA. Into each upper well was added 1x10^4 cells. The lower wells contained FGF-2 (12.5 ng/ml for HME and RAOSMC; 25 ng/ml for PmT-EC and HMEC-C) or HGF (12.5 ng/ml for HME, PmT-EC, and RAOSMC; 5 ng/ml for HMEC-C). The cells were incubated in the chamber for 6-12h hours at 37°C, after which cells attached to the membrane were fixed in methanol and stained with Hematoxylin. Six replicate samples were used in each experiment, and experiments were performed at least twice. Migrating cells were analyzed using a light microscope, and reported as numbers of migrating cells x10^2 per mm². For analyses of cell proliferation, HME, HMEC-C, and RAOSMCs were either pretreated or not for 24h with FGF-2 (25 ng/ml for HME; 50 ng/ml for HMEC-C and RAOSMCs) or HGF (25 ng/ml for HMEC-C; 50 ng/ml for HME and RAOSMCs). Next, 1x10^4 cells were added to each well of 24-well plates and incubated for 2h at 37°C. After cell attachment, the medium was replaced with fresh medium containing FGF-2 (25 ng/ml for HME; 10 ng/ml for HMEC-C; 50 ng/ml for RAOSMCs) or HGF (1 ng/ml for HME; 25 ng/ml for HMEC-C; 50 ng/ml for RAOSMCs). Cell proliferation was assayed after 24h, 48h, or 72h using the WST-1 assay according to manufacturer’s instructions (Roche).

Albumin-alginate microcapsules

Albumin-alginate microcapsules were prepared using a modified version of the previously described interfacial cross-linking method. Briefly, 4% (w/v) human serum albumin (HSA, LFB) and 2% (w/v) propylene glycol alginate (PGA, ISP) were dissolved in a phosphate buffer pH 7.4. This aqueous phase was emulsified in cyclohexane (SDF) containing 2% (w/v) sorbitan trioleate (Sigma), at a stirring speed of 2000 rpm. Then, a 2.5% (w/v) solution of terephthaloyl chloride (Acros) in a chloroform-cyclohexane (1:4 v/v) mixture was added to
the emulsion and the cross-linking reaction was allowed to develop for 30 min. The reaction was stopped by dilution of the reaction medium. The microcapsules were separated from the organic phase by centrifugation, and washed successively with cyclohexane, with ethanol (Charbonneaux-Brabant) containing 2% (w/v) polysorbate (Sigma), with 95% (v/v) ethanol and finally thrice with pure water. Diameter measurements were performed using laser diffraction (Particle Sizer LS200, Beckman-Coulter). After staining with methylene blue, the microparticles were observed with a light microscope (Olympus, BH-2) equipped with interferential phase contrast. SEM observations (JSM-5400LV, JEOL) were made after alcohol dehydration of microcapsule suspension followed by Au/Pd coating. Finally, the microcapsules were freeze-dried in a Freezone 6 (LabConco, condenser temperature: -45°C, pressure < 0.5 mbar).

**In vitro growth factor release**

Lyophilised microcapsules were loaded with growth factors by imbibitions\(^4\), using 1 µg growth factor per mg microcapsules (approximately 35’000 particles), during a 1h incubation at +4°C. The growth factor-loaded microcapsules were resuspended at 4 mg microcapsules/mL in extracellular fluid mimetic release buffer (EFM-RB; 5 mM KCl, 125 mM NaCl, 20 mM Hepes, 1.5 mM MgCl\(_2\), 1.5 mM CaCl\(_2\), pH 7.4) and incubated under continuous rotation (6 rpm) for 40 days at 37°C. Every other day the tubes were centrifuged (300g, 8 min) to pellet the microcapsules. A sample of the supernatant was collected and stored at -80°C. The initial volume in the test tube was restored by addition of fresh EFM-RB to simulate unlimited diffusion conditions. The growth factor-release was quantified by ELISA according to the manufacturer’s instructions (HGF and PDGF-BB, RnD systems; FGF-2, Invitrogen). Data are presented as mean amount (ng) of growth factor released per day per mg of microcapsules (n=3).
Corneal angiogenesis assay

The mouse corneal assay was performed as previously described\(^5\). Briefly, micropellets (0.35 x 0.35 mm) of sucrose and aluminum sulfate coated with Hydron polymer type NCC (IFN Sciences, New Brunswick, NJ), containing 280 ng rhHGF and 40 ng rhFGF-2 (Phamacia & UpJohn, Milan, Italy) or 80 ng of rhFGF-2 used alone, were surgically implanted into corneas at 1.0 to 1.4 mm from the corneal limbus in 7- to 8-week-old C57Bl/6 male mice (n=5 mice per group). The eyes were examined on day 5, 25, day 40, and around 1 year after pellet implantation.

Matrigel plug model

Growth factor-reduced matrigel (BD, Bedford, Massachusetts, USA) was used to evaluate angiogenic responses. Briefly, 500 µL matrigel was mixed with 0.125, 0.5, or 1 mg microcapsules loaded with growth factors as above. Controls contained matrigel mixed with buffer or microcapsules without growth factors. The matrigel mixture was subcutaneously injected to form dorsal plugs in male Balb/c mice anesthetized by intraperitoneal injection of ketamine (90 mg/kg, Bayer, France) and xylazine (3.6 mg/kg). After 3 weeks, matrigel plugs were harvested, snap-frozen embedded in Tissue-Tek (Sakura Finetek, Torrance, USA) and stored at -80°C.

Chronic heart failure model

Myocardial infarction (MI) was induced in anesthetized (ketamine, 3.6 mg/kg; isoflurane gas, 2%), mechanically ventilated male Wistar rats by ligation of the proximal left coronary artery following a left thoracotomy (n=22 rats/group) as previously described\(^6\).\(^7\). Rats with infarcts encompassing 20-50% of the left ventricle (LV) were included in the study. Immediately after, microcapsules loaded with growth factors as outlined above were injected in three spots
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(23 µl/spot) along the infarct border zone on the right anterior side of the LV free wall adjacent to the septum. The total amount of growth factor administered per heart was 125 ng HGF or 500 ng FGF-2 alone or the combination of both. Controls were injected with the same numbers of microcapsules without growth factor. The thorax was closed in three layers and rats allowed to recover on a heating pad before being returned to their cages (2-3 rats/cage). At the time of sacrifice, rats were given a lethal dose of anaesthesia (Sodium Methohexital) followed by rapid excision of the heart through a ventral thoracotomy. Hearts were arrested in diastole by immersion in ice-cold saturated potassium chloride buffer. Lungs and hearts were weighed and the LV dissected before being cut transversally at the level of the papillary muscles and snap-frozen embedded in Tissue-Tek and stored at -80C.

Histochemistry

MI size and collagen density were determined in 10 µm thick serial heart cryosections fixed in Carnoy’s fixative and stained with Sirius Red as previously described\(^{6}\). Slides were examined and photographed under a light microscope (Zeiss) at 40x magnification. Collagen-rich areas were identified using Image Pro Plus (version 6.3). Collagen content was calculated as percentage of collagen area to total area of the image (n=14-15 animals/group). Infarct size was analyzed in videomicrophotographs of the sections using Adobe Photoshop (CS3 extended version 10) to delineate epicardial and endocardial LV perimeters and infarct perimeter. Infarct size was calculated as: total infarction perimeter/(epicardial LV perimeter + endocardial LV perimeter) x 100. For immunohistochemical analyses, 10 µm cryosections were post-fixed in acetone and stained according to standard protocols using rat antimouse CD31 (PECAM-1, 1:100, BD); biotinylated mouse antirat CD31 (PECAM-1, 1:100, BD); mouse antihuman SMA-FITC (smooth muscle α actin, 1:200, Sigma); rabbit anti human Ki67 (1:500, Novocastra Laboratories, Newcastle, UK); and WGA-A488 (wheat germ agglutinin,
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1:100, Invitrogen). Secondary reagents included: streptavidin (SA)-Fluoprobe 547 (1:5000, Interchim, France), SA-Cy5 (1:1000, GE Healthcare Life Sciences, Uppsala, Sweden), donkey anti-rabbit Cy3 (1:300, Jackson Immunoresearch Laboratories, Inc., West Grove, USA), or Vectastain® ABC kit containing HRP-conjugated anti-rat IgG (Vector Laboratories, Burlingame, USA) used with DAB substrate kit (Vector Laboratories) for peroxidase staining. Sections were counterstained for 2 min with Hoescht’s dye (1:10’000, Sigma). Micrographs (n=2-4 sections/animal, 5-15 animals/group) were captured using 10x, 20x or 40x objectives on a fluorescence microscope (Zeiss AxioImager Z1) equipped with an Apotome, or using a 2.5x objective on a light microscope (Leica). Images were processed by an operator blinded to the treatment groups using Image Pro-Plus, AxioVision (version 4.6), or Adobe Photoshop image analysis software. Vessel density and vessel maturity were quantified as the number of CD31+ vessels and SMA+ vessels per mm² respectively. Matrigel plug vessel content was calculated as percentage of vascularized area to total section area. Endothelial proliferation was analyzed by Ki67 and CD31 double labeling, and presented as number of Ki67+ endothelial cells per mm². Cardiomyocyte sizes were measured in WGA-stained sections. Vessel to cardiomyocyte ratio was calculated as number of vessels to number of cardiomyocytes per mm².

Semi-quantitative fluorescence microscopy

Angiogenic growth factor receptor protein expression levels were determined in small and larger blood vessels in histological sections from matrigel plugs implanted with microcapsules loaded with either FGF-2 or HGF, as above. Briefly, 10 µm thick acetone-fixed cryosections were double labeled for either c-Met (rabbit antimouse c-Met, 1:300, sc162, Santa Cruz Biotech, USA) or FGFR-1 (rabbit anti human FGFR1, 1:100, Sigma), in conjunction with CD31 (biotinylated rat antimouse CD31, 1:100, BD). Secondary reagents included a donkey
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antirabbit-Cy3 (1:300, Jackson Immunoresearch Laboratories) and SA-FITC (1:200, Sigma). Following counterstaining with Hoescht’s dye (1:10’000) and mounting in Vectashield, the sections were viewed using a Leica SP5 TCS X inverted confocal microscope at 40x (NA = 1.25) and images were acquired in a sequential mode. Confocal settings were kept strictly identical between series. In addition, homogeneity of field illumination and power laser output was verified prior to and after experiments to ensure stable conditions of acquisition. Images were analyzed using Bitplane Imaris software (version 6.10) to determine maximal relative levels of signal intensity in regions of interest (ROI) centered on individual blood vessels. In total 3-5 animals per group, and for each animal 5-7 images, each including 7-50 ROI, were analyzed.

Confocal imaging of growth factor-loaded microcapsules

Twenty µg of rhFGF-2 or rmHGF were fluorescently labeled using an Alexa-555 kit according to the manufacturer’s instructions (Microscale protein labeling kit, Invitrogen). Lyophilised microcapsules were loaded with the fluorescent growth factors, using 1 µg growth factor per mg microcapsules as above. In a first in vitro experiment, the growth factor-loaded microcapsules were resuspended at 4 mg microcapsules/mL in extracellular fluid mimetic release buffer (EFM-RB, as above). Following over night incubation, the microcapsules were embedded in matrigel, and imaged using a Leica SP5 TCS X inverted confocal microscope at 20x. In a second in vivo experiment, the growth factor-loaded microcapsules were immediately injected in rat hearts in three spots (23 µl/spot) along the infarct border zone on the right anterior side of the LV free wall adjacent to the septum as detailed above for the MI model (n=2-4 rats/group). Microcapsules without growth factors served as negative controls. At 1 or 3 weeks post-MI rats were killed and hearts collected and snap-frozen. Cryosections of 30 µm thickness were imaged using a Leica SP5 TCS X inverted
confocal microscope at 20x. Confocal settings were kept strictly identical between series. Images were processed using Leica LAS AF software (version 2.2.0).

**MRI**

Cardiac perfusion was assessed by arterial spin labeling MRI using a 4.7T small animal magnet (Biospec 47/40 advanced II, Brucker, Ettlingen, Germany) equipped with a gradient insert (BGA12S 400mT/m) and a transmit/recieve radiofrequency coil in quadrature mode with 86 mm internal diameter (Brucker). Briefly, animals were anesthetized with methohexital and placed in a supine position on a warming cradle. ECG signal were monitored by placing two subcutaneous electrodes on each side of the chest connected to a small animal monitoring and gating system (1025-S-50 model, SA Instruments Inc., NY, USA) for MRI synchronization. After optimization of the radio frequency signal, the perfusion sequence was run in the short-axis plane allowing determination of myocardial tissue perfusion. Global as well as slice-selective spin inversion recovery T1* (fitted time constant) maps were acquired. During acquisition 32 signal averages were performed resulting in an imaging duration of about 20 min per animal. Regional perfusion in the treated area of the LV was calculated as $P=\frac{0.95}{T1_{\text{internal}}} \times \frac{T1*_{\text{global}}}{T1*_{\text{selective}}}-1$ of the ROI where $T1_{\text{internal}}$ is the signal in the blood. Perfusion images were analyzed with ParaVision 5.0 software (Brucker) by two independent observers.

**Echocardiography**

Animals (n=14-15 rats/group, and 8 age-matched sham-operated rats) were examined at 1 month and 3 months post-MI by transthoracic echocardiography. 2D images and 2D-guided M-mode, Doppler M mode, and pulsed-wave Doppler recordings were obtained from parasternal short axis (level of papillary muscles) views, using a Vivid 7 Ultrasound (GE
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Healthcare) echograph with a M12L linear probe operated at 14 MHz, and analyzed using Echopac PC software. LV end-diastolic diameter (EDD) and end-systolic diameter (ESD) were measured by the leading-edge convention, and used to calculate fractional shortening (FS) through the equation $FS = \frac{((EDD-ESD)/EDD) \times 100}{1}$. Velocity-time integral (VTI) was measured at the level of the pulmonary artery by pulse-wave Doppler. LV free wall AW end-diastolic thickness (AWT ED) and AW end-systolic thickness (AWT ES) were measured in TM mode captured in short-axis views, and used to calculate LV Anterior Wall fractional thickening (AW FT) by the equation $AW\ FT = \frac{((AWT\ ES-AWT\ ED)/AWT\ ED) \times 100}{1}$. Rate-corrected Velocity of circumferential shortening (VCFc) was calculated as $VCFc = \frac{\%\ FS}{LVET\times(R-R)}$, where $LVET= LV$ ejection time (ms) and $R-R = ECG\ R-R$ interval. The mean of three consecutive cardiac cycles was used for all measurements in each animal. Measurements, performed by a single echocardiographer blinded to the treatment groups, were made in accordance with the conventions of the American Society of Echocardiography.

**Western blot**

HMEC-C cells were seeded at $1\times10^5$ per well in 6-well plates ($n=3-4$ samples/condition). After 24 hours, cells were stimulated or not with FGF-2 (50 ng/ml) or HGF (25 ng/ml). At different times (0, 1, 5, 10, 15, 30, 60 and 120 min), cellular lysates were prepared in cold lysis buffer containing sodium dodecyl sulfate (1%), Tris-HCl (10 mmol/L, pH 7.4), aprotinin (5 µg/ml), leupeptin (2.5 µg/ml), and sodium orthovanadate (1 mmol/L). Lysates were immediately frozen in liquid nitrogen, and stored at -80°C. For western blot analyses of rat hearts ($n=4-5$ rats/group), the LV treated zone was rapidly dissected and snap-frozen. Tissue homogenates were obtained by mechanical disruption in phosphoSafe Extraction Reagent (Novagen) lysis buffer. Protein concentrations of cell or tissue lysates were verified by Bradford assay. For MAPK analyses in cell culture homogenates, and FGFR-1, c-Met, and
Akt analyses in tissue homogenates, samples were immunoprecipitated with protein A/G plus agarose vol/vol (Santa Cruz Biotechnology Inc., USA), and 20 µg protein per sample was used for blotting. For Akt analyses in cell culture homogenates, 40 µg protein per sample was used. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Mini Gel Protean III System, Bio-Rad Laboratories, Hercules, USA) and transferred to Hybond ECL membranes (Amersham Biosciences) during 45 minutes at 100 V (Minitrans-blot Cell, Bio-Rad Laboratories, Hercules, USA). Membranes were incubated with the following primary antibodies: anti-human MAPK (monoclonal; Cell Signaling Technology, Danvers, USA), anti-human Akt (polyclonal; Cell Signaling Technology), anti-phospho-MAPK (monoclonal; Cell Signaling Technology), and anti-phospho-Akt (polyclonal; Cell Signaling Technology), antihuman FGFR-1 (Cell Signaling Technology), anti phospho-FGFR-1 (Cell Signaling Technology), antihuman cMet (Cell Signaling Technology), and antiphospho-c-Met (Cell Signaling Technology). Membranes were washed and incubated with goat anti-rabbit HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, USA). Proteins were visualized with the use of a Chemiluminescence kit (Lumi Light, Roche). Densities of the specific bands were estimated on a densitometer analyzer using BioCapt and Bio-Profil (Bio-ID) software. Results are presented as the fold increase over control (time point = 0) of the ratio of the density of the band for phospho-MAPK, phospho-Akt, phospho-FGFR-1, or phospho-c-Met to the density of the band for MAPK, Akt, FGFR-1, or c-Met in the same sample, respectively.

**RT-PCR**

HME and HMEC-C were stimulated or not with FGF-2 (25 ng/ml for HME; 50 ng/ml for HMEC-C) or HGF (50 ng/ml for HME; 25 ng/ml for HMEC-C) for 24 hours, after which cell pellet were harvested and stored at -80°C. Cardiac LV samples were rapidly excised and
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RNA was extracted using either the acidic phenol/chloroform procedure or a Qiagen RNA isolation kit (Qiagen Inc.). The quantity and quality of RNA was assessed with NanoDrop™ ND 1000 Spectrophotometer (NanoDrop Technologies). Reverse transcriptase (RT) was performed during 1h at 37°C using 1 µg total RNA in the presence of 2.5 µg hexameric random probes (oligo pd(N)6, 100 pmol/µl), 10 mM dNTP, 40 U RNAse out, and 200 U reverse transcriptase (Mo-oney Murine Leukemia Virus Reverse Transcriptase, Life Technologies). Real-time PCR was performed on a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using a commercially available mix containing Taq DNA polymerase, SYBR-Green I, and MgCl₂ (FastStart DNA Master SYBR Green I kit; Roche). The following primers were used:

<table>
<thead>
<tr>
<th>Target gene</th>
<th>sense</th>
<th>antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>murine FGFR-1</td>
<td>5'-CAA CAA GAC AGT TGG CCC TGG G-3'</td>
<td>5'-CGG TGC AGT AGA TAA TGA TC-3'</td>
</tr>
<tr>
<td>murine c-Met</td>
<td>5'-CTG AAG GAA ACC CAA GAT G-3'</td>
<td>5'-AAA CAC CCC GAA GAG AAT G-3'</td>
</tr>
<tr>
<td>murine 18S</td>
<td>5'-GTG GAG CGA TTT GTC TGG TT-3'</td>
<td>5'-CGC TGA GCC AGT CAG TGT AG-3'</td>
</tr>
<tr>
<td>rat FGFR-1</td>
<td>5'- GAA GAG CGA CTT CCA TAG CC-3'</td>
<td>5'- ACA CAG TTA CCT GTC TGC GG-3'</td>
</tr>
<tr>
<td>rat c-Met</td>
<td>5'- CAG ACG CCT TGT ATG AAG T-3'</td>
<td>5'- CAT AAG TAG CGT TCA CAT GG-3'</td>
</tr>
<tr>
<td>rat beta-MHC</td>
<td>5'-CAC CAA CAA CCC CTA CGA TT-3'</td>
<td>5'-AGC ACA TCA AAG GGC CTA TC-3'</td>
</tr>
<tr>
<td>rat ANP</td>
<td>5'-CAC AGA TCT GAT GGA TTT CAA GA-3'</td>
<td>5'-CGC TTC ATC GGT CTG CTC-3'</td>
</tr>
<tr>
<td>rat BNP</td>
<td>5'-GTC AGT CGC TTG GGC TGT-3'</td>
<td>5'-CCA GAG CTG GGG AAA GAA G-3'</td>
</tr>
<tr>
<td>rat 18S</td>
<td>5'-GTG GAG CGA TTT GTC TGG TT-3'</td>
<td>5'-CGC TGA GCC AGT CAG TGT AG-3'</td>
</tr>
<tr>
<td>human FGFR-1</td>
<td>5'-CCA AGA AGG TGT GGA ACC TG-3'</td>
<td>5'-TCA CAT TGT CCT CTG TCA CCA-3'</td>
</tr>
<tr>
<td>human c-Met</td>
<td>5'-CAG AGA CTT GGC TGC AAG AA-3'</td>
<td>5'-GGC AAG ACC AAA ATC AGC A-3'</td>
</tr>
<tr>
<td>human β₂-microglobulin</td>
<td>5'-TTC TGG CCT GGA GCC TAT C-3'</td>
<td>5'-TCA GGA AAT TTG ACT TTC CAT TC-3'</td>
</tr>
</tbody>
</table>

For each sample 0.5 or 1 µg of RNA was reverse transcribed in duplicates, and for each reaction 2 PCRs were carried out. For each condition 3-6 cell or tissue samples were
analyzed, resulting in 12-24 RT-PCRs being carried out per gene for each condition. Data were analyzed using the Light Cycler software. Differences in relative expression levels were calculated according to the $\Delta\Delta Ct$ (cycle threshold) method by Pfaffer et al.

**Statistics**

Data are presented as mean ± SEM. Student's $t$-test (two-tailed) was used to compare two groups of independent samples. For multiple comparisons, data distribution was analyzed by D’Agostino and Pearson’s omnibus normality test using GraphPad Prism software (version 5.0). For data sets with normal distribution, one-way analysis of variance (ANOVA) was employed, followed by Tukey’s post-hoc test, except for echocardiographic data where repeated measurements two-way ANOVA was used followed by Bonferroni post-hoc test. For data sets with non Gaussian distribution (AWT ED, AWT ES, AWT FT, FS, VCFc, EF and EDV at 1 month, and EDV at 3 months), nonparametric Kruskal-Wallis analyses were used followed by Dunn’s post-hoc test. A $p<0.05$ was considered significant.
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References


### Supplementary Tables

#### Table 1

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<th>Sham</th>
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<th>HGF vs H±F</th>
<th>FGF-2 vs H±F</th>
<th>F±H</th>
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<td>Weight (g)</td>
<td>378 ± 15</td>
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<td>416 ± 11 ns</td>
<td>396 ± 9 ns</td>
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<tr>
<td>AWT ED (mm)</td>
<td>2,1 ± 0,1***</td>
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<td>1,1 ± 0,1 ns</td>
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<td>1,3 ± 0,1</td>
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<tr>
<td>AWT ES (mm)</td>
<td>3,5 ± 0,1 $$$</td>
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<td>AW FT (%)</td>
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<td>LVESD (mm)</td>
<td>2,9 ± 0,2***</td>
<td>7,8 ± 0,2</td>
<td>7,7 ± 0,2 # #</td>
<td>7,1 ± 0,2* ns</td>
<td>6,8 ± 0,2**</td>
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<td>FS (%)</td>
<td>54 ± 3***</td>
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<td>17 ± 1 ns</td>
<td>19 ± 2 ns</td>
<td>22 ± 2**</td>
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<td>HR (bpm)</td>
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<td>412 ± 11 ns</td>
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<tr>
<td>VTI pulm (cm)</td>
<td>6,1 ± 0,2</td>
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<td>5,8 ± 0,2 ns</td>
<td>5,7 ± 0,2 ns</td>
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<td>VCFc (circ/s)</td>
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<td>EDV (mL)</td>
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<td>ESV (mL)</td>
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<td>1,0 ± 0,1 # #</td>
<td>0,8 ± 0,1* ns</td>
<td>0,7 ± 0,1**</td>
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</table>

**Supplementary Table 1:** Echocardiography measures at 1 month post-MI. *AWT ED*, anterior wall thickness end-diastolic; *AWT ES*, anterior wall thickness end-systolic; *AW FT*, anterior wall fractional thickening; *LVEDD*, left ventricular end-diastolic diameter; *LVESD*, left ventricular end-systolic diameter; *FS*, fractional shortening; *HR*, heart rate; *VTI*, velocity time integral; *VCFc*, rate-corrected velocity of circumferential fiber shortening; *EF*, ejection fraction; *EDV*, end-disatolic volume; *ESV*, end-systolic volume. RM-ANOVA: *P*<0.05; **P*<0.01; ***P*<0.001 vs control; #P*<0.05, ##P*<0.01 vs F+H. Nonparametric test: $$$P*<0.001 vs control.
Local delivery of HGF and FGF-2 in combination prevents CHF

Table 2: Sham Control HGF vs H±F FGF-2 vs H±F F±H

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<td><strong>AWT ED</strong> (mm)</td>
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<td><strong>FS (%)</strong></td>
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<td><strong>VTI pulm</strong> (cm)</td>
<td>0,7 ± 0,1***</td>
<td>2,0 ± 0,1</td>
<td>1,6 ± 0,1</td>
<td>ns</td>
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**Supplementary Table 2:** Echocardiography measures at 3 month post-MI. AWT ED, anterior wall thickness end-diastolic; AWT ES, anterior wall thickness end-systolic; AW FT, anterior wall fractional thickening; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; HR, heart rate; VTI, velocity time integral; VCFc, rate-corrected velocity of circumferential fiber shortening; EF, ejection fraction; EDV, end-diastolic volume; ESV, end-systolic volume. RM-ANOVA: *P<0.05; **P<0.01; ***P<0.001 vs control; #P<0.05 vs F+H.
Local delivery of HGF and FGF-2 in combination prevents CHF

Legends of Supplementary Figures

**Supplementary Figure 1:** Migration of Murine Embryonic Microvascular Endothelial cells (PmT-EC). FGF-2 or HGF-pretreated PmT-EC stimulated with HGF or FGF-2 assayed in Boyden chambers for 12 hours. Migrating cells per field are given (n = 6 per group). ***$P<0.001$.

**Supplementary Figure 2:** Albumin-alginate microcapsules for sequential slow-release of FGF-2 and HGF. Microcapsules have a mean diameter of 100 µm as observed by laser diffraction measurement. Image inset shows methylene blue-stained microcapsules before freeze-drying (A, scale bar 100 µm). Microcapsules observed by scanning electron microscopy at 500x (left, scale bar 50 µm) or 7500x (right, scale bar 5 µm) display a continuous, pleated surface (B). *In vitro* growth factor release rate evaluated every other day for 6 weeks. Amounts are given as ng growth factor released per day per mg of microcapsules (C). Confocal microscopy images of FGF-2 (D) or HGF (E) localization on the microcapsules. Left image, 3D rendering; Center image, 2D rendering of fluorescence signal; and Right image, overlay of fluorescence and transmitted light signals. Magnification 20x, scale bar 100 µm.

**Supplementary Figure 3:** Albumin-alginate microcapsules for slow-release of angiogenic growth factors. Microcapsule-release of PDGF-BB evaluated *in vitro* during 6 weeks (A). Effects on HME migration using “fresh” FGF-2, HGF, PDGF-BB, or the same concentration of growth factors released from microcapsules were assayed in Boyden chambers for 6h (B). Six replicate samples were used in each experiment, and experiments were performed at least twice. Microcapsules loaded with FGF-2 or HGF were stained with Giemsa solution to
Local delivery of HGF and FGF-2 in combination prevents CHF
determine their *in vitro* degradation rate (C). Matrigel plug vessel content following microcapsule-delivery or bolus naked-protein delivery of FGF-2 or HGF was analyzed (D). Microcapsule-delivery of HGF (125 ng, ‘+ capsules’) or FGF-2 (500 ng, ‘+ capsules’), as compared with HGF (125 or 1000 ng, ‘-capsules’), or FGF-2 (500 or 1000 ng, ‘-capsules’) administered without microcapsules. Vessel numbers per field is shown. *P<0.05, **P<0.01.

**Supplementary Figure 4:** Growth factor release by albumin-alginate microcapsules *in vivo*. Confocal microscopy images of rat cardiac sections containing microcapsules without growth factors (control) or loaded with fluorescently labeled (*red*) HGF or FGF-2 at 1 or 3 weeks following intramyocardial injection into the viable LV border zone. Images show overlay of fluorescence and transmitted light signals. Magnification 20×, scale bar 50 µm. At 3 weeks the HGF and FGF-2 signals were reduced, suggestive of growth factor liberation coinciding with microcapsule degradation.

**Supplementary Figure 5:** Angiogenic effects at 1 month post-MI of intramyocardial delivery of angiogenic growth factors. Schematic illustration of LV free wall treated area (A). Immunohistochemical analysis at 1 month post-MI following microcapsule-delivery of FGF-2, HGF, or the combination, in the LV infarct border zone. (Data obtained at 3 months post-MI are shown in Fig. 3). Vascular density (B) and mature vessel density (C) is presented as the number of CD31+ and SMA+ vessels per mm², respectively. Proliferating endothelial cells per mm² was determined by Ki67 and CD31 double labeling (D). *P<0.05; **P<0.01; ***P<0.001 versus MI control; #P<0.05, ##P<0.01; ###P<0.001 for other comparisons as indicated. RV, right ventricle; LV, left ventricle.
Supplementary Figure 6: Arteriogenic responses following intramyocardial delivery of angiogenic growth factors. Histochemical morphometric analyzes (lumen sizes of SMA+ vessels analyzed at 10x) of the rat coronary microcirculation at 1 or 3 months post-MI (n=8-15 rats/group, 3-4 serial sections per rat). Quantification of microvessels (diameters less than 15 µm) at 1 (A) or 3 months (C) post-MI. Quantification of small arterioles (vessel diameters between 15-50 µm) at 1 (B) or 3 months (D) post-MI. *P<0.05; **P<0.01; ***P<0.001 versus MI control; #P<0.05, ##P<0.01; ###P<0.001 for other comparisons as indicated.

Supplementary Figure 7: Expression analyses of markers of cardiac hypertrophy and dysfunction. Quantitative real-time PCR analyses were performed to determine mRNA levels of β–MHC (A), ANP (B) and BNP (C) in the viable zone of the LV at 3 weeks post-MI. *P<0.05 versus MI control.

Supplementary Figure 8: Targeted intramyocardial delivery using microcapsules. Analysis of intramyocardial distribution of fluorescently labeled albumin-alginate microcapsules at different time points following injection. Microcapsules appear as flattened red bands or dots in sections of hearts arrested in diastole. Cell nuclei in blue. Scale bar 100 µm. The LV area occupied by microcapsules was calculated for each time point to estimate their dispersion in the heart (n=2-3 rats/time point, 15-18 sections per heart). *P<0.05; **P<0.01.

Supplementary Figure 9: Myocardial infarct sizes. Myocardial infarct sizes (% of LV perimeter) were evaluated at 3 months post-MI. Mean ± SEM are outlined together with individual data points.
Supplementary Figure 10: Activation of cell signaling *in vivo*. Akt (A, B), FGFR-1 (C, D) and c-Met (E, F) activation levels assayed by Western blot in samples from the viable zone of the LV at 3 weeks post-MI. Results are presented as the ratio of density of the band for phospho-Akt, phospho-FGFR-1, or phospho-c-Met in each experiment to the density of the band for Akt, FGFR-1, or c-Met in the same sample, respectively (n=4-5 rats/group). *P<0.05 versus MI control.
Supplementary Fig. 1

![Graph showing the number of migrating cells per mm² with stimulation and pretreatment conditions: FGF, HGF, and various combinations. The graph indicates a significant increase in cell migration with FGF pretreatment.](image-url)
Supplementary Fig. 2

A

![Graph showing volume (%) vs. microcapsule diameter (μm)]

B

![Scanning electron microscopy images of microcapsules]

C

![Graph showing growth factor release (ng/day) over time (days)]

D

![Images showing different growth factor release under orange light]
Supplementary Fig. 3

A

Growth factor release (ng/d) vs. Time (Days)

B

Migrating cells/mm² vs. stimulation capsules

C

Images of FGF and HGF treatments over time (Days)

D

Vascular density vs. HGF and FGF capsules

* and ** indicate statistical significance.
Supplementary Fig. 4

1 wk | 3 wks

control

HGF

FGF-2
Supplementary Fig. 5

A. Diagram showing different areas of the heart: Nontreated LV area, Treated LV area, Infarced area, RV.

B. Bar graph showing vascular density with treatments: Sham, Con, HGF, FGF, H + F.

C. Bar graph showing mature vessel density with treatments: Sham, Con, HGF, FGF, H + F.

D. Bar graph showing proliferating endothelial cells with treatments: Sham, Con, HGF, FGF, H + F.
Supplementary Fig. 6

A. SMA+ microvessels

B. Small arterioles

C. SMA+ microvessels

D. Small arterioles
Supplementary Fig. 7

A

Relative β-MHC levels

Sham  Con  H + F

B

Relative ANP levels

Sham  Con  H + F

C

Relative BNP levels

Sham  Con  H + F
Supplementary Fig. 9

[Graph showing myocardial infarct size for control, HGF, FGF-2, and FGF+ HGF groups]
성장인자조합을 지속적으로 심근내에 주입하는 새로운 혈관신생요법은 심부전을 예방한다

백 상 홍 교수 가톨릭대학교 서울성모병원 순환기내과

Summary

배경
치료적 혈관신생술은 심장질환 치료법 중 좋은 전략이다. 관상동맥의 측부혈관 존재는 관상동맥질환의 좋은 예후를 보여주지만, 관상동맥 환자의 66-75% 정도는 관상동맥의 측부혈관이 충분하지 않은 실정이며, 심근경색 환자의 30% 정도는 심근관류가 충분하지 않다는 증거는 관상동맥질환에 신생혈관 촉진과 같은 치료법이 필요하다는 것을 시사한다. 만성 심부전증은 오늘날에도 여전히 치료가 어렵고, VGF나 FGF-2(Fibroblast growth factor-2)와 같은 단일 성장인자를 투여한 실험에서도 결과는 부정적이었다. 가장 중요한 문제는 성장인자를 통한 치료법이 대용량의 성장인자를 필요로 하는 경제적인 제한성이라는 것이다. 또한, 대부분의 실험적 접근에서 성장인자 투여 농도는 정상적인 농도보다 훨씬 높은 10-100μg/kg이므로, 이는 정상적인 총 성장인자의 농도가 아닌 혈관신생 치료를 극대화하기 위해 산출된 결과로 허혈조직 내 존재하는 성장인자의 농도보다 훨씬 높다는 제한점이 있다. 기존의 성장인자 치료법은 성장인자 병용투여로 대체할 수 있는데, 이는 단일투여에 비해 혈관의 성숙이나 안정화를 높이는 방법이다. 그러므로 본 연구에서는 FGF-2와 HGF(hepatocyte growth factor)의 병용투여를 새로운 albumin-alginate microcapsule을 이용하여 만성 심부전증 모델에서의 심장 기능의 향상 효과를 분석하고자 하였다.

방법 및 결과
FGF-2와 HGF의 상승작용으로
혈관세포 이주 및 증식 촉진으로 얻은 혈관성장 유도
FGF-2와 HGF의 이동능과 증식능을 murine HME(heart microvascular endothelial cell), murine embryonic microvascular EC(endothelial cell), HMEC-C(human microvascular cardiac endothelial cell), SMC(smooth muscle cell)를 이용하여 확인한 결과, HGF, FGF-2의 단독투여에 비하여 병용투여가 증식 및 이동능의 특징이 두드러졌고, corneal angiogenesis assay에서 HGF는 1주
일 만에 혈관 신생반응을 보였으나 1개월 후에 사라졌고, FGF-2는 1달 동안 생성이 진행되다가 1년 만에 사라졌지만, 병용투여에서는 혈관을 1년 동안 유지하였다.

Albumin-alginate microcapsule로 성장인자들을 지속적, 서서히 유리
일정하고 지속적인 FGF-2와 HGF의 분비를 위하여 albumin-alginate microcapsule을 개발하였는데, 이들의 지름은 100μm의 거친 구형이고, 가운데 액체 핵을 둘러싸는 형태로 제작되었으며, HGF와 FGF-2의 분비를 6주간 지속시켰다.

FGF-2와 HGF 지속적 유리가 혈관생성에 미치는 영향
Matrigel plug assay에서 CD31로 모세혈관밀도를 분석하고, SMA(α-smooth muscle actin)을 측정하여 분석하여, plug의 section을 통하여 혈관이 생성된 네이어를 측정한 결과, HGF, PDGF-BB(platelet-derived growth factor-BB)의 단독투여는 완전한 혈관을 만들지 못하였지만, FGF-2와의 병용투여에서 완전한 혈관의 생성과 함께 plug내 혈관 밀도를 증가시켰다.

FGF-2와 HGF 근육내 전달이 혈관생성을 촉진하고, 심근경색에 의한 심비대와 섬유화를 예방
취 심근경색 모델을 이용하여 만성 심부전(chronic heart failure, CHF)을 측정하여, 혈관 생성 정도를 면역조직화학법으로 확인하였다. 단독투여군에 비해 병용투여군에서 혈관의 숫자, SMA+가 향상된 혈관의 숫자, 증식하고 있는 혈관 내피세포의 개수가 더욱 증가하였다. 심근의 사이즈는 병용투여군에서는 감소하였으나, 오히려 FGF-2의 단독투여군에서는 감소하지 않았다. 혈관·심근세포의 비율이 병용투여군에서 정상 대조군과 유사한 패턴을 보였고, 혈관조직 방법으로 microcapsule의 분해능을 확인하였다. 투여 6시간 후에 전체 LV(left ventricular)의 8% 정도를 차지하고 있던 microcapsule이 2주째 4.5%까지 감소하였다.

FGF-2와 HGF 심근내 투여가 심근경색 후 국소 심근관류와 심기능 개선
심근관류는 병용투여군에서 증가하고, 심장의 전측벽 두께가 회복되었으며, 확장기말 심초음파 검사의 결과를 확인하였다. HGF와 PDGF-BB의 단독투여는 완전한 혈관을 만들지 못하였지만, FGF-2와의 병용투여에서 완전한 혈관의 생성과 함께 plug내 혈관 밀도를 증가시켰다. 심장의 전측벽 두께가 회복되었으며, 확장기말 심초음파 검사의 결과를 확인하였다. HGF와 PDGF-BB의 단독투여는 완전한 혈관을 만들지 못하였지만, FGF-2와의 병용투여에서 완전한 혈관의 생성과 함께 plug내 혈관 밀도를 증가시켰다.

혈관신생의 분자학적 기전
FGF의 경우, p-Akt phospho-Akt는 1분 후 발현이 증가하고, 30분 뒤부터 다시 감소하며, HGF는 30분에 최고 조의 발현을 하였는데, 병용투여는 1시간이 지나도 발현을 지속하였다. Erk여기서는 MAPK(mitogen-activated protein kinase)의 경우 병용투여군에서 발현이 증가하며, 단독투여군에 비하여 전주간의 차이는 거의 변화가 없었다. 심근경색 모델에서 약물투여가 종료된 후 3주 후에 심장조직에서 Akt의 발현을 확인하면, 병용투여군은 대조군에 비해 Akt의 활성을 증가하였다. 심장의 사이즈는 병용투여군에서는 감소하였으나, 오히려 FGF-2의 단독투여군에서는 감소하지 않았다. 혈관·심근세포의 비율이 병용투여군에서 정상 대조군과 유사한 패턴을 보였고, 혈관조직 방법으로 microcapsule의 분해능을 확인하였다. 투여 6시간 후에 전체 LV(left ventricular)의 8% 정도를 차지하고 있던 microcapsule이 2주째 4.5%까지 감소하였다.

결론
FGF는 조직 재생에 중요한 역할을 담당하고 있는 물질로 HGF의 단독투여는 심장의 섬유화와 심근비대를 예방하지만, 혈관 신생에 있어서 제한적인 영향을 미친다. HGF의 단독투여는 심장의 기능을 부분적으로 개선하는 반면에, FGF-2의 단독투여는 안정화된 혈관의 생성을 증가시켰다.
가시키는 데 실패하였고, 심장의 섬유화나 심근비대를 역제하는 데 영향력이 미미하다. 하지만 이들은 심근 확장기말 직경의 분석 결과에서 심장의 국소적 기능 항상에 긍정적인 영향을 미친다. 또한 FGF-2는 세포 간의 대사작용을 통해 심장의 보호 기능에 직접적으로 연관이 있다. 심장 섬유화는 심장의 기능을 손상시키고, 이는 HGF의 TGF-beta 신호전달 기전의 억제로 인해 감소한다. FGF-2는 심장의 콜라겐 농도를 감소시켜 심장의 섬유화를 예방할 수 있다. HGF와 FGF-2는 Akt와 MAPK의 신호전달기전을 통하여 EC의 증식 및 apoptosis를 예방하는데, 이러한 신호전달기전은 FGFR-1과 c-Met 신호전달기전을 통하여 이루어진다. 본 연구 결과는 FGF-2와 HGF의 병용투여로 심근교환으로 유도된 심장의 섬유화 및 기능부진을 예방하고, 심혈관질환의 임상적 접근에 있어 치료적 혈관생성을 중요한 역할을 담당하고 있음을 시사한다.

관상동맥질환, 허혈성 심근경색, 그리고 태어협성 심부전증은 근본적인 치료가 어려우므로 치료적 혈관신생술과 같은 새로운 치료법을 요구한다. 다양한 연구들의 긍정적인 결과에도 불구하고, 기존의 치료법들은 아직도 심장의 혈관 성장에서 정상적인 기능을 하는 혈관을 생성하는 데 어려움이 따른다. 성장인자를 통한 혈관신생의 치료법은 지금까지 그 영향이 미미하였고, 추가적으로 더 정확한 조절인자의 발굴과 더불어 투여 방법의 개선, 최대한의 효과를 나타내기 위한 투여 용량의 정립 그리고 투여 기간에 대한 연구가 요구되었다. 그래서 기존의 방법이 개선된 새로운 치료법 및 전달 방법의 개발이 급한 실정인데, 저자들이 개발한 지속적인 분비가 가능한 microcapsule 전달 방법은 심장의 기능을 강화시킬 뿐만 아니라, 심장의 섬유화를 예방하는 가능성을 보였다. 이러한 결과는 만성 심부전증 환자의 치료제 개발에 중요한 역할을 담당할 수 있을 것으로 사료된다.

상기의 연구 결과는 심장질환의 혈관신생을 통한 심장기능의 회복에 미세순환기의 작용에 대한 기전과 심장기능의 회복을 보여준 중요한 연구이고, 지속적인 분비가 가능한 microcapsule 전달 방법은 심장의 기능을 강화시킬 뿐만 아니라 심장의 섬유화를 예방하는 가능성을 보였다. 이러한 결과는 만성 심부전증 환자의 치료제 개발에 중요한 역할을 담당할 수 있을 것으로 사료된다. 그리고 줄기세포나 전구세포는 선천적으로 다양한 cytokine과 성장인자를 분비하므로, 이러한 줄기세포들이 성장인자나 cytokine을 조절할 수 있는 연구가 더 필요할 것으로 사료된다. 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자의 추가 도입과 세포치료의 병용 그리고 유전자.
Arteriogenic Therapy by Intramyocardial Sustained Delivery of a Novel Growth Factor Combination Prevents Chronic Heart Failure

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Background— Therapeutic angiogenesis is a promising approach for the treatment of cardiovascular diseases, including myocardial infarction and chronic heart failure. We aimed to improve proangiogenic therapies by identifying novel arteriogenic growth factor combinations, developing injectable delivery systems for spatiotemporally controlled growth factor release, and evaluating functional consequences of targeted intramyocardial growth factor delivery in chronic heart failure.

Methods and Results— First, we observed that fibroblast growth factor and hepatocyte growth factor synergistically stimulate vascular cell migration and proliferation in vitro. Using 2 in vivo angiogenesis assays (n=5 mice per group), we found that the growth factor combination results in a more potent and durable angiogenic response than either growth factor used alone. Furthermore, we determined that the molecular mechanisms involve potentiation of Akt and mitogen-activated protein kinase signal transduction pathways, as well as upregulation of angiogenic growth factor receptors. Next, we developed crosslinked albumin-alginate microcapsules that sequentially release fibroblast growth factor-2 and hepatocyte growth factor. Finally, in a rat model of chronic heart failure induced by coronary ligation (n=14 to 15 rats per group), we found that intramyocardial slow release of fibroblast growth factor-2 with hepatocyte growth factor potently stimulates angiogenesis and arteriogenesis and prevents cardiac hypertrophy and fibrosis, as determined by immunohistochemistry, leading to improved cardiac perfusion after 3 months, as shown by magnetic resonance imaging. These multiple beneficial effects resulted in reduced adverse cardiac remodeling and improved left ventricular function, as revealed by echocardiography.

Conclusion— Our data showing the selective advantage of using fibroblast growth factor-2 together with hepatocyte growth factor suggest that this growth factor combination may constitute an efficient novel treatment for chronic heart failure. (Circulation. 2011;124:1059-1069.)

Key Words: angiogenesis ■ capsules ■ heart failure ■ remodeling

The presence of coronary collaterals is a favorable prognostic factor in coronary artery disease, associated with reduced myocardial infarct size and increased patient survival.1 However, the fact that an estimated 66% to 75% of coronary artery disease patients have insufficient coronary collaterals presents an incentive for therapeutic stimulation of arterial blood vessel growth, ie, collaterogenesis or arteriogenesis.2 In addition, ≈30% of myocardial infarction (MI) patients display inadequate myocardial perfusion (ie, the no-reflow phenomenon),3 suggesting that therapeutic stimulation of smaller-caliber blood vessel growth, ie, angiogenesis, also is a valid treatment approach for coronary artery disease.4 Chronic heart failure (CHF), an increasingly common consequence of MI and other cardiomyopathies, is characterized by extensive cardiac hypertrophy and fibrosis. It has been suggested that the switch between reversible physiological and irreversible pathological cardiac hypertrophy depends on angiogenesis.5 Thus, therapeutic stimulation of arteriogenesis and/or angiogenesis is an attractive common target in cardiovascular diseases.

Clinical Perspective on p 21

At the turn of the century, several clinical trials of therapeutic angiogenesis for cardiovascular diseases such as coronary artery
disease were initiated. Today, 10 years later, there is still no approved treatment for patients who are poor candidates for surgical revascularization or have distal diffuse ischemic disease. Indeed, double-blind controlled clinical trials in coronary artery disease patients, based on administration of vascular endothelial growth factor or fibroblast growth factor-2 (FGF-2), have failed to demonstrate a durable effect.6,7 The main problems of current proangiogenic approaches include the limited duration of the therapy achieved with bolus delivery of naked proteins or gene delivery vectors, the high costs associated with protein therapy because of the general need for large doses, the transient effects of treatment likely due to the generation of unstable blood vessels that regress over time, and important safety issues related to the possibility of inadvertent stimulation of angiogenesis in distant, dormant micrometastases or in diabetic retinopathy.8,9 Responses to these challenges include the proposition that single growth factor therapy be replaced by growth factor combinations to stimulate the generation of mature and stable blood vessels, including large-caliber collateral arteries,10 or gaining control over growth factor release rates using delivery vectors that allow prolonged angiogenic stimulation.11-13 Of note, most experimental approaches have involved delivery of supraphysiologic doses of angiogenic growth factors, often in the range of 10 to 100 μg/kg. However, it seems that it is not the total growth factor dose that determines the outcome of angiogenic therapy; rather, local microenvironmental concentration gradients are important for achieving functional revascularization responses.14

Here, we report a new therapeutic strategy specifically designed to stimulate angiogenesis and arteriogenesis in the failing heart based on targeted, intramyocardial, sequential slow release of a novel angiogenic growth factor combination, FGF-2 and hepatocyte growth factor (HGF), using albumin-alginate microcapsules.

Methods

A detailed description of all methods and reagents used for the experiments is provided in the online-only Data Supplement.

Cell Migration and Proliferation

Briefly, cells were pretreated with FGF-2 or HGF and then added to the upper wells of a modified Boyden 48-well chamber. The lower wells contained FGF-2 or HGF. Cells migrated across the membrane during 6 to 12 hours at 37°C. Results are reported as number of migrating cells per 1 mm² (n=6). For analyses of cell proliferation, heart microvascular endothelial cells (HMVECs), human microvascular cardiac endothelial cells (HMCECs), and rat aortic smooth muscle cells (SMCs) were pretreated with FGF-2 or HGF, added to 24-well plates, and stimulated with FGF-2 or HGF. Cell numbers were estimated with the WST-1 assay.

Corneal Angiogenesis Assay

The mouse corneal assay was performed as previously described in C57Bl/6 mice and as approved by the North Stockholm Animal Ethics Committee. Briefly, sucrose aluminum sulfate micropellets containing recombinant human HGF and/or FGF-2 were implanted into mice corneas (n=5 per group). The eyes were examined on days 5, 25, and 40 and ~1 year after pellet implantation.

Albumin-Alginate Microcapsules

Albumin-alginate microcapsules were prepared with a modified version of the previously described interfacial cross-linking method14 and as outlined in the Methods section in the online-only Data Supplement.

In Vitro Growth Factor Release

Briefly, lyophilized microcapsules were loaded with growth factors by immobition, with 1 μg growth factor per 1 mg microcapsules (~35,000 particles). Growth factor release after incubation in extracellular fluid mimic release buffer was quantified by ELISA. Data are presented as mean amount (nanograms) of growth factor released per day per 1 mg microcapsules (n=3).

Matrigel Plug Model

Growth factor–reduced Matrigel was used to evaluate angiogenic responses as outlined in the Methods section in the online-only Data Supplement and in accordance with National Institutes of Health guidelines, European Communities Council directives (86/609/EEC), and French National legislation (ethical approval No. 76-114). Briefly, 500 μL Matrigel was mixed with microcapsules loaded with growth factors as above. Controls contained Matrigel mixed with buffer or microcapsules without growth factors. The Matrigel mixture was subcutaneously injected in male Balb/c mice (n=5 per group). After 3 weeks, Matrigel plugs were harvested and snap-frozen.

Chronic Heart Failure Model

We induced MI in anesthetized, mechanically ventilated male Wistar rats by ligation of the proximal left coronary artery after a left thoracotomy (n=22 rats per group) as previously described18,19 and in accordance with National Institutes of Health guidelines, European Communities Council directives (86/609/EEC), and French National legislation (ethical approval No. 76-114). Immediately after ligation, microcapsules loaded with growth factors, as above, were injected in 3 spots (23 μL per spot) along the infarct border zone on the right anterior side of the left ventricular (LV) free wall adjacent to the septum. The total amount of growth factor administered per heart was 125 ng HGF or 500 ng FGF-2 alone or in combination. Controls were injected with the same numbers of microcapsules without growth factor. A. The time of death, hearts were arrested in diastole by immersion in ice-cold saturated potassium chloride buffer.

Histochemistry

Collagen density and M1 size were determined in heart cryosections stained with Sirius Red as previously described16 and as outlined in the Methods section in the online-only Data Supplement. Collagen content was calculated as percentage of collagen area to total area of the image (n=14 to 15 animals per group). Infarct size was calculated as follows: total infarction perimeter/(epicardial LV perimeter + endocardial LV perimeter)×100. For immunohistochemical analyses, cryosections were stained according to standard protocols as detailed in the Methods section in the online-only Data Supplement. Micrographs were processed by an operator blinded to the treatment groups with Image Pro-Plus, AxioVision, or Adobe Photoshop image analysis software. Vessel density and vessel maturity were quantified as the number of CD31⁺ vessels and smooth muscle active-positive (SMA⁺) vessels per 1 mm², respectively. Matrigel plug vessel content was calculated as percentage of vascularized area to total section area. Endothelial proliferation was analyzed by Ki67 and CD31 double labeling and presented as number of Ki67⁺ endothelial cells (ECs) per 1 mm². Cardiomyocyte sizes were measured in wheat germ agglutinin–stained sections. The ratio of vessel to cardiomyocyte was calculated as the number of vessels to the number of cardiomyocytes per 1 mm².

Semiquantitative Fluorescence Microscopy

Angiogenic growth factor receptor protein expression levels were determined in Matrigel plugs implanted with microcapsules loaded with FGF-2 or HGF as above and detailed in the Methods section in the online-only Data Supplement. Briefly, sections were double labeled for either c-Met or FGF receptor-1 (FGFR-1) in conjunction
with CD31. Micrographs obtained with a Leica SP5 TCS X inverted confocal microscope were analyzed with Bitplane Imaris software to determine maximal relative levels of signal intensity in regions of interest centered on individual blood vessels.

Confocal Imaging of Growth Factor Distribution
Recombinant human FGF-2 or recombinant mouse HGF (20 μg) was fluorescently labeled with an Alexa-555. Lyophilized microcapsules were loaded with fluorescent growth factors as above. Microcapsules were either imaged directly by confocal microscopy or injected into the viable zone of the LV after coronary ligation in rats as above. Cardiac samples retrieved at 1 or 3 weeks after MI were imaged with a Leica SP5 TCS X inverted confocal microscope. Images were processed with Leica LAS AF software.

Magnetic Resonance Imaging
Cardiac perfusion was assessed by arterial spin-labeling magnetic resonance imaging with a 4.7-T small animal magnet ( Biospec 47/40 advanced II, Bruker, Ettlingen, Germany). Briefly, the perfusion sequence was run in the short-axis plane, allowing determination of myocardial tissue perfusion. Global and slice-selective spin inversion recovery T1* (fitted time constant) maps were acquired.20 Perfusion images were analyzed with ParaVision 5.0 software (Bruker) by 2 independent observers, and regional perfusion in the treated area of the LV was calculated as described.21

Echocardiography
Animals (n=14 to 15 rats per group and 8 age-matched sham-operated rats) were examined at 1 and 3 months after MI by transthoracic echocardiography as previously described.18 Measurements performed by a single echocardiographer blinded to the treatment groups were made in accordance with the conventions of the American Society of Echocardiography and as detailed in the Methods section in the online-only Data Supplement.

Western Blot
The HMECs and HMEC-Cs were stimulated with FGF-2 or HGF as detailed in the Methods section in the online-only Data Supplement. Cardiac samples from the viable zone of the LV after coronary ligation in rats as above were collected at 3 weeks after MI and snap-frozen. Samples were either immunoprecipitated or separated directly by gel electrophoresis followed by membrane transfer for blotting of specific proteins using anti-human total or phosphorylation-specific antibodies as indicated. Results are presented as fold increase over control (time point=0 for cells; MI controls for rats) of the ratio of the band density for the phosphorylated form versus the total amount of the specific protein in the same sample.

Real-Time Polymerase Chain Reaction
The HMECs and HMEC-Cs were stimulated with FGF-2 or HGF as detailed in the Methods section in the online-only Data Supplement. Cardiac samples were collected at 3 weeks after MI. Real-time polymerase chain reaction was performed on a LightCycler (Roche Molecular Biochemicals) with a commercially available mix (FastStart DNA Master SYBR Green I kit; Roche). The primer sequences listed in the Methods section in the online-only Data Supplement. Differences in relative expression levels were calculated according to the ΔΔCt (cycle threshold) method by Pfaffer et al.

Statistics
Data are presented as mean±SEM. The Student t-test (2 tailed) was used to compare 2 groups of independent samples. For multiple comparisons, 1-way ANOVA was used followed by the Tukey post hoc test. Echocardiographic data were analyzed by repeated measures 2-way ANOVA followed by Bonferroni posthoc test. Data sets with nonparametric Kruskal-Wallis followed by the Dunn post hoc test. Values of P<0.05 were considered significant.

Results
Fibroblast Growth Factor-2 and Hepatocyte Growth Factor Synergistically Stimulate Vascular Cell Migration and Proliferation and Induce Stable Blood Vessel Growth
We evaluated the effects of FGF-2 and/or HGF in vitro using murine HMEs, murine embryonic microvascular ECs, HMEC-Cs, and rat aortic SMCs. Whereas both FGF-2 and HGF used alone enhanced EC and SMC migration and proliferation (Figure 1), HGF pretreatment significantly potentiated EC motility response to FGF-2 (Figure 1A and 1B). Reciprocally, pretreatment with FGF-2 enhanced HGF-induced migration (Figure 1A and 1B and Figure I in the online-only Data Supplement). Similarly, in SMCs, FGF-2 strikingly increased motility responses to HGF, although HGF did not alter FGF-2-induced migration (Figure 1C). Furthermore, pretreatment of HMEC-Cs (Figure 1D) or HMEs (Figure 1E) with FGF-2 significantly increased HGF-induced cell proliferation. Conversely, pretreatment of HMEC-Cs (Figure 1G) or SMCs (Figure 1F) with HGF significantly increased cell proliferation responses to FGF-2. These data show that FGF-2 and HGF synergistically stimulate both EC and SMC migration and proliferation, suggesting that this growth factor combination may generate mature and thus stable blood vessels in vivo. To investigate this possibility, we applied the corneal angiogenesis assay. Whereas HGF used alone induced a potent angiogenic response within 1 week as previously reported,15 most new blood vessels had regressed within 1 month (data not shown). In contrast, the blood vessels induced by FGF-2 used alone or in combination with HGF remained largely intact throughout the first month after initial remodeling (Figure 1H). However, whereas after ~1 year only a few vessels remained in FGF-2–implanted corneas, considerably more extensive vascular networks persisted in corneas implanted with HGF and FGF-2.

Sequential Slow Release of Growth Factors by Albumin-Alginate Microcapsules
Next, we aimed to obtain an injectable particulate growth factor delivery system to achieve spatiotemporally controlled release inside the myocardium. Alginate, a naturally occurring polysaccharide, is suitable for the delivery of positively charged proteins such as FGF-2 and HGF because it bears negatively charged carboxylic groups available for electrostatic interactions.22 Indeed, ionically cross-linked alginate hydrogels have been widely used for angiogenic growth factor delivery23 but generally display uncontrolled degradation, leading to unpredictable release kinetics.24 However, covalently cross-linking polysaccharides to proteins in a microcapsule membrane prevents hydrolysis-driven dissolution and delays protease-driven degradation, resulting in more stable particles with reproducible drug release rates.16,17 Thus, we developed microcapsules containing a thin, covalently cross-linked human serum albumin and propylene glycol alginate membrane surrounding a liquid center. Laser diffraction measurements and microscopic observations revealed that these albumin-alginate microcapsules had a mean diameter of 100 μm and were roughly spherical (Figure IIA in the...
Dehydration caused a partial and reversible collapse, resulting in the appearance of a very pleated surface in desiccated microcapsules as observed by scanning electron microscopy (Figure IIB in the online-only Data Supplement). The microcapsules were assayed in vitro for the release of angiogenic growth factors under conditions approximating the in vivo tissue environment. Whereas FGF-2 release from the microcapsules began immediately, the release of HGF and another angiogenic growth factor, platelet-derived growth factor (PDGF)-BB, was delayed for \( \geq 1 \) week (Figures IIC and IIIA in the online-only Data Supplement). Furthermore, whereas PDGF-BB release lasted 4 weeks, that of FGF-2 and HGF lasted 6 weeks. We confirmed that the growth factors released from the microcapsules retained their full bioactivity using an in vitro assay (Figure IIIB in the online-only Data Supplement).

To investigate the stability of the microcapsules, we analyzed their morphology during in vitro incubation. Although FGF-2–loaded microcapsules started to disintegrate within 1 week, those loaded with HGF remained largely intact for 3 to 4 weeks (Figure IIIC in the online-only Data Supplement). Furthermore, to determine the growth factor localization, we performed confocal analyses of microcapsules loaded with fluorescently labeled FGF-2 or HGF (Figure IID and IIE in the online-only Data Supplement). The results show that although both growth factors bound to the microcapsule surface layer, confirming their interactions with the cross-linked protein-polysaccharide membrane, FGF-2 was also found in the liquid center of the microcapsule. These findings may in part explain why the microcapsules display different release profiles for FGF-2 and HGF.

**Sustained Delivery of Fibroblast Growth Factor-2 and Hepatocyte Growth Factor Enhances Angiogenic and Arteriogenic Responses**

To determine whether our slow-release system would influence the angiogenic effect of FGF-2 or HGF in vivo, we compared treatment with growth factor–loaded microcapsules and naked growth factors using the mouse Matrigel plug model. Growth factor delivery by microcapsules was found to be 3 to 6 times more potent to induce angiogenesis compared with bolus delivery of growth factors (Figure IIID in the online-only Data Supplement).

The reciprocal stimulatory interactions observed between FGF-2 and HGF in vascular cells indicated that these growth factors may cooperatively regulate vessel growth. To further
investigate this possibility, microcapsules containing FGF-2 and/or HGF were injected in Matrigel plugs in mice. In each case, we used the lowest dose of growth factor resulting in a substantial angiogenic effect. Whereas each growth factor used alone induced a moderate angiogenic response, FGF-2 and HGF used in combination synergistically stimulated angiogenesis and arteriogenesis, as evidenced by the increased vascular density (Figure 2A and 2C), vascular maturity (Figure 2D), and vascularized area (Figure 2E) compared with single growth factor treatments. The most potent angiogenic growth factor combination described to date is the association of FGF-2 and PDGF-BB.12 To compare these growth factor combinations, microcapsules containing FGF-2 and/or PDGF-BB were injected in Matrigel plugs. We found that whereas the vessel density induced by the combination of FGF-2 and PDGF-BB (Figure 2B, 2F, and 2I) was moderately greater than that induced by FGF-2 in combination with HGF, the number of mature vessels did not differ (Figure 2G and 2J). However, whereas FGF-2 together with PDGF-BB resulted in only 30% of the total plug area being vascularized, the combination of FGF-2 and HGF notably resulted in a Matrigel plug vessel content of ≈80% (Figure 2E and 2H). These results reveal our microcapsules to be particularly efficient for growth factor delivery in vivo. Furthermore, in agreement with the in vitro data, the combination of FGF-2 and HGF synergistically induced angiogenesis and arteriogenesis at a level comparable to or surpassing that of the most potent angiogenic growth factor combination currently described.12

Intramyocardial Delivery of Fibroblast Growth Factor-2 and Hepatocyte Growth Factor Stimulates Angiogenesis and Arteriogenesis and Prevents Myocardial Infarction-Induced Cardiac Hypertrophy and Fibrosis

To evaluate the effect of FGF-2 in combination with HGF in a setting of cardiovascular disease, we performed a randomized, blinded experiment in rats surviving coronary artery ligation (n=102) or sham surgery (n=11). The experimental MI model leads to the development of CHF within 3 months.18 We found that the albumin-alginate microcapsules...
Displayed slow release of HGF or FGF-2 in vivo after intramyocardial injection in rats (Figure IV in the online-only Data Supplement). Thus, we used these microcapsules for cardiac delivery of angiogenic factors by local injection into the viable free wall of the myocardium bordering the LV infarct zone immediately after MI (Figure VA in the online-only Data Supplement). At 1 or 3 months after MI, the angiogenic and arteriogenic cardiac effects were evaluated by immunohistochemistry. At 1 month after MI, untreated controls exhibited myocardial vessel rarefaction, including reduced levels of mature blood vessels, compared with healthy shams (1552±71 versus 2489±93 vessels per 1 mm²; Figure VB and VC in the online-only Data Supplement). The reduction was limited mainly to SMA⁺ microvessels with lumen diameters inferior to 15 μm (Figure VI A and VI B in the online-only Data Supplement). As a result of an inherent compensatory angiogenic response, evidenced by increased EC proliferation (Figure VD in the online-only Data Supplement), the total vessel (1848±57 vessels per 1 mm²) and mature vessel densities were slightly improved in untreated control hearts at 3 months (Figure 3A through 3D). Monotherapy with FGF-2 resulted in a further increase in angiogenesis and arteriogenesis locally in the treated LV area, leading to slightly augmented vessel density (1809±83 vessels per 1 mm²) and significantly increased mature vessel density at 1 month compared with controls. However, the effects were lost at 3 months (1839±84 vessels per 1 mm²; Figure 3A through 3E). Monotherapy with HGF, on the other hand, showed limited arteriogenic effects at 1 month, tended to increase EC proliferation and vascular density at 3 months (2107±80 vessels per 1 mm²; Figure 3A, 3C, and 3E). In contrast, the combination therapy induced a potent angiogenic (1919±66 vessels per 1 mm²) and arteriogenic response, again strictly limited to the treated LV zone, with more than a doubling of the number of proliferating ECs (Figure VD in the online-only Data Supplement) and 3 times more mature blood vessels compared with untreated controls at 1 month after MI (Figure VC in the online-only Data Supplement). The increase in SMA⁺ vessels was due mainly to an increase in microvessels (Figure VI A and VI B in the online-only Data Supplement). Strikingly, by 3 months, the myocardial vessel density in the combination group had attained normal sham levels (2505±106 vessels per 1 mm²; Figure 3A and 3C). Moreover, the mature vessel density in
the group treated by the combination even surpassed that of sham (Figure 3B and 3D). Furthermore, whereas the numbers of SMA+ microvessels had decreased, there was a significant increase in the number of small arterioles with lumen diameters between 15 and 50 μm (Figure VIC and VJD in the online-only Data Supplement), suggestive of vascular remodeling.

Next, the extent of MI-induced cardiomyocyte hypertrophy and fibrosis was evaluated by histological analyses of cardiomyocyte sizes and collagen density, respectively. At 1 month, cardiomyocyte sizes were not significantly increased in MI controls compared with sham, although the mRNA level of the maladaptive cardiomyocyte hypertrophic marker β-myosin heavy chain was found to be increased, together with significantly higher expression levels of atrial natriuretic peptide and a tendency for increased brain natriuretic peptide (Figure VII in the online-only Data Supplement). In contrast, FGF-2 used alone or in combination with HGF tended to stimulate cardiac hypertrophy (data not shown), although it did not reach significance. This is in line with previous studies suggesting a direct stimulatory role of angiogenesis on cardiac hypertrophy.25 However, the cardiomyocyte hypertrophy was not associated with increased β-myosin heavy chain, suggesting, together with the increased vessel density observed, that the cardiomyocyte growth may have been adaptive. At 3 months, MI controls and FGF-2–treated rats showed equally enlarged cardiomyocytes and increased levels of collagen deposition compared with sham (Figure 3F and 3G). In contrast, both HGF alone and the combination treatment significantly reduced cardiac fibrosis and hypertrophy at 3 months. In agreement, the ratio of LV weight to body weight that was significantly increased in MI controls compared with sham was reduced by the combination treatment (data not shown).

Importantly, the coordinated decrease in cardiomyocyte sizes and increase in blood vessel density generated by the combination treatment resulted in a normalization of the ratio of cardiomyocytes to vessel (Figure 3H).

We also verified the cardiac distribution of the slow-delivery vehicles using fluorescently labeled microcapsules. Hearts were evaluated histologically at 6 hours and 1 or 2 weeks after intramyocardial injection. We found that the microcapsules were confined in a small area surrounding the injection points, extending maximally 1 to 2 mm into the subepicardial myocardium (Figure VIII). The microcapsules spread over an area representing ~8% of a cross section of the LV at the papillary muscle level after 6 hours, progressively decreasing to ~6% after 1 week and ~4.5% after 2 weeks. These findings confirm a localized microcapsule distribution centered around the points of injections, in agreement with the observed restricted LV effects of the therapies, and a comparable timeline of microcapsule degradation in the heart as seen in vitro.

**Interrmyocardial Delivery of Fibroblast Growth Factor-2 and Hepatocyte Growth Factor Improves Regional Cardiac Perfusion and Cardiac Function After Myocardial Infarction**

To assess whether the blood vessels induced by the angiogenic therapies resulted in a functional improvement in cardiac perfusion, magnetic resonance imaging was used. Our results, obtained 3 months after MI, demonstrate that whereas untreated controls displayed significantly reduced cardiac perfusion compared with healthy sham-operated animals, only the combination treatment increased regional cardiac perfusion in the treated area of the LV (Figure 4A).

Next, to investigate whether the multiple beneficial myocardial alterations induced by the angiogenic therapies correlated with improved cardiac function, even though myocardial infarct sizes were similar in all groups (Figure IX in the online-only Data Supplement), echocardiographic analyses were carried out at 1 and 3 months after MI. As previously reported,16 untreated controls displayed severe cardiac dysfunction (Figure 4B through 4D and Tables I and II in the online-only Data Supplement), characterized by LV wall thinning at 1 month (Figure 4C), and progressive LV dilation associated with the development of CHF (Figure 4B and 4D; LV end-systolic [LVEDD] and end-diastolic [LVESD] diameters in Tables I and II in the online-only Data Supplement). Both regional and global cardiac contractility were reduced, as evidenced at 1 month by a decreased fractional shortening (FS; 16±1% for control versus 54±3% for sham; Figure 4E) and velocity of circumferential fiber shortening (Figure 4F).

Monotherapy with HGF only slightly improved LV parameters by 3 months, as evidenced by a decreased LVEDD and end-systolic volume (Table II in the online-only Data Supplement) and a tendency for increased FS and velocity of circumferential fiber shortening (Figure 4E and 4F) compared with untreated controls. Monotherapy with FGF-2, on the other hand, significantly reduced LV dilation (Figure 4B and 4D; LVEDD and LVESD in Tables I and II in the online-only Data Supplement) and, by 3 months after MI, increased both FS (Figure 4E) and velocity of circumferential fiber shortening (Figure 4F).

In contrast to these moderate effects of monotherapies, the combination of FGF-2 and HGF reduced LV dilation (Figure 4B and 4D; LVESD and LVEDD in Table I in the online-only Data Supplement) and LV dysfunction already at 1 month after MI, as shown by an increased FS (22±2%; Figure 4E) compared with untreated controls. This was associated with a tendency for increased velocity of circumferential fiber shortening (Figure 4F), LV anterior end-systolic wall thickness (Table I in the online-only Data Supplement), and wall thickening (Figure 4C). At 3 months, the combination treatment group displayed a significant further increase in LV wall thickness (Table II in the online-only Data Supplement), and wall thickening (Figure 4C). Moreover, the LV dilation was further reduced (Figure 4D; LVEDD and LVESD, Table II in the online-only Data Supplement) and associated with a marked recovery of LV function, evidenced by an increased FS (Figure 4E) and velocity of circumferential fiber shortening (Figure 4F) compared with both untreated controls and HGF-treated animals, indicating that the development of CHF was partially prevented.

**Molecular Mechanisms of the Angiogenic Synergy**

To investigate potential molecular mechanisms behind the observed synergy between FGF-2 and HGF, the effects on
different signaling pathways were analyzed in ECs. The levels of phospho-Akt (p-Akt) and phospho-42/44 mitogen-activated protein kinases (p-MAPKs) were assayed in HMEC-Cs after stimulation with FGF-2 and/or HGF. We found that FGF-2 induced maximal Akt activation within 10 to 15 minutes (1.7 ± 0.5-fold induction over control) but p-Akt levels returned to baseline after 30 minutes (Figure 5A and 5C). In turn, HGF induced maximal Akt activation within 30 minutes (1.6 ± 0.5-fold). The increased levels of p-Akt persisted up to 1 hour, consistent with the potent antiapoptotic effects of HGF.26 Notably, the combination of FGF-2 with HGF induced a prolonged Akt activation lasting >2 hours, with a significantly increased maximal activation level (2.2 ± 0.2-fold). Next, we found that FGF-2 induced maximal p42/44-MAPK activation within 15 minutes (1.8 ± 0.6 fold; Figure 5B and 5D). In addition, HGF activated p42/44-MAPK, with a maximal effect after 30 minutes (2.4 ± 0.8-fold) persisting for >1 hour. The combination of FGF-2 and HGF resulted in a strikingly potent MAPK activation within 10 to 15 minutes (5.8 ± 1.0-fold). However, the signal duration was only slightly prolonged compared with single growth factor stimulation. To verify that synergistic activation of cell signaling pathways also may occur in vivo, we assayed the levels of p-Akt in cardiac samples obtained at 3 weeks after MI. We found that the combination treatment led to significantly increased Akt activation compared with MI controls (Figure XA and XB in the online-only Data Supplement).

Another mechanism by which dual growth factor treatment may produce synergistic effects involves the induction of growth factor receptor levels. For instance, the potent angiogenic synergy between FGF-2 and PDGF-BB is due in part to an upregulation in vascular cells of FGFR-1 by PDGF-BB and of PDGF receptors by FGF-2.12,27 To investigate whether similar mechanisms may operate in the combination of FGF-2 with HGF, real-time polymerase chain reaction analyses were performed in vascular cells. Indeed, we found that HGF stimulation significantly induced the expression of both FGFR-1 (Figure 5F) and the HGF receptor c-Met (Figure 5E) in murine HMEs. In a reciprocal manner, FGF-2 stimulation potently increased c-Met mRNA levels (Figure 5E) while displaying a weaker effect on FGFR-1 levels (Figure 5F). Similar results were obtained in human HMEC-Cs (Figure 5G and 5H). Moreover, we found that FGF-2 induced the expression of c-Met in rat SMCs (data not shown). Next, to
confirm these findings in vivo, FGFR-1 and c-Met protein levels were assayed by semiquantitative immunofluorescence analyses of mouse Matrigel plugs. We found that although vascular c-Met levels were comparable after 3 weeks of stimulation with either FGF-2 or HGF, the FGFR-1 levels were increased in blood vessels induced by HGF compared with FGF-2 (Figure 5I and 5J).

To extend these findings to the heart, we assayed by Western blot the levels of phosphorylated FGFR-1 or c-Met in the treated LV peri-infarct zone at 3 weeks after MI. We found that FGF-2 treatment used alone or together with HGF resulted in increased levels of activated FGFR-1 compared with MI controls (Figure XB and XC in the online-only Data Supplement). Similarly, HGF treatment used alone significantly increased phospho–FGFR-1 levels in the heart. Reciprocally, FGF-2 used alone increased the levels of phospho–c-Met to levels similar to those with HGF treatment used alone or together with FGF-2 (Figure XE and XF in the online-only Data Supplement). Taken together, this finding suggests that whereas FGF-2 may have induced HGF and/or c-Met expression, HGF may have induced FGF-2 and/or FGFR-1 expression in the heart, in line with our in vitro findings in vascular cells.

Discussion

Current strategies of therapeutic angiogenesis or arteriogenesis in cardiovascular diseases, including CHF, have proved insufficient. Our study shows that therapeutic angiogenesis, based on targeted intramyocardial albumin-alginate microcapsule delivery of a synergistic combination of growth factors, results in potent stimulation of mature blood vessel growth, prevention of cardiac remodeling, and enhanced myocardial perfusion and cardiac function in a rat model of post-MI CHF.

HGF plays an important role in tissue regeneration. It displays considerable prosurvival effects in many cell types, including cardiomyocytes. In addition, HGF potently stimulates angiogenesis. During tissue ischemia, HGF and its receptor, c-Met, are upregulated in vascular cells and cardiomyocytes. Most recently, encouraging results have been reported for a double-blind controlled HGF gene therapy trial for chronic limb ischemia, suggesting the utility of this particular growth factor for regenerative medicine. Interestingly, in the present study, we found that HGF monotherapy at a dose at least 1 to 2 orders of magnitude lower than most experimental studies, while efficiently preventing cardiac fibrosis and hypertrophy, had limited angiogenic effects. Thus, although the structural antiremodeling effects were indeed considerable, the fact that HGF monotherapy merely showed weak amelioration of cardiac function suggests that stimulation of angiogenesis and arteriogenesis plays a key role in prevention of cardiac dysfunction. However, whereas FGF-2 monotherapy failed to induce a stable increase in blood vessel density and further showed no effects on cardiac fibrosis or hypertrophy, it did successfully improve regional cardiac function, as evidenced by decreased LVESD and increased FS. This suggests that mechanisms other than stimula-
tion of angiogenesis were at play. Indeed, FGF-2 may have direct cardioprotective effects involving decreased cell-to-cell metabolic coupling in cardiomyocytes through inhibition of connexin-43 gap junctions.33 Thus, it seems probable that the improvement in both regional and global cardiac function observed with the combination of FGF-2 and HGF is multifactorial in origin and not due only to potent induction of angiogenesis leading to a normalized ratio of cardiomyocytes to vessel and enhanced regional cardiac perfusion.

The cardiac fibrosis occurring during postinfarction remodeling causes increased ventricular wall stiffness and reduced cardiac relaxation rates, contributing to the cardiac dysfunction. HGF has been shown to reduce cardiac fibrosis, in part via suppression of transforming growth factor-β signaling.32 Similarly, in our study, we found that HGF used alone or in combination with FGF-2 resulted in an impressive reduction of cardiac collagen content. This antifibrogenic effect of HGF may have played an important role in the observed cardiac functional improvement induced by the combination treatment.

With regard to the potential molecular mechanisms behind the observed angiogenic synergy, we found that the combination of FGF-2 and HGF significantly potentiated the activation of Akt and MAPKs compared with single growth factors. This may contribute to the synergistic stimulation of cellular migration and proliferation observed in vitro and to the potent angiogenic effects in vivo. Moreover, we found evidence, both in vitro and in vivo, that HGF induces FGFR-1 levels in ECs, which should lead to enhanced FGF-2–induced signaling. Similar results were found in human ECs in which FGF-2 also reciprocally increased c-Met levels.

Previous studies have indicated some further potential means of angiogenic interactions, including HGF induction in SMCs by FGF-2. Moreover, HGF induces vascular endothelial growth factor production in SMCs and has been suggested to act in synergy with vascular endothelial growth factor to induce EC migration.33 However, another study reported that HGF and vascular endothelial growth factor did not synergistically stimulate angiogenesis in vivo and further that vascular endothelial growth factor was not required for the angiogenic effects of HGF.34 Interestingly, the proangiogenic signal of angioptocin-1 has been shown to depend on induction of HGF in ECs,35 suggesting that HGF is indeed a key mediator of vascular maturation similar to PDGF-BB.

Whereas angiogenesis generally requires prolonged stimulation of vascular cells, the in vivo half-life of most angiogenic growth factors is very limited, illustrating the need to incorporate sustained delivery systems for protein therapy approaches.36 In this study, we devised albumin-alginate microcapsules to gain spatiotemporal control over growth factor release. These microcapsules released active growth factors over a significant period of time, showing different release kinetics for FGF-2 versus HGF. This sequential release pattern, generating an initial strong angiogenic signal through FGF-2 followed by a potent arteriogenic signal via HGF, might be ideal to induce mature blood vessel formation. Furthermore, our sustained release system not only increased the efficacy of treatment, permitting the use of remarkably low doses of growth factors, but also resulted in the confinement of the angiogenic effect to a well-defined area of the LV. Both of these aspects should be instrumental in considerably reducing growth factor leakage to the circulation and thus the risk for serious side effects of treatment, including stimulation of angiogenesis at distant sites potentially harboring micrometastases.

Conclusions

Our data provide proof of principle that localized sustained delivery of low doses of a specific combination of angiogenic growth factors is sufficient to generate stable and functional blood vessels in the heart. Moreover, dual delivery of FGF-2 and HGF reduced MI-induced cardiac fibrosis and remodeling and partially prevented cardiac dysfunction. These findings may provide conceptual guidelines for future clinical trials of therapeutic angiogenesis for the treatment of cardiovascular diseases.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Therapeutic stimulation of blood vessel growth, including microvascular growth (angiogenesis) and collateral growth (arteriogenesis), is an attractive common target in cardiovascular diseases. Such novel therapies may in particular benefit coronary artery disease patients with advanced-stage or small-vessel disease that responds poorly to standard pharmaceutical or surgical revascularization treatments or patients who develop chronic heart failure. However, despite significant preclinical efforts, there is still no available treatment to stimulate cardiac vessel growth, and clinical studies have so far been inconclusive. Indeed, current approaches for therapeutic angiogenesis or arteriogenesis result in the generation of unstable or nonfunctional blood vessels. Thus, there is an urgent need for new treatments and delivery methods to improve the efficacy of therapeutic blood vessel growth. In the present study, using a microparticulate slow-release system, we have evaluated targeted intramyocardial delivery of a growth factor combination of fibroblast growth factor-2 and hepatocyte growth factor in a rat model of myocardial infarction. We show that the combination treatment induces angiogenesis and arteriogenesis, resulting in enhanced cardiac perfusion at 3 months after myocardial infarction. Furthermore, the cardiac remodeling and dysfunction associated with the development of heart failure were partially prevented by fibroblast growth factor-2 and hepatocyte growth factor used in combination. Our data suggest that local intramyocardial delivery of fibroblast growth factor-2 together with hepatocyte growth factor in our slow-release formulation may constitute an efficient novel treatment to stimulate a durable angiogenic and arteriogenic response in the heart. Furthermore, on the basis of this study, we believe that this therapy might prevent the development of chronic heart failure in patients.