Repair of Infarcted Myocardium by an Extract of Geum japonicum with Dual Effects on Angiogenesis and Myogenesis

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Background: It has become apparent recently that cardiac myocytes can divide after myocardial infarction, a circumstance that challenges the orthodox view that myocytes may be terminally differentiated. Replacement of the necrosed heart tissue by newly regenerated functional myocardium is a therapeutic ideal, but attempts to reconstitute functional myocardia and coronary vessels have been less successful.

Methods: We isolated a fraction containing 5 compounds from the Chinese herb Geum japonicum, which stimulates the processes of angiogenesis and cardiomyogenesis. We investigated these dual properties in both ex vivo and in vivo systems.

Results: We observed that this bioactive fraction displayed favorable dual actions on early angiogenesis and cardiomyogenesis in acute myocardial infarction in an animal model. Our results demonstrated that application of this bioactive fraction showed pronounced effects on limiting infarct size by 35%–45%, stimulating early development of new blood vessels in 24 h, and regenerating myocardium, replacing ~49% of the total infarction volume after 2 weeks. Echocardiographic studies demonstrated marked improvement of left ventricular function within 2 days after infarction, and the improvement was sustained for >1 month.

Conclusions: The properties of this bioactive fraction appear to be entirely novel and represent a new approach for the treatment of ischemic heart disease.

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Repair and replacement of infarcted myocardium after an infarction has been an unrealistic therapeutic ideal until recently, because cardiac myocytes were considered to be terminally differentiated cells, comparable to neurons in their inability to regenerate and replace damaged tissue. Although evidence now exists for adult neurogenesis (1), the concept of regeneration of cardiac myocytes remains controversial (2). However, Beltrami et al. and others have provided evidence that a population of myocytes within the myocardium can and does replicate after infarction, challenging the dogma that the heart is a postmitotic nonregenerating organ (3–7). The location of the newly regenerated cardiac myocytes occurs exclusively in the border zone adjacent to the infarct and in distant tissues where the blood supply is largely maintained (3–7). Because myocytes in the infarcted area die in a few hours and ischemic damage destroys the vascular and nonvascular components of the interstitial tissue, regeneration of new myocardium replacing the infarcted myocardium in the central region through growth of a subpopulation of cardiac myocytes alone would seem unlikely. Angiogenesis is also required. Although angiogenic growth factors such as vascular endothelial growth factor,5 acidic fibroblast growth factor, basic fibroblast growth factor (VEGF) and platelet-derived growth factor have been studied, these are slow acting (8–17), whereas

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5 Nonstandard abbreviations: VEGF, vascular endothelial growth factor; HCAEC, human coronary artery endothelial cells; MRF, myocardial repair fraction; BrdU, 5-bromo-2′-deoxyuridine; HPF, high-power field; MHC, myosin heavy chain; LVFS, left ventricular fractional shortening; DMSO, dimethyl sulfoxide.
myocardial necrosis caused by coronary occlusion occurs rapidly, within a matter of hours (15–19). Despite the relative ischemic condition, fibrous tissue grows rapidly, replacing the infarcted myocardium, but it also blocks space for any newly regenerated myocyte replacement. Therefore, any repair strategy after acute myocardial infarction must protect surviving cardiomyocytes against continuing hypoxia and must enhance blood and nutrient delivery. The most effective way to rescue affected cells is to reestablish at an early stage a new blood supply network to the infarcted area that would allow circulating stem cells, nutrients, and growth factors to be delivered to the infarction site. Thus, the synergistic effects of both early angiogenesis and cardiomyogenesis are required to create an effective repair after a myocardial infarction. In this study, we assessed the effect of a bioactive fraction of the herb *Geum japonicum*, an ancient Chinese herbal remedy (20), on both angiogenesis and myogenesis.

**Materials and Methods**

**BIOASSAY-GUIDED ISOLATION OF THE ACTIVE COMPOUNDS FROM *Geum japonicum***

*Geum japonicum*, collected from Guizhou Province of China in August 2004, was dried and percolated with methanol at room temperature for 7 days. The extract was then dried under reduced pressure to yield a powder residue. The dried powder was suspended in H₂O and then dried under reduced pressure to yield a powder methanol at room temperature for 7 days. The extract was collected from Guizhou Province of *Geum japonicum*, an ancient Chinese herbal remedy (15–19), on both angiogenesis and myogenesis.

**IN VITRO BIOASSAY FOR MYOGENIC ACTIVITY**

A C₂C₁₂ myoblast cell line was used to examine the myogenic activity during the process of bioassay-guided fractionation of *Geum japonicum*. The cells were made in DMEM containing 10% fetal bovine serum and cultured in a 96-well culture plate. The myogenic effect of the test samples was determined. The dose effect of the test samples in different concentrations was also studied. We used the same volume of 5% DMSO for the controls. We maintained all cultures at 37 °C and 5% CO₂ for 36 h. To determine the extent of cell proliferation, we used BrdU labeling solution to label the proliferated cells, and the incorporation of BrdU was quantified as described above.

**MYOCARDIAL INFARCTION ANIMAL MODEL AND TREATMENT PROTOCOL**

To evaluate the effects of MRF in a heart infarction model, 152 male, 250–300-g Sprague-Dawley rats were used. Myocardial infarction was induced by ligation of the left anterior descending coronary artery. MRF made up in 5% DMSO (0.1 mL, containing 0.3 mg MRF) was injected into the distal myocardium (the ischemic region) of the ligated artery in 60 rats immediately after the ligation (test group). Another 60 rats were injected with an equivalent amount of 5% DMSO at the same location and timing as the control group. For sham ischemia, thoracotomy was performed on 16 rats without LAD ligation. An additional 16 rats were set as an untreated control group.

Twenty rats from the test group, 20 rats from the control group, and 4 rats from sham group were killed on day 2 and day 7, respectively, postinfarction, together with 4 untreated rats. The remaining 20 rats from the test...
group, 20 rats from the control group, 8 rats from the sham group, and 8 rats from the untreated control group were subjected to echocardiography measurements on day 2 and day 30 postinfarction and were killed after the second echocardiography measurement on day 31. At different times, the hearts of the killed rats were removed, washed with phosphate-buffered saline, and photographed. All the specimens harvested were sectioned for histologic and immunohistochemical analyses.

**ESTIMATION OF INFARCT SIZE**

The left ventricles of experimental rats killed on days 2, 7, and 31 were removed and cut from apex to base in 3 transverse slices and embedded in paraffin. We stained thin sections (10-μm thickness) of the left ventricle with Masson’s trichrome, which labels collagen blue and myocardiun red. These sections were digitized, and all blue staining was quantified morphometrically. The volume of infarct (mm³) of a particular section could be calculated based on the thickness of the slice. The volumes of infarcted tissue for all sections were added to yield the total volume of the infarct for each particular heart. The mean value was calculated for each rat. A blinded pathologist performed all studies. Infarct size was expressed as percentage of total left ventricular area.

**ANGIOGENIC ASSESSMENT IN INFARCT REGION**

On day 2 postinfarction, we determined vascular density from histology sections by counting the number of vessels within the infarct area using a light microscope under a high-power field (HPF) (40×). Seven random and non-overlapping HPFs within the infarct field were used to count all newly formed vessels in each section of all MRF-treated hearts and control hearts. The number of vessels in each HPF was averaged and expressed as the number of vessels per HPF. Vascular counts were performed by 2 investigators in a blinded fashion.

**ASSESSMENT OF REGENERATING CARDIOMYOCYTES AND MYOCARDIUM**

The heart sections from test group on days 7 and 31 postligation were used to evaluate the volume of the regenerating myocardium in the central area of the infarct. We stained the sections immunohistochemically with both monoclonal rat-specific anti-Ki67 (Dako) and polyclonal antimyosin heavy chain (MHC) (Santa Cruz, sc-20641) antibodies sequentially to identify the regenerating myocardium. Specific secondary antibody conjugated with alkaline phosphatase (Santa Cruz) was used to visualize the positively stained nuclei. The numbers of newly regenerated myocytes on the border zones of the infarct area were determined by counting all cells positively stained by both anti-Ki67 and MHC concomitantly with the morphology of cardiac myocytes in each section (50 μm apart). The numbers of newly regenerated myocytes for ~100 sections covering particular infarct volumes were added to yield the total number of these regenerating myocytes for each particular heart in these border zones. The mean value was calculated for each experimental group.

The regenerating, myocardial-like clusters in the central infarct region were delineated in the projected field by a grid containing 42 sampling points. Approximately, 30–60 calculating points along the border of a particular regenerating myocardium were selected in each section. This grid defined an uncompressed tissue area of 62 500 μm² that was used to measure the selected 30–60 calculating points in each section. The shapes and volumes of regenerating myocardium in the central infarct area were determined by measuring in each of the ~100 sections (50 μm apart), the shapes and areas occupied by the regenerating myocardium and section thickness. Integration and calculation with these variables produced a stereo structure and yielded the volume of a particular regenerating myocardium in the central area of the infarct in each section. Values and stereo structure of all sections of a particular tissue block were added and computed to obtain the total volume and the full stereo structure of the regenerating myocardium. Only the cell clusters with the morphology of myocardium in the central area of the infarct and with positive staining by both anti-Ki67 and MHC were included.

**ECHOCARDIOGRAPHY ASSESSMENT DURING THE ACUTE AND CHRONIC PHASE**

In all, 32 Sprague–Dawley experimental rats received baseline echocardiography before the experimental procedure. Echocardiography was recorded under controlled anesthesia using an S10-MHz phased-array transducer and a GE VingMed Vivid 7 system. M-mode tracing and 2-dimensional echocardiography images were recorded from the parasternal long- and short-axis views. The short-axis view was at the papillary muscle level. Left ventricular end-systolic and end-diastolic dimensions, as well as systolic and diastolic wall thickness, were measured from the M-mode tracings by using the leading-edge convention of the American Society of Echocardiography. For each M-mode measurement, at least 3 consecutive cardiac cycles were sampled. Left ventricular ejection fraction and fractional shortening (FS) were derived from a left ventricular cross-sectional area in 2-dimensional short axis view:

\[
\text{ejection fraction} = \frac{\text{LVDA} - \text{LVSA}}{\text{LVDA}} \times 100,
\]

where LVDA and LVSA correspond to left ventricle areas in diastole and in systole (17). Standard formulae were used for echocardiographic calculations (21).

**STATISTICS**

All morphometric data were collected blindly, and the code was broken at the end of the experiment. Results are presented as mean (SD) computed from the mean measurements obtained from each heart. Statistical significance for comparison between 2 measurements was de-
determined using the unpaired 2-tailed Student \( t \)-test. Values of \( P < 0.05 \) were considered to be significant.

**Results**

**ISOLATION OF MRF FROM Geum japonicum**

Bioassays of the partitioned fractions demonstrated that \( n \)-butanol fraction derived from the methanol extract of *Geum japonicum* (Fig. 1A) not only significantly enhanced the proliferation of cultured C2C12 cells and HCAECs up to \( \sim 2 \)-fold, compared with DMSO control cells (Fig. 1, A and B; \( P < 0.05 \) at 20 \( \mu \)g/mL and \( P < 0.01 \) at 80 \( \mu \)g/mL) in vitro, but also promoted rapid revascularization within 24 h and muscle fiber regeneration in muscle injury animal models. The active \( n \)-butanol soluble fraction was further resolved into 7 subfractions by liquid column chromatography with a column of Sephadex LH-20 (Fig. 1A). Activity tests with the C2C12 cell culture system and a muscle injury animal model demonstrated that fraction 6 not only showed more substantial proliferative effect on C2C12 growth ex vivo than control (\( P < 0.01 \) at 5 \( \mu \)g/mL).

**Fig. 1. Bio-assay guided isolation of MRF from Geum japonicum.**

(A), the diagram of bioassay-guided isolation of MRF. (A,B), dose-dependent (20–80 \( \mu \)g/mL) effect of \( n \)-butanol fraction (GJ-B) on proliferations of HCAECs (a) and C2C12 (b) in vitro. (A-C), dose–response effects on the growth of C2C12 cells in culture with all resolved 7 fractions (GJ-B1, . . . , GJ-B7) from GJ-B. (A-D), angiogenesis induced by GJ-B3 within 48 h (B3 black arrowheads) and the myotube regeneration enhanced by GJ-B6 in severely damaged muscles within 48 h postinjury (B6 blue circles).
and \(P < 0.01\) at 30 \( \mu \text{g/mL}\) (Fig. 1, A-C), but also enhanced myogenesis in a skeletal muscle injury animal model (Fig. 1, A-D), and fraction 3 displayed a potent effect on rapid angiogenesis in muscle injury, but almost no effect on myogenesis (Fig. 1, A-D). The structural analysis of the MRF by nuclear magnetic resonance spectroscopic analysis demonstrated that MRF consisted of 5 compounds, including 23-hydroxytormentic acid, 5-desgalloylstachyurin, Gemin A, tellimagrandin II, and niga-ichigoside F1, which were reported to have antinociceptive and antiinflammatory effects (22, 23).

**INFARCT SIZE**

Seven days postinfarction, the area (~3–5 mm in diameter) distal to the ligation site became pure white because of ischemic necrosis in the control animals (Fig. 2C). We observed this change in the hearts of all control animals. By contrast, the corresponding areas in MRF-treated hearts were red in appearance, probably because of neovascularization (Fig. 2A). Histologic observations revealed that the infarct size in MRF-treated hearts \([n = 17, 16 (3.8)\%]\) was, on average, one third to one half times smaller than that in the control \([n = 15, 30 (7)\%; P < 0.001]\)

![Fig. 1. Continued](image)

**Fig. 2. MRF-mediated effects on limiting infarct size and early revascularization.**

(C), white appearance of the distal region of the ligation in control hearts (circled area). (A), the corresponding areas in MRF treated hearts were red (circled area). (B), The smaller infarct size in MRF treated hearts (arrowheads). (D), Bigger infarct size in control hearts.
as calculated by the percentage of the average infarct volume in total left ventricle volume (Fig. 2, B and D).

CAPILLARY DENSITY AND MYOGENESIS
Two days after infarction and MRF treatment, numerous vessels filled with blood cells were observed in the whole infarct zones (Fig. 3, A-C and E). In addition to these newly formed functional vessels, some of the newly formed vessels were still in their early stage of regenerating development and appeared as lumen structures without filling of blood cells (Fig. 3A). The capillary density in the infarct area of the MRF treated myocardium was ~9 per high-power field (40× HPF), derived from 7 randomly selected view fields of each slide and 17 slides in total from 17 MRF-treated hearts on day 2. In contrast, fewer blood vessels (~2 per HPF) with inflammatory cell infiltration were observed in the controls on day 2.

On day 7, many newly formed blood vessels (7/HPF) filled with blood cells remained in the infarction area, including the central areas and the border regions of the infarct in MRF-treated animals (Fig. 3, B, C, and E). More interestingly, numerous Ki67 and MHC positively stained cells organized into several cell clusters or myocardial-like tissue in the central infarct zone in MRF-treated hearts were seen (Fig. 3, C and D). In the high power field, this myocardial-like tissue showed the typical morphology of myocardium (Fig. 3E), but these individual cells were smaller than surviving cardiac myocytes and the newly regenerated cardiac myocytes along the border areas. These newly formed myocardial-like tissues occupied ~49.3% of the total infarct volume on day 7 postinfarction. In contrast, in DMSO treated control hearts, only a few Ki67-positive cardiac myocytes (0–3) in each section of ~100 slices for a particular infarcted heart were found, and the infarction site was occupied mainly by fibrous tissue (Fig. 3F). Immunohistochemical staining with monoclonal antibody against Ki67 positively stained the newly regenerated nuclei of cells, and polyclonal anti-MHC immunohistochemical staining stained only the cytoplasm of cardiac myocytes. By combining these dual immunohistochemical staining procedures with morphologic analysis, we could reliably identify the newly regenerated cardiac myocytes or myocardium (Fig. 3D). The nuclei of the undamaged cardiomyocytes surrounding the infarct areas were negatively stained for Ki67, but the cytoplasm was positively stained for MHC. Numerous Ki67-positive cells with the morphology of myocardium were also found along the border on the infarction site forming highly organized cell clusters that replaced the necrosed myocardium. Approximately 10–60 Ki67-positive cardiac myocytes were also found along the proximal zone of surviving myocardium adjacent to the infarction border in each section of ~100 slices for a particular infarcted heart, making ~5000 newly regenerated cardiac myocytes scattered among this region. The distal zone of surviving myocardium did not show any Ki67-positive

Fig. 3. Early revascularization and myocardial regeneration of the infarcted myocardium after MRF treatment.
(a), in MRF treated MI hearts, formation of numerous vessels (red arrowheads) and lumens (blue arrowheads) 2 days postinfarction. (B), On day 7 postMRF treatment, many vessels (black arrowheads and black circles) and some myocardial-like cell clusters (blue circles). (C), Ki67 positively stained dark-brown nuclei (blue circles) of the regenerating myocardium. The unaffected cardiomyocytes (N) were Ki67 negative. (D), both Ki67 and MHC positively stained regenerating myocardium (blue circle). (E), the typical myocardial morphology (light blue arrowheads) of the regenerating myocardial-like clusters (blue circle in C) with capillary vessels (black arrowheads). (F), fibrous tissue replacement (fib) in control 7 days postinfarction.
cells (Fig. 3, C and D). By contrast, the infarct zones in the control hearts were occupied mainly by fibrous tissues (Fig. 3F).

One month later, the morphologic changes remained similar to those observations on day 7 post infarction. Many Ki67- and MHC-positive myocytes with the phenotype of healthy cardiac myocytes were found scattered in the surviving myocardium along the border zones of the infarction. Numerous Ki67- and MHC-positive myocyte-like cells were highly organized into myocardial-like tissue that replaced the infarcted myocardium and further reduced the infarct volume by ~53%–60%.

**FUNCTIONAL EVALUATION**

These histologic changes were accompanied by measurable functional improvement. Left ventricular ejection fraction in the MRF-treated group improved substantially on day 2 [65.44 (2.41)] and at 1 month [61.13 (3.44)] postinfarction, compared with that on day 2 [54.10 (2.27)] and at 1 month [52.30 (2.10)] postinfarction in the control hearts \( (P = 0.001 \text{ and } P = 0.03, \text{ respectively}) \). Left ventricular shortening in the MRF-treated group was significantly higher than in the control group [31.72 (1.67) vs 24.41 (1.36); \( P = 0.002 \text{ on day 2 and } [29.44 (2.32) \text{ vs } 23.49 (1.21); \( P = 0.03 \text{ at } 1 \text{ month postinfarction. Left ventricular end-diastolic dimension in MRF-treated hearts was smaller compared with that in the nontreated control group on day 2 and 1 month postinfarction (Fig. 4 and Table 1).}

**Discussion**

The results of these studies have demonstrated that MRF isolated from this Chinese herbal medicine has substantial dual actions on angiogenesis and cardiomyogenesis postinfarction. After a single injection of MRF applied locally, right after LAD artery ligation, we found that the ischemic damaged heart tissues were undergoing active neovascularization within the first 24–48 h postinfarction, forming many functional blood vessels in the whole infarct field, although newly regenerating cardiac myocytes could be observed only 3–4 days postinfarction in the central and border areas of the infarct or within the surviving myocytes adjacent to the infarct border. By day 7, we saw many newly regenerating myocyte-like cells,

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**Table 1.** Echocardiographic results expressed as mean (SD).

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<th></th>
<th>After 2 days</th>
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<th>After 1 month</th>
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<tbody>
<tr>
<td></td>
<td>EF, %</td>
<td>FS, %</td>
<td>EF, %</td>
</tr>
<tr>
<td>Normal, n = 4</td>
<td>72.53 (4.78)</td>
<td>37.16 (4.02)</td>
<td>69.24 (4.98)</td>
</tr>
<tr>
<td>Sham, n = 4</td>
<td>76.07 (2.97)</td>
<td>40.02 (2.69)</td>
<td>72.25 (3.73)</td>
</tr>
<tr>
<td>MRF, n = 13</td>
<td>65.44 (2.41)</td>
<td>31.72 (1.67)</td>
<td>61.13 (3.44)</td>
</tr>
<tr>
<td>Control, n = 14</td>
<td>54.10 (2.27)</td>
<td>24.41 (1.36)</td>
<td>52.30 (2.18)</td>
</tr>
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EF, ejection fraction; FS, fractional shortening. The distribution of 3 groups of rats in EF and FS after 2 days and after 1 month of MRF treatment. After 2 days treatment, left ventricular ejection fraction in the MRF-treated group was significantly higher than in control group \((P = 0.001).\) FS in the MRF-treated group was significantly higher than that in control group \((P = 0.002).\) After 1 month follow-up, left ventricular ejection fraction in the MRF group was still significantly higher than that in control group \((P = 0.03).\) FS was also significantly higher in the MRF-treated group compared with the control group \((P = 0.03).\)
clustered together, forming myocardial-like tissue in the whole infarct zone. In addition, we observed many average sized, Ki67 positively stained myocytes among the unaffected myocytes adjacent to the border areas of the infarct, indicating that these newly regenerated myocytes might be derived from different sources than the regenerating myocardium in the central infarct, where the myocytes were of smaller size.

The exact mechanisms by which MRF promotes early reestablishment of the blood supply network and functional myocardium regeneration in the whole infarct zone are not clear from these preliminary studies. MRF may directly affect vascular endothelial and myogenic cells by promoting their proliferation and/or regeneration. It may also indirectly stimulate healing of infarcted heart by increasing the production of other growth factors, such as VEGF, or by increasing the action of growth factors delivered to the wound by platelets or macrophages. Furthermore, MRF-mediated active neovascularization in an early stage of the infarction may allow effective homing and differentiating of circulating stem cells, and may increase the delivery rate of systemic growth factors and necessary components for effective healing to the infarcted area, enhancing removal of metabolic products or growth-inhibiting factors, or inducing alteration in growth-factor receptors (7, 9, 15, 17). According to the morphologic features and the location of the newly regenerating myocardium-like tissue, the newly regenerated myocardium may come from 2 sources. One source is a subpopulation of pre-existing cardiomyocytes that are not terminally differentiated and possess the ability to divide when given the appropriate signal (3–6), such as the normally sized Ki67 positively stained cardiac myocytes scattered along the edge of the infarct or other native progenitor cells, such as the recently described isl1+ cardioblasts, which are present in postnatal hearts (24). The other source is circulating stem cells, which may be attracted to the location of the infarct and may be induced to differentiate into cardiac myocytes or myocardium by MRF. It is also possible that inhibition of fibrosis would allow myocyte regeneration to occur. The possibility that an external biological agent can stimulate myogenesis and repair postinfarction comes from the recent work of Gnecchi et al. (25), who have demonstrated that an extract of Akt-modified mesenchymal stem cells can also promote myocardial repair through a paracrine action, presumably by stimulating resident cardiac stem cells, or possibly myocytes themselves, to divide. Direct application of MRF to the ischemic region of the heart may also increase survival potential of the viable myocytes at risk (the myocytes surrounding the infarct zone), which was confirmed by substantially decreased apoptotic nuclei in the infarct zone compared with those in control hearts (unpublished data). The smaller sizes of the infarcts in MRF-treated hearts were probably also the consequence of the increased survival and reduced apoptotic death of the cardiomyocytes at risk.

Whatever the mechanisms, our experiments provide proof that cardiac myocytes can divide, the infarction can be repaired by the newly regenerated myocardium, and the function of the infarcted heart can be substantially improved in the early stages after infarction and onwards, making myocardial regeneration a real possibility. Thus, MRF appears to have highly beneficial effects in promoting both early reestablishment of the impaired blood supply network and regeneration of functional myocardium, replacing the necrosed heart tissues in myocardial infarction.

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