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Hyaluronan Mixed Esters of Butyric and Retinoic Acid Drive Cardiac and Endothelial Fate in Term Placenta Human Mesenchymal Stem Cells and Enhance Cardiac Repair in Infarcted Rat Hearts*[S]

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We have developed a mixed ester of hyaluronan with butyric and retinoic acid (HBR) that acted as a novel cardiogenic/vasculogenic agent in human mesenchymal stem cells isolated from bone marrow, dental pulp, and fetal membranes of term placenta (FMhMSCs). HBR remarkably enhanced vascular endothelial growth factor (VEGF), KDR, and hepatocyte growth factor (HGF) gene expression and the secretion of the angiogenic, mitogenic, and antiapoptotic factors VEGF and HGF, priming stem cell differentiation into endothelial cells. HBR also increased the transcription of the cardiac lineage-promoting genes GATA-4 and Nkx-2.5 and the yield of cardiac marker-expressing cells. These responses were notably more pronounced in FMhMSCs. FMhMSC transplantation into infarcted rat hearts was associated with increased capillary density, normalization of left ventricular function, and significant decrease in scar tissue. Transplantation of HBR-preconditioned FMhMSCs further enhanced capillary density and the yield of human vWF-expressing cells, additionally decreasing the infarct size. Some engrafted, HBR-pretreated FMhMSCs were also positive for connexin 43 and cardiac troponin I. Thus, the beneficial effects of HBR-exposed FMhMSCs may be mediated by a large supply of angiogenic and antiapoptotic factors, and FMhMSC differentiation into vascular cells. These findings may contribute to further development in cell therapy of heart failure.

Loss of cardiomyocytes due to myocardial infarction or hereditary cardiomyopathies may represent causative factors in the progression toward heart failure. In mice, bone marrow-derived stem cells have been found to enhance functional recovery in infarcted hearts (1, 2), suggesting that delivery of these cells may contribute to the regeneration of myocardial tissue. These observations triggered the launch of numerous clinical studies to assess the effect of bone marrow cell injection into human infarcted hearts. However, the initial observations and the scientific underpinning of the human trials have been challenged by a number of studies using mouse bone marrow hematopoietic cells expressing LacZ or green fluorescent protein to enable detection in recipient hearts. In these studies little or no reporter activity could be seen after direct stem cell injection into the damaged heart or continuous cell transfusion into the bloodstream of infarcted mice (3, 4). These controversies prompt further investigations assessing whether cell populations retaining a therapeutic potential for damaged hearts may be identified in human bone marrow or alternative sources. In this regard, human mesenchymal stem cells (hMSCs)[2] of bone marrow (BMhMSCs) display ease of isolation, high expansion potential, genetic stability, and potential to enhance repair in many vital tissues (5). BMhMSCs differentiate into cardiomyocytes when cocultured with adult rat ventricular cardiomyocytes (6) or injected into healthy (7) or infarcted (8, 9) animal hearts, suggesting that BMhMSCs may afford effective cell therapy of heart failure. Moreover, hMSCs have been found to express vascular endothelial growth factor (10, 11) and a broad

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

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[2] The abbreviations used are: hMSC, human mesenchymal stem cell; BMhMSC, bone marrow hMSC; DPhMSC, dental pulp hMSC; FMhMSC, fetal membrane hMSC; HA, hyaluronan; BU, butyric acid; RA, retinoic acid; HBR, HA mixed esters with BU and RA; ES, embryonic stem; MHC, sarcomeric myosin heavy chain; vWF, von Willebrand factor; Cx43, connexin 43; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SH, Src homology; TRITC, tetramethylrhodamine isothiocyanate; TnI, troponin I; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; DS, degree of substitution; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LV, left ventricle.

spectrum of arteriogenic cytokines promoting *in vitro* and *in vivo* vasculogenesis through paracrine mechanisms (12, 13). These effects, which are referred to as trophic effects, have been recently proposed as a major contribution to the therapeutic potential of MSCs in experimental models of myocardial infarction (14, 15) and dilated cardiomyopathy (16).

Nevertheless, the use of bone marrow-derived cells is potentially associated with high degree of viral infection and significant decline in cell viability and differentiation with age. Besides bone marrow, the human dental pulp includes clonogenic, rapidly proliferating stem cells (DPhMSCs) sharing an immunophenotype similar to BMhMSCs (17). Fetal membranes may also represent tissues of particular interest for their role in preventing fetus rejection and their early embryologic origin, which may entail progenitor potential. Phenotypic and gene expression studies revealed mesenchymal stem cell-like profiles in both amnion and chorion cells of fetal membranes (FMhMSCs) (18, 19) that were positive for neuronal, pulmonary, adhesion, and migration markers (18). However, albeit hMSCs may encompass cells committable to cardiovascular lineages, it is well established that cardiac differentiation is an extremely low yield process even in embryonic stem (ES) cells. Hence, affording high-throughput of cardiogenesis in hMSCs isolated from bone marrow and alternative sources would have obvious therapeutic potential.

We have recently shown that a mixed ester of hyaluronan with butyric and retinoic acids (HBR) remarkably increased the yield of cardiomyocytes derived from mouse ES cells (20). Here, we investigated whether HBR may commit BMhMSCs, DPhMSCs, or FMhMSCs into cardiovascular lineages and/or enhance their potential as trophic mediators and whether, in the affirmative, transplantation of HBR-pretreated hMSCs into infarcted rat hearts may result in enhanced cardiovascular repair.

MATERIALS AND METHODS

Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from BioWhittaker Cambrex (Walkersville, MD). Culture flasks were from Falcon BD (Bedford, MA). SH2, SH3, and SH4 were kindly provided by Dr. Mark Pittenger (Osiris Therapeutics, Baltimore, MD). Anti-CD29 and anti-CD166 were from Ancell (Bayport, MN). Anti-CD14, anti-CD34, anti-CD44, and anti-CD45 were from BD Biosciences. The monoclonal anti-CD44 antibody A3D8 and the isotype-matched control antibody (IgG1) were from Sigma. Primary antibodies against von Willebrand factor (vWF) and connexin 43 (Cx43) were from Dako Cytomation (Glostrup, Denmark) and Zymed Laboratories Inc. (South San Francisco, CA), respectively. SuperScript II RT and Trizol reagent were from Invitrogen. Antibodies against human mitochondria were from Chemicon (Temecula, CA). Goat anti-mouse (TRITC)- and fluorescein isothiocyanate-conjugated antibodies were from ICN Cappel (Aurora, OH). SYBR Green fast start kit (Lightcycler® FastStart DNA Master^{PLUS} SYBR Green I) was from Roche Diagnostics. AmpliTaq was from Applied Biosystems (Foster City, CA). The MF20 antibody, raised against sarcomeric myosin heavy chain (MHC), was from Developmental Studies Hybridoma Bank (The University of Iowa). Monoclonal mouse anticardiac troponin I (TnI) (clone mAb20) was from Covance (Berkeley, CA). Enzyme immunoassays for human vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) were from R&D Systems Inc. (Minneapolis, MN). Ficoll-Histopaque, penicillin, streptomycin, amphotericin B, α-sarcomeric actinin primary antibody (clone EA.53), and all the other chemicals were from Sigma.

Synthesis of HBR—The glycoconjugate HBR is an ester between the hydroxyl groups of hyaluronan (HA) and the carboxyl groups of both butyric acid (BU) and retinoic acid (RA). The procedure for the synthesis and characterization of HBR and the related chemical structure are reported in detail elsewhere (20). The primary hydroxyl group in position 6 of the *N*-acetyl-D-glucosamine residues in the polysaccharide backbone is the most reactive toward esterification. The degree of substitution (DS) was considered as the number of the esterified OH groups for each repeating unit of hyaluronic acid (GlcNAc-GlcUA dimer). All the synthesized HBR exhibited a DS with BU (DS_{BU}) ranging between 0.05 and 1.5, whereas the DS with RA (DS_{RA}) was between 0.002 and 0.1. The DS_{BU}/DS_{RA} ratio was at least 6. The average molecular mass of HBR, referred to as the average molecular mass of sodium hyaluronate, was determined by high performance size-exclusion chromatography and ranged between 10,000 and 30,000 daltons (20). In the HBR used in the present study, DS_{BU} and DS_{RA} were 1.44 and 0.032, respectively.

Cell Isolation—According to the policy approved by the local ethical committee, all tissue samples were obtained after informed consent.

BMhMSCs—Bone marrow was collected from healthy volunteers in heparinized tubes, diluted 1:3 with PBS, and layered over a Ficoll-Histopaque gradient (1.077 g/ml). The low density mononuclear cells were washed twice in PBS, counted, and plated at $1 \times 10^6/cm^2$ in culture flasks in DMEM supplemented with 20% heat-inactivated FBS and antibiotics (200 units/ml penicillin, 100 μg/ml streptomycin) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 1 week the non-adherent cells were removed by replacing the medium supplemented by 10% FBS. When the cultures were near confluence (after 2–3 weeks), the cells were detached by treatment with 0.08% trypsin, EDTA (Sigma) and maintained and subcultured for up to 6–7 passages.

DPhMSCs—Vital human molars were obtained from adult subjects during a routine dental extraction. The tooth was immersed in physiological solution containing antibiotics to eliminate any contamination by the germs present in the oral cavity. Soon after extraction, the dental crown was fractured in several parts by means of pliers (bone forceps) under sterile conditions, and the pulp was uncovered. The tissue fragments were suspended in DMEM in the presence of 25% FBS, antibiotics (200 units/ml penicillin, 100 μg/ml streptomycin), and 2.5 μg/ml amphotericin B. This procedure was followed by incubation at 37 °C in a humidified atmosphere containing 5% CO_2, allowing cells to slip down from the explants and expand up to confluence. The cell layer was removed by enzymatic digestion (0.08% trypsin, EDTA) at 37 °C and further expanded in DMEM containing 10% FBS and antibiotics.

FMhMSCs—Term placenta obtained from caesarian sections were rapidly rinsed in PBS containing penicillin and streptomycin and used immediately. Pieces from fetal membranes were minced and digested for 10 min in DMEM with 0.25% trypsin, EDTA, 10 units/ml DNase I, and 0.1% collagenase. Tissues were pipetted vigorously up and down, avoiding foam for 5 min; larger pieces of tissue were allowed to settle under gravity for 5 min. Each supernatant was transferred to a fresh tube, neutralized with FBS, then spun at 1500 rpm for 10 min. Each pellet was resuspended in 5 ml of DMEM containing 20% FBS, 10 units/ml penicillin, and 100 μg/ml streptomycin. FMhMSCs were seeded into 25-cm^2 flasks and grown at 37 °C in 5% CO_2. Non-adherent cells were removed after 1 week, and medium (with 10% FBS) was changed subsequently every 4 days.

Flow Cytometry Analysis—The fibroblast-like cells obtained from bone marrow, dental pulp, and fetal membrane cultures at the same doubling passage were harvested by treatment with 0.08% trypsin, EDTA and incubated with 1 μg/10^6 cells of fluorescein isothiocyanate-conjugated antibodies for 40 min at 4 °C in the dark. The antibodies used were SH2, SH3, SH4, anti-CD166, anti-CD14, anti-CD34, anti-CD44, and anti-CD45. After washing, cells were analyzed on a flow cytometer (FACSCalibur, BD Biosciences) by collecting 10,000 events, and the data were analyzed using the Cell Quest Software (BD Biosciences). Flow cytometry analysis was also exploited to assess the percentage of BMhMSCs, DPhMSCs, and FMhMSCs expressing α-sarcomeric actinin. Cells were harvested by treatment with 0.08% trypsin, EDTA. After a fixation/permeabilization step, hMSCs were incubated with a primary antibody directed against α-sarcomeric actinin (1 μg/10^6 cells) for 1 h at 4 °C and with 1 μg of fluorescein isothiocyanate-conjugated secondary antibody for 1 h at 4 °C in the dark. After washing, cells were analyzed on a flow cytometer (FACSAria, BD Biosciences, San Jose, CA) by collecting 10,000 events, and the data were analyzed using the FACS Diva software (BD Biosciences).

Gene Expression—Total RNA was isolated by the Trizol reagent (Invitrogen) following the manufacturer's instructions. For RT-PCR, cDNA was synthesized in a 21-μl reaction volume with 2 μg of total RNA and SuperScript II RT. To assess GATA-4 and Nkx-2.5, 0.2 μg of cDNA were used for real-time RT-PCR performed with a Lightcycler system (Roche Diagnostics) and with the SYBR Green fast start kit (Lightcycler® Fast-Start DNA Master^{PLUS} SYBR Green I). Primers used in real-time RT-PCR were as follows: GATA-4 forward 5′-TGGCCTGTCATCTCACTACG-3′ and reverse 5′-TAGCCTTGTGGGGAGAGCTT-3′; Nkx-2.5 forward 5′-CAAGTGTGCGTCTGCCTTT-3′ and reverse 5′-GCGCACAGCTCTTTCTTTTC-3′; VEGF forward 5′-AGAAGGAGGAGGGCAGAATC-3′ and reverse 5′-ACACAGGATGGCTTGAAGATG-3′; KDR forward 5′-CTGCAAATTTGGAAACCTGTC-3′ and reverse 5′-GAGCTCTGGCTACTGGTGATG-3′; HGF forward 5′-ATTTGGCCATGAATTTGACCT-3′ and reverse 5′-ACTCCAGGGCTGACATTTGAT-3′; GAPDH forward 5′-CAGCCTCAAGATCATCAGCA-3′ and reverse 5′-TGTGGTCATGAGTCCTTCCA-3′. The reaction mixture (20 μl) contained 4 μl of Master SYBR Green I mix (TaqDNA polymerase, buffer, deoxynucleoside trisphosphate mix, $MgCl_2$, and SYBR Green I dye) and 0.5 μM concentrations of each primer to which 2 μl of cDNA was added. Data were normalized using GAPDH as an index of cDNA content after reverse transcription. Amplification included initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at 55–65 °C for 6–10 s, and extension at 72 °C for 10 s, performed at a temperature transition rate of 20 °C/s. Fluorescence was measured at the end of each extension step. Specificity of the product was determined by a melting curve analysis, conducted after completion of the cycling process with the aid of a temperature ramp (from 55–95 °C at 0.1 °C/s) and continuous fluorescence monitoring, confirmed by gel electrophoresis. Samples were run in duplicate, and the average threshold cycle (C_t) value was used for calculations. Relative quantification of mRNA expression was calculated with the comparative C_t method using the "Delta-delta method" for comparing relative expression results between treatments in real time PCR (21).

MyoD mRNA was determined by conventional RT-PCR using AmpliTaq under the following conditions; hot start for 10 min at 95 °C, 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s. Primers were: forward 5′-CGACGGCATGATGGACTAC-3′ and reverse 5′-AGGCAGTCTAGGCTCGACAC-3′.

Isolation of Stem Cell Nuclei and Nuclear Run-off Transcription Assay—Isolation of nuclei and assessment of nuclear purity were performed as detailed elsewhere (20, 22). Only freshly isolated nuclei were used in each experiment. Nuclear run-off experiments were carried out as previously described (20). Briefly, nuclear RNA was isolated by using guanidine thiocyanate and acid phenol extraction followed by purification on RNAMATRIX^{TM}. Equal counts of ^{32}P-labeled RNA (about 5·10^6 cpm) were then subjected to a solution hybridization RNase protection assay and were hybridized for 12 h at 55 °C in the presence of unlabeled antisense GATA-4, Nkx-2.5, HGF, and GAPDH mRNA. To generate these cRNA probes, cDNA fragments of human GATA-4 (237 bp), Nkx-2.5 (245 bp), HGF (177 bp), or GAPDH (574 bp) genes were inserted into a pCRII-TOPO vector. Transcription of plasmids linearized with BamHI generated antisense strands of Nkx-2.5 and GAPDH mRNA, whereas transcription of plasmids linearized with XbaI produced an antisense strand of GATA-4 and HGF mRNA. Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide non-denaturing gel. Autoradiographic exposure was for 48 h.

Assay for Angiogenic and Growth Factors—To investigate whether cell exposure to HBR may affect the production of angiogenic, antiapoptotic, and mitogenic factors, hMSCs were cultured for 14 days in the absence or presence of 2 mg/ml HBR. Then the levels of VEGF and HGF were measured in conditioned medium at defined time intervals by enzyme immunoassay (Quantikine human VEGF and HGF immunoassays, R&D Systems, Inc.).

Immunostaining—Immunofluorescence analyses were performed as previously described (22). Briefly, cells were washed with PBS and fixed with 4% formaldehyde at room temperature for 1 h. After fixation, cells were washed three times with PBS and permeabilized with 0.2% Triton X-100 at room tempera-

ture for 15 min. Aspecific antibody binding sites were blocked by incubating with 1% bovine serum albumin for 1 h at 37 °C. Then cells were labeled for 1 h at 37 °C in the presence of mouse monoclonal antibodies directed against MHC (MF20), α-sarcomeric actinin, Cx43, and vWF. Excess primary antibody was removed by 3 washes with PBS, and the cells were stained at 37 °C for 1 h with fluorescein-conjugated goat IgG. Microscopy was performed with a Bio-Rad Microradians confocal microscope.

Myocardial Infarction, Stem Cell Implantation, and Functional Evaluation of Myocardial Performance—Protocols were approved by an institutional review board. Myocardial infarction was produced in male Wistar rats at 2 months of age, according to previously described protocols (23). Briefly, under ether anesthesia, the thorax was opened through a thoracotomy performed at the fourth left intercostal space, the heart was rapidly exposed and the left anterior descending coronary artery was ligated with 6-0 mononylon thread during continuous subcutaneous electrocardiographic monitoring for ST changes and arrhythmias. After left anterior descending coronary artery occlusion, 1×10^6 culture-expanded hMSCs resuspended in 100 μl of sterile saline solution (PBS) were injected into the viable myocardium bordering the infarct using an insulin syringe with a 30-gauge needle. The ischemic zone was identified by the pale color of the myocardium. Infarcted rats that were injected with 100 μl of PBS, and sham-operated rats in which left anterior descending coronary artery was not occluded were used as controls. Transthoracic echocardiography was performed before thoracotomy and 28 days thereafter in sedated rats with an Esaote Megas equipped with a 7.5-MHz linear transducer. Short and long axis two-dimensional views and M-mode at the level of infarction were analyzed in real-time and recorded on a magnetooptic disk for off-line analysis. Anterior and posterior end-diastolic and end-systolic wall thicknesses and left ventricular (LV) internal dimensions were measured, as recommended by the American Society of Echocardiography (24, 25). Systolic ventricular function and global contractility were quantified such as fractional shortening (%) and ejection fraction (%). Fractional shortening was calculated from the composite LV internal, diastolic and LV internal, systolic dimensions from M-mode short-axis views. LV ejection fraction and cardiac output were calculated from a long-axis view.

Tissue Immunohistochemistry—Hearts were arrested in diastole and fixed as described (26) and then cut into 5-μm sections; the sections were used for immunohistochemical studies to localize the human transplanted cells using the antibodies against human mitochondria protein (27, 28). Cardiac lineage-committed cells were identified by antibodies directed against Cx43 and TnI. Endothelial lineage-committed cells were identified with an antibody directed against human vWF. Briefly, the sections were dewaxed with xylene and rehydrated through decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked by a 10-min incubation at room temperature with absolute methanol containing 3% H_2O_2. Antigen retrieval was performed by immersion of the slides in a jar containing citrate buffer (pH 6) and treatment at 120 °C, 1 atm, for 21 min. After cooling and washing, the samples were incubated overnight at 4 °C in a moist chamber with the antibodies against human mitochondria (1:100, Chemicon), vWF (1:50, Dako Cytomation), Cx43 (1:200, Zymed Laboratories Inc.), and TnI (1:400, Covance). Monoclonal antibody tissue labeling was revealed by using a non-biotin polymeric system (Super Sensitive Polymer HRP IHC Detection Systems; Biogenex, San Ramon, CA). After washing, the slides were incubated for 20 min at room temperature with Super Enhancer reagent, washed in PBS, and then incubated for 30 min at room temperature with super sensitive horseradish peroxidase polymeric complexes. Then the slides were incubated with diaminobenzidine tetrahydrochloride peroxidase substrate solutions, rinsed in distilled water, and then counterstained with hematoxyline. The samples were dehydrated, mounted, and viewed in a light microscope using the Image Pro Plus®. To demonstrate that the same transplanted human cells were also positive for Cx43 or TnI, we performed a double immunofluorescent staining. Formalin-fixed, paraffin-embedded tissue sections were dewaxed in xylene and then rehydrated in a graded series of ethanol. To recover tissue antigenicity, the slides were immersed in citrate buffer (pH 6) and treated at 120 °C, 1 atm, for 21 min. After cooling and washing, the samples were incubated in a blocking solution containing 2% rabbit serum and 1% bovine serum albumin in PBS for 30 min at room temperature to reduce the unspecific binding. Afterward, the tissue samples were first incubated with anti-human mitochondria at 4 °C overnight in a moist chamber, washed, and then labeled with polyclonal rabbit anti-mouse-fluorescein isothiocyanate (1:100, Dako Cytomation) for 45 min at 37 °C in the dark; then the slides were washed several times to remove any unbound secondary antibody, treated with the blocking solution, and incubated at 4 °C overnight with anti Cx43 (1:200) or anti TnI (1:400) monoclonal antibodies. After overnight incubation, the slides were washed and incubated for 45 min at 37 °C in the dark with goat anti-mouse (TRITC)-conjugated antibody (1:100, ICN Cappel). After washing, the slides were mounted, and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole using the kit "Pro long antifade reagent" (Molecular Probes, Milano, Italy). Negative controls were performed omitting the primary antibodies.

Data Analysis—The statistical analysis of the data were performed by using a one-way analysis of variance and the Bonferroni test assuming a p value less than 0.05 as the limit of significance.

RESULTS

Flow cytometry revealed that BMhMSCs, DPhMSCs, and FMhMSCs were positively stained with SH2 (supplemental Fig. 1), which identifies an epitope of endoglin (CD105), the transforming growth factor-β receptor III present on endothelial cells, erythroblasts, monocytes, and connective tissue stromal cells, facilitating the enrichment of stromal progenitors from bone marrow (29). Like BMhMSCs, DPhMSCs and FMhMSCs were recognized by the SH3 and SH4 antibodies (supplemental Fig. 1), which identify epitopes on culture-expanded stromal cells and bind CD73, a molecule involved in B-cell activation (30). Each group of hMSCs was positive for CD29 (supplemental Fig. 1), the β-subunit of an integrin family behaving as the

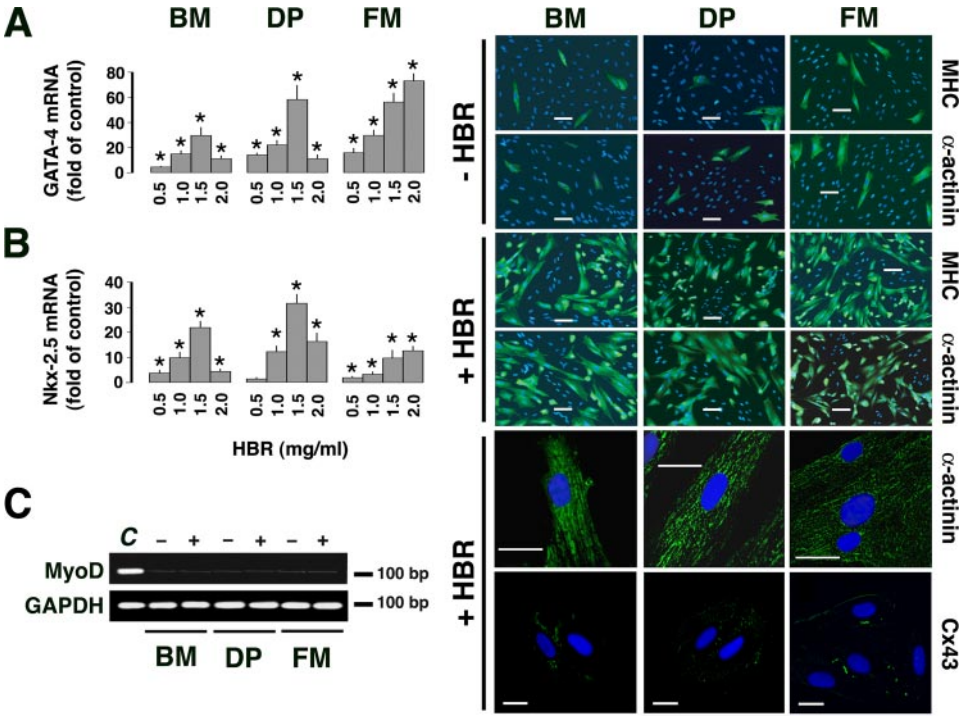


FIGURE 1. **HBR primes the expression of cardiac lineage-promoting transcripts without affecting skeletal myogenesis.** GATA-4 (*A*) and Nkx-2.5 (*B*) mRNA expression were measured by real-time RT-PCR. Cells were treated for 7 days in the absence or presence of HBR. The abundance of each mRNA in untreated cells was defined as 1, and the amounts of GATA-4 or Nkx-2.5 mRNA from HBR-treated cells were plotted relative to that value (mean ± S.E.; $n = 6$). *, significantly different from untreated cells (controls). MyoD mRNA expression (*C*) was assessed by conventional RT-PCR. Cells were treated for 7 days in the absence (−) or presence (+) of 1.5 mg/ml HBR (BMhMSCs (*BM*) and DPhMSCs (*DM*)) or 2 mg/ml HBR (FMhMSCs (*FM*)). Human skeletal muscle mRNA (*c*) was used as a positive control (ethidium bromide-stained agarose gels, representative of four separate experiments). For immunofluorescence analyses cells were cultured for 14 days in the absence (−*HBR*) or presence (+*HBR*) of 1.5 mg/ml HBR and were stained with monoclonal antibodies directed against sarcomeric MHC or α-sarcomeric actinin (*α-actinin*). *Scale bars* are 100 μm. Sarcomeric-like organization of α-sarcomeric actinin staining (*α-actinin*), and Cx43 expression were only evident in each cell population that had been cultured for 14 days in the presence of 1.5 mg/ml HBR. *Scale bars* are 20 μm. Nuclei are labeled by 4′,6-diamidino-2-phenylindole (*blue*).

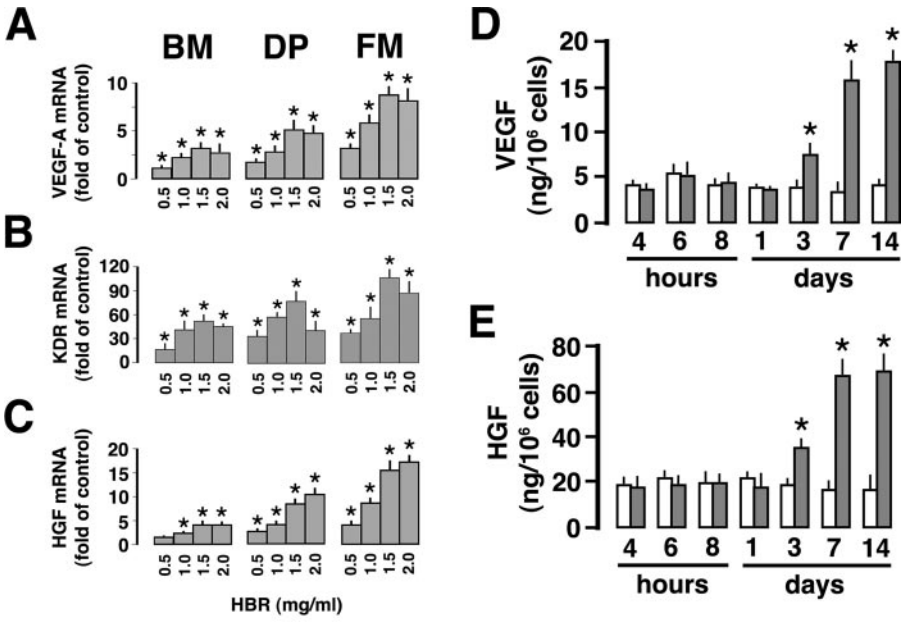
major receptor for extracellular matrix molecules. Cells also expressed CD166, an hMSC marker not found in hematopoietic precursors (31), and were uniformly positive for the CD44 hyaluronate receptor (supplemental Fig. 1). Conversely, antigen profiles were negative for the hematopoietic markers CD14 and CD34 and the leukocyte common antigen CD45 (supplemental Fig. 1). These observations and the comparative analysis of surface antigen expression (supplemental Table 1) indicate that both DPhMSCs and FMhMSCs can be regarded as alternative sources for hMSC-like elements.

In each hMSC population HBR dose-dependently increased the expression of GATA-4 and Nkx-2.5 mRNA, encoding a zinc finger and a homeodomain essential for cardiogenesis in different animal species (32, 33), including humans (34). Real-time RT-PCR revealed that the HBR effect peaked at 1.5 mg/ml in both BMhMSCs and DPhMSCs, reaching a maximum at 2.0 mg/ml in FMhMSCs (Fig. 1, *A* and *B*). In BMhMSCs exposed for 7 days to HBR, both GATA-4 and Nkx-2.5 mRNA were subjected to more than a 20-fold increase, as compared with untreated cells. The transcriptional response to HBR was even more remarkable in DPhMSCs and FMhMSCs (Fig. 1). HBR did not affect MyoD mRNA, a transcript involved in skeletal myogenesis (Fig. 1*C*). Both MHC and α-sarcomeric actinin were expressed in a limited number (1–5%) of untreated hMSCs (Fig. 1). Interestingly, in each hMSC population HBR remarkably increased the yield of cells expressing each cardiac marker protein (Fig. 1). Most of HBR-treated hMSCs connected with neighboring positively stained cells. Cardiac lineage commitment was further inferred from the observation that HBR exposure was associated with a sarcomeric-like organization of α-sarcomeric actinin staining (Fig. 1) and resulted in Cx43 expression along regions of intimate cell-to-cell contact (Fig. 1). Neither sarcomeric-like features nor Cx43 expression was detected in untreated cells (not shown). Flow cytometry analysis confirmed that treatment with HBR consistently increased the percentage of α-sarcomeric actinin-positive cells. After 14 days of exposure to 2 mg/ml HBR, 36.80 ± 1.50% of FMhMSCs expressed α-sarcomeric actinin, whereas the percentage of positive cells was 30.20 ± 0.85% in DPhMSCs or 16.12 ± 0.80% in BMhMSCs (mean ± S.E.; $n = 4$). Under the same experimental conditions, the percentages of Cx43-expressing cells in BMhMSCs, DPhMSCs, and FMhMSCs were 13.20 ± 1.40, 22.8 ± 1.65, and 33.0 ± 2.4% (mean ± S.E.; $n = 4$), respectively.

Real-time RT-PCR provided evidence that exposure to HBR dose-dependently increased the expression of VEGF-A mRNA (Fig. 2*A*) and that of the KDR gene (Fig. 2*B*), encoding a major VEGF receptor. HBR also remarkably enhanced the expression of HGF mRNA (Fig. 2*C*). These genes have been shown to play a pivotal role in both endothelial tissue formation (vasculogenesis) and sprouting of new blood vessels from pre-existing vessels (angiogenesis) (35–38). The HBR effect on gene expression was more pronounced in FMhMSCs than in BMhMSCs or DPhMSCs.

Each cell population secreted consistent amounts of angiogenic and antiapoptotic factors, including VEGF and HGF. Similar to mRNA expression, the relative protein levels assessed during a 14-day period of culture were higher in FMhMSCs (Fig. 2, *D* and *E*) than in BMhMSCs or DPhMSCs (data not shown). In FMhMSCs, the concentration of each growth factor was significantly higher in the supernatant of HBR-treated cells than in the culture medium from untreated controls (Fig. 2, *D* and *E*). Time-course analyses revealed that the stimulatory effect was evident after 3 days and progressively increased persisting throughout an overall period of 14 days (Fig. 2, *D* and *E*).

Undifferentiated hMSCs showed no appreciable staining for vWF, a marker for endothelial specification, but after 14



% of vWF-expressing cells

	BMhMSCs	DPhMSCs	FMhMSCs
- HBR	N.D.	N.D.	N.D.
+ HBR	18.0 ± 2.1	26.2 ± 1.5	41.5 ± 3.7
+ VEGF	55.6 ± 6.0	66.0 ± 7.3	70.0 ± 8.0

FIGURE 2. **HBR induces a gene program of angiogenesis and enhances the secretion of angiogenic, antiapoptotic, and mitogenic factors, leading to endothelial lineage commitment.** VEGF-A (*A*), KDR (*B*), and HGF (*C*) gene expression were assessed by real-time RT-PCR. Cells were cultured for 7 days in the absence or presence of HBR. The abundance of each mRNA in untreated cells was defined as 1, and the amounts of VEGF-A, KDR, or HGF mRNA from HBR-treated cells were plotted relative to that value (mean ± S.E.; $n = 6$). *, significantly different from untreated cells (controls). *D* and *E*, time-course analysis of VEGF and HGF, respectively, released by FMhMSCs cultured in the absence (*white bar*) or presence (*gray bar*) of 2 mg/ml HBR (mean ± S.E.; $n = 6$). *, significantly different from untreated cells. *F*, immunofluorescence analysis of vWF expression was investigated in BMhMSCs (*BM*), DPhMSCs (*DP*), and FMhMSCs (*FM*) cultured for 14 days in the presence of 1.5 mg/ml HBR (*scale bars* are 20 μm). No appreciable staining for vWF was observed in cells cultured without HBR. The figure reports the percentage (mean ± S.E.; $n = 4$) of HBR-treated hMSCs exhibiting the typical granular patterning of vWF staining. For comparison, the effect of VEGF (50 ng/ml for 14 days) on vWF expression is also reported. *N.D.*, not determined.

days of HBR treatment vWF expression was clearly evident, and Weibel-Palade bodies were also visible (Fig. 2*F*). The percentage of vWF-expressing cells was higher in FMhMSCs as compared with DPhMSCs or BMhMSCs (Fig. 2*F*). Similar percentages of both α-sarcomeric actinin- and vWF-positive cells were observed when BMhMSCs, DPhMSCs, or FMhMSCs were treated for 14 days with HBR and then cultured in the absence of HBR for additional 14 days (supplemental Fig. 2).

We investigated whether CD44, a hyaluronan binding molecule highly expressed in BMhMSCs and DPhMSCs as well as FMhMSCs, may be involved in the differentiating response to HBR. Cell culture in the presence of a CD44-specific blocking monoclonal antibody (A3D8) resulted in the complete suppression of the ability of HBR to commit FMhMSCs into both cardiac and endothelial lineages (supplemental Fig. 3). Results from competition experiments indicated that the cardiogenic action of HBR was also dose-dependently counteracted by HA (supplemental Fig. 3). To further dissect the hMSC response to HBR, nuclear run-off experiments were designed to assess whether HBR may have affected the rate of transcription of cardiogenic/angiogenic genes and whether, in the affirmative, it may have acted as a unit or after hydrolysis of hyaluronan-grafted moieties. Supplemental Fig. 4 shows that nuclei isolated from FMhMSCs that had been cultured for 7 days with HBR exhibited a consistent increase in the transcription rate of GATA-4, Nkx-2.5, and HGF genes as compared with nuclei isolated from untreated cells. In separate experiments nuclei were isolated from untreated cells and subsequently incubated with HBR or exposed to HA, BU, or RA administered alone or in combination. Although nuclear exposure to HBR or HA failed to trigger a transcriptional response, the incubation with BU or RA enhanced GATA-4, Nkx-2.5, and HGF gene transcription (supplemental Fig. 4). The transcription rate of these genes was further enhanced when nuclei were exposed to a combination of BU and RA (supplemental Fig. 4).

We next investigated whether hMSC treatment with HBR *in vitro* before cell delivery *in vivo* may represent a novel strategy for the rescue of damaged hearts. To this end FMhMSCs, which exhibited the most pronounced commitment to cardiovascular lineages and enhanced release of trophic factors in response to HBR, were cultured for 14 days in the absence or presence of this mixed ester and subsequently transplanted into infarcted rat hearts. Four weeks after myocardial infarction, the non-transplanted group showed indices of cardiac failure (Fig. 3). In marked contrast, echocardiographic analysis of infarcted rats receiving FMhMSCs non-exposed to HBR revealed a recovery to essentially normal indices of cardiac function (Fig. 3). A similar normalization was observed after transplantation of HBR-treated cells (Fig. 3). Injection of pretreated cells resulted in significantly higher values of left ventricle to body weight ratio and diastolic thickness as compared with untreated FMhMSCs (Fig. 3). No significant recovery of cardiac function was observed 1 or 2 weeks after the injection of untreated or HBR-exposed FMhMSCs (not shown).

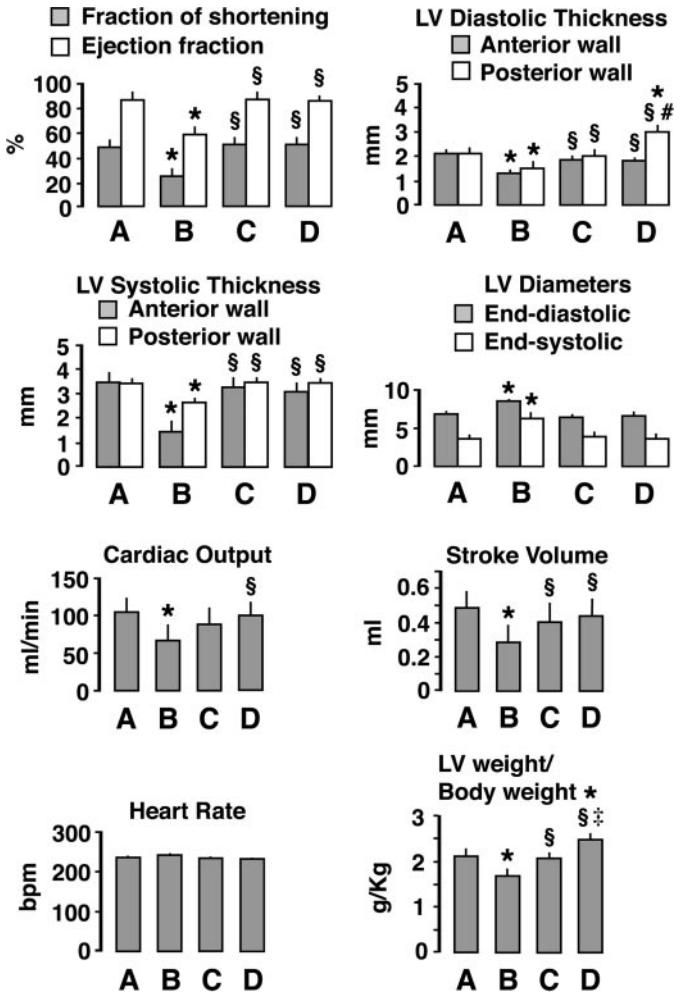


FIGURE 3. **Assessment of cardiac function in infarcted rat hearts transplanted with FMhMSCs cultured in the absence or presence of HBR.** Transthoracic echocardiography was performed in normal sham-operated rats without left anterior descending coronary artery occlusion (*A*) and 28 days after myocardial infarction in animals receiving saline as a placebo (*B*) or FMhMSCs previously cultured for 14 days in the absence (*C*) or presence (*D*) of 1.5 mg/ml HBR. *, significantly different from *A*; §, significantly different from *B*; # in *D*, posterior wall thickness is significantly different from anterior wall thickness; ‡, significantly different from *C*. Data in each group are the mean ± S.E. from six individual animals (one-way analysis of variance and Bonferroni test, assuming a *p* value less than 0.05 as the limit of significance). *bpm*, beats per minute.

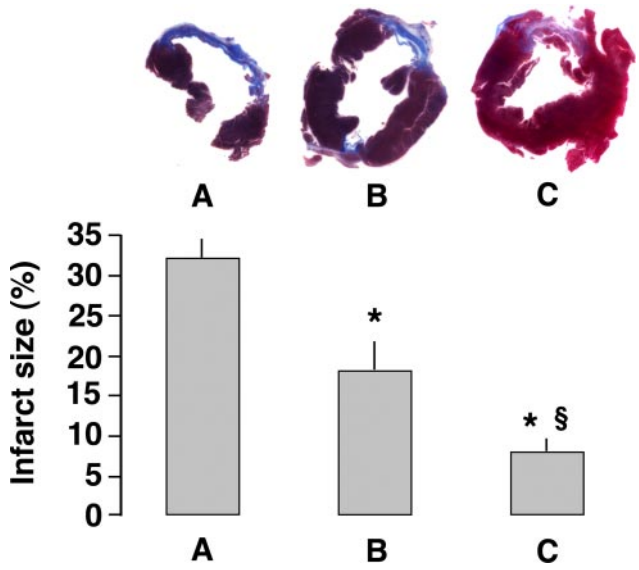


FIGURE 4. **Impact of FMhMSC injection on infarct size.** *Upper panel*, representative example of scar formation 4 weeks after myocardial infarction in rats treated with saline as a placebo (*A*) or FMhMSCs previously cultured for 14 days in the absence (*B*) or presence (*C*) of 1.5 mg/ml HBR. Macroscopic infarct size was assessed in each group by nitro blue tetrazolium staining. 3-mm-thick transverse slices cut through the short axis of both ventricles at midseptal level were processed for light microscopy, stained with picro-Mallory trichrome (*upper panel*), and digitalized through a video camera connected with the microscope. *Lower panel*, infarct sizes (*blue-stained* areas) were quantified by using image Pro Plus Version 8 (Media Cybernetics). *, significantly different from *A*; §, significantly different from *B*. Data in each group are the mean ± S.E. from six individual animals (one-way analysis of variance and Bonferroni test, assuming a *p* value less than 0.05 as the limit of significance).

Gross pathologic examination of ischemic myocardium after nitro blue tetrazolium staining revealed that FMhMSC transplantation substantially decreased the percentage of left ventricle occupied by fibrosis. Further remarkable reduction in scar tissue formation was observed after injection of HBR-treated FMhMSCs. Interestingly, in this group the unstained tissue (possibly reflecting infarct scarring) was only confined to a limited area in the subendocardial zone, which also exhibited regions of viable tissue. Picro-Mallory trichrome staining and quantitative analyses showed that the infarct area was significantly lower in animals receiving HBR-treated FMhMSCs than in the group transplanted with untreated cells (Fig. 4).

To assess whether injected FMhMSCs may differentiate into endothelial cells, sections from infarct regions of each experimental group were incubated in the presence of human-specific anti-vWF antibody. No staining was detected in samples from placebo-treated animals (Fig. 5). On the contrary, vWF-positive cells were observed in cardiac samples from infarcted animals injected with both untreated or HBR-exposed cells (Fig. 5). However, the number of vWF-expressing FMhMSCs was remarkably higher in sections from hearts receiving HBR-pretreated cells than in samples with untreated FMhMSCs (Fig. 5). Moreover, a consistent number of capillaries containing vWF-positive FMhMSCs was observed in samples harvested from hearts injected with HBR-pretreated cells, whereas no evidence for organization of vWF-stained FMhMSCs into capillary structures was found in sections from hearts transplanted with untreated cells (Fig. 5).

Fig. 5 also shows that the density of capillary vessels lacking cells positively stained with anti-human vWF was significantly increased at the infarct border zone of animals injected with untreated FMhMSCs as compared with infarcted tissue receiving saline as a placebo. The density of capillaries negative for anti-human vWF staining was further augmented after transplantation of HBR-pretreated FMhMSCs (Fig. 5).

Immunofluorescence analysis also provided evidence that engrafted HBR-pretreated cells concomitantly expressed Cx43 or cardiac TnI with a human-specific anti-mitochondria antibody (Fig. 6) and that the number of positive FMhMSCs exceeded that observed in hearts transplanted with untreated cells. However, this low yield of cardiac marker-expressing cells appeared to lack complete myogenic differentiation with mature sarcomeric organization.

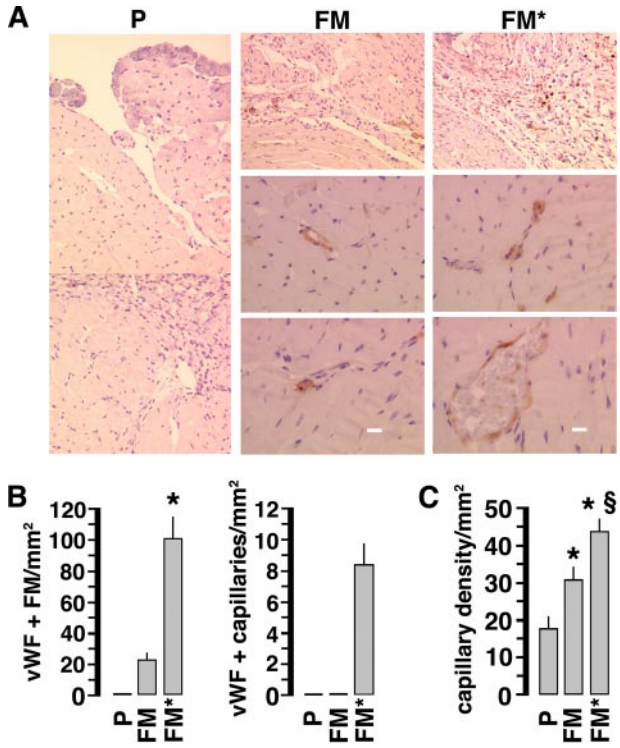


FIGURE 5. **Capillary density and development of transplanted FMhMSCs into endothelial cells at the infarcted region.** Sections were obtained from border zone 4 weeks after myocardial infarction. *A*, expression of vWF was assessed by immunohistochemistry with an anti-human vWF antibody. Although no staining was detected in samples from placebo-treated animals (*P*), a consistent number of vWF-positive cells was observed in the tissue harvested from animals injected with FMhMSCs previously cultured for 14 days in the absence (*FM*) or presence (*FM**) of 1.5 mg/ml HBR. Organization into erythrocyte-containing capillary-like structures (*lower right*) was observed in samples from hearts transplanted with HBR-treated cells. Light microscopy, 20× (original magnification). *Scale bars* are 20 μm. *B*, quantitative analysis of vWF-expressing cells. The number of FMhMSCs positively stained with anti-human vWF antibody and the density of capillary vessels containing vWF-positive FMhMSCs were counted in randomly selected five-high-power fields of each section and averaged. *, significantly different from FM. *C*, density of capillary vessels lacking positive staining with anti-human vWF antibody at the infarct border zone. *, significantly different from placebo-treated animals; §, significantly different from FM. Data are the mean ± S.E. from sections of six individual animals.

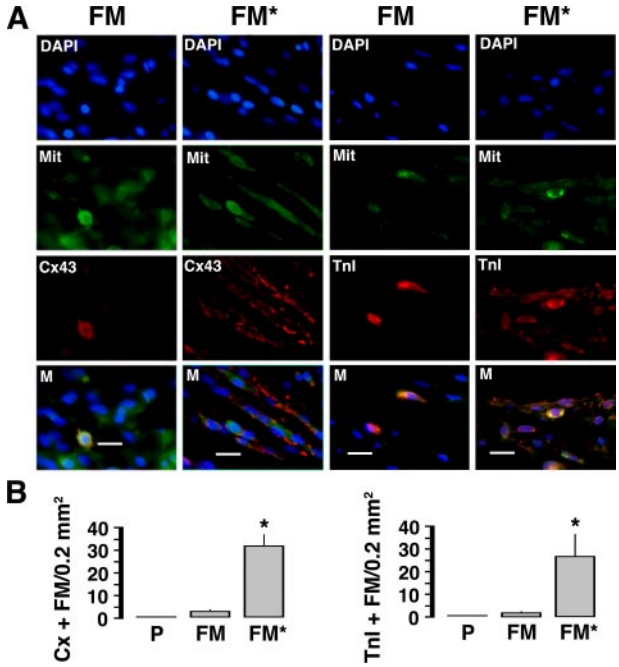


FIGURE 6. **Development of transplanted FMhMSCs into Cx43- and cardiac troponin I-positive cells.** *A*, 4 weeks after myocardial infarction, Cx43 or TnI expression were investigated in samples from rats injected with FMhMSCs previously cultured for 14 days in the absence (*FM*) or presence (*FM**) of 1.5 mg/ml HBR. Sections were double-stained with human-specific anti-mitochondrial antibody (*Mit*, green fluorescence), and antibody was directed against Cx43 or cardiac TnI (*red fluorescence*). Double-labeled cells are shown in merged (*M*) images. Nuclei were labeled by 4′,6-diamidino-2-phenylindole (*DAPI*, *blue signal*). Light microscopy, 100× (original magnification). *Scale bars* are 20 μm. *B*, quantitative analysis of the number of Cx43- or TnI-expressing FMhMSCs. Positive cells were counted in randomly selected five-high-power fields of each section and averaged. *, significantly different from FM. Data are the mean ± S.E. from sections of six individual animals.

DISCUSSION

During myocardial infarction, cardiomyocyte loss is associated with hibernation of underperfused heart cells. Pluripotent hMSCs differentiate into a variety of cells, including cardiomyocytes and endothelial cells and may hold promises for both replacing the lost heart cells (cardiomyogenesis) and restoring perfusion of the hibernating cells (vasculogenesis or angiogenesis). It is also becoming increasingly evident that hMSCs may secrete large amounts of angiogenic, antiapoptotic, and mitogenic factors and that the improvement of cardiac function and reduction in scar formation observed in animal models of myocardial infarction after hMSC transplantation may be mediated by the supply of these trophic factors (12–14). In this regard bone marrow-derived mesenchymal stem cells overexpressing Akt have been shown to inhibit ventricular remodeling and restore cardiac function mainly through paracrine mechanisms involving an increase in the expression of VEGF, fibroblast growth factor-2, and HGF genes and enhanced secretion of their related products (39, 40). Consonant with these observations, monolayered adipose tissue-derived mesenchymal stem cells repaired scarred myocardium in infarcted rat hearts, acting as trophic mediators for paracrine angiogenic pathways (41).

Despite these attractive properties of hMSCs, their potential as a cell therapeutic for cardiac repair is limited by age and disease states affecting the collection of sufficient healthy autologous bone marrow-derived cells for transplantation. A potential use of stem cells in cardiac repair is also hampered by the low yield of cardiac lineage commitment in both adult and ES cells. Hence, the identification of pluripotent cells transplantable in an allogenic setting and the development of differentiating molecules enhancing both stem cell commitment to cardiovascular lineages and secretion of trophic factors may prove rewarding in sight of cardiac cell therapy.

Here we show that DPhMSCs and FMhMSCs, like BMhMSCs, exhibit a low yield commitment to a myocardial-like lineage *in vitro*. HBR increased the expression of cardiac lineage-promoting genes without affecting MyoD mRNA. This observation seems to exclude a generalized activation of repressed genes. The ability of HBR to enhance cardiogenesis *in vitro* is also inferred by the observation that the mixed ester enhanced the amount of cells expressing MHC and α-sarcomeric actinin and by the finding that Cx43-positive cells only developed from HBR-treated hMSCs. Interestingly, exposure to HBR remarkably enhanced the transcription of genes tightly involved in

vasculogenesis and angiogenesis and led to the development of vWF-expressing endothelial cells. The attainment of cardiomyocyte- and endothelial-related features was a long-lasting differentiating response, as suggested by the prolonged persistence of markers for both lineages in high percentages of hMSCs even after HBR withdrawal. The present finding that FMhMSCs secreted large amounts of VEGF and HGF and that HBR consistently increased the secretion of each trophic mediator is worthy of consideration. In fact, besides VEGF, HGF has not only angiogenic but also cardioprotective effects, including antiapoptotic, mitogenic, and antifibrotic activities (42, 43). HGF gene transfer into the myocardium improved myocardial function and geometry (44), in particular because of antifibrotic effects through inhibition of transforming growth factor-β expression. Therefore, taking the present findings together, HBR appears to encompass both differentiating features for hMSC commitment to cardiovascular lineages and the potential to afford growth factor-mediated paracrine regulation.

Akin to these considerations and taking into account that *in vitro* responses to HBR were considerably more accentuated in FMhMSCs than in BMhMSCs or DPhMSCs, we designed separate *in vivo* experiments to assess whether FMhMSCs may be used to repair infarcted rat hearts and whether their rescuing potential may be enhanced by cell pretreatment with HBR. Notably, untreated FMhMSCs led to normalization of left ventricular function and remarkable reduction in infarct size and scar tissue. The observation that these cells exhibited a very low yield of myocardial commitment and failed to differentiate into endothelial cells spontaneously *in vitro* prompt the hypothesis that FMhMSCs may have acted as trophic mediators to rescue the infarcted myocardium. Such a hypothesis is supported by the ability of FMhMSCs to spontaneously secrete large amounts of angiogenic, antiapoptotic, and mitogenic factors *in vitro*. The possibility that these factors may have protected the infarcted myocardium in a paracrine fashion is inferred by the finding that FMhMSC transplantation was followed by a significant increase in the density of capillary vessels lacking positive staining against human vWF, likely reflecting an angiogenic response of the recipient tissue to the injected FMhMSCs. Although cardiac performance was similarly recovered after injection of untreated or HBR-exposed cells, the transplantation of HBR-pretreated FMhMSCs led to increased left ventricle to body weight ratio and diastolic thickness as well as further reduction in infarct size as compared with untreated cells. The finding that *in vitro* exposure of FMhMSCs to HBR enhanced the secretion of VEGF and HGF suggests that the beneficial effects exerted *in vivo* by HBR-preconditioned cells may be due at least in part to enhanced cardioprotection through growth factor-mediated paracrine patterning. In this regard the injection of HBR-treated cells was followed by a significant increase in density of capillaries negative for anti-human vWF compared with the capillary density observed in the tissue receiving untreated cells. Enhanced *in vivo* release of VEGF and HGF by HBR-pretreated FMhMSCs may have contributed both to the observed angiogenic response and the reduction of infarct size and scar formation through antiapoptic and antifibrotic activities.

The current data also show that in the hearts injected with HBR-exposed cells, the number of FMhMSCs positively stained with a human-specific anti-vWF antibody remarkably exceeded the number of vWF-positive cells detected in samples from the untreated group. It is worthy to note that a consistent organization of vWF-positive FMhMSCs into capillary structures was only observed in hearts transplanted with HBR-treated cells. Hence, the endothelial lineage commitment primed by HBR *in vitro* was retained within the transplanted cells in the infarcted myocardium, suggesting that HBR-treated cells may also contribute to neovascularization and heart rescue through their ability to generate capillary-like structures. The relative contribution of growth factor-mediated paracrine mechanisms and differentiation into vascular lineages by HBR-treated FMhMSCs within the infarcted heart remains to be established and is the subject for further investigations. Some of the transplanted cells that had been pretreated with HBR also exhibited a colocalization of Cx43 and cardiac TnI with a human mitochondrial protein. Nevertheless, these cells lacked mature sarcomeric organization, and their role in restoring the cardiac performance within the infarcted tissue remains questionable.

The molecular dissection of mechanisms underlying HBR-mediated responses *in vitro* and *in vivo* remains to be fully elucidated. The finding that CD44 was expressed in more than 95% of BMhMSCs, DPhMSCs, and FMhMSCs and that cell exposure to a CD44-specific blocking monoclonal antibody abrogated HBR-mediated cardiac and endothelial commitment indicate that engagement of the hyaluronan CD44 receptor may represent a major initial step in the signaling cascade triggered by the mixed ester. The possibility that HBR uses a hyaluronan-related uptake system for cell entry is further suggested by results of competition experiments showing that HA dose-dependently decreased the yield of cells committed to both cardiac and endothelial lineages in the presence of the glycoconjugate. Nuclear run-off experiments indicate that the action of HBR was mediated at the transcriptional level. However, the transcription rate of GATA-4, Nkx-2.5, and HGF was unaffected after a direct exposure of nuclei isolated from undifferentiated cells to HBR. On the contrary, the transcription of these cardiogenic/angiogenic genes was increased by nuclear exposure to BU or RA and was additively enhanced by a mixture of the two. These results prompt the hypothesis that, at least at nuclear level, HBR may have acted after the hydrolysis of its grafted moieties.

In conclusion, we show the possibility of using a novel glycoconjugate to enhance both vascular differentiation and the secretion of angiogenic, antiapoptotic, and mitogenic factors from FMhMSCs. These cells do not induce allogeneic or xenogeneic lymphocyte proliferation and actively suppress lymphocyte responsiveness (18). Moreover, FMhMSC transplantation in neonatal swine and rats resulted in human microchimerism in various organs and tissues (18), suggesting that FMhMSCs may be administered as allogeneic grafts without the need of bone marrow harvests on recipient subjects. The current findings may contribute to further development in cell therapy for heart failure.

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