Title: Mesenchymal Stem Cells Utilize Integrin β1 not CXCR4 for Myocardial

Migration and Engraftment

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ABSTRACT

Recent evidence have demonstrated the importance of bone marrow derived mesenchymal stem cells (BM-MSCs) in the repair of damaged myocardium. The molecular mechanisms of engraftment and migration of BM-MSC in the ischemic myocardium are unknown. In this study, we developed a functional genomics approach towards the identification of mediators of engraftment and migration of BM-MSC within the ischemic myocardium. Our strategy involves microarray profiling (>22000 probes) of ischemic hearts, complemented by RT-PCR and FACS of corresponding adhesion molecule and cytokine receptors in BM-MSCs to focus on the co-expressed pairs only. Our data revealed 9 complementary adhesion molecules and cytokine receptors, including integrin β1, integrin α4, and CXCR4. To examine their functional contributions, we first blocked selectively these receptors by pre-incubation of BM-MSCs with specific neutralizing antibodies, then administered these cells intramyocardially. A significant reduction in the total number of BM-MSC in the infarcted myocardium was observed after integrin β1 blockade, but not integrin α4 or CXCR4 blockade. The latter observation is distinctively different from that reported for hematopoietic stem cells (HSC). Thus, our data show that BM-MSCs utilize a different pathway from HSCs for intramyocardial trafficking and engraftment.
INTRODUCTION

Cardiac repair and remodeling following ischemic injury involves myocyte hypertrophy, collagen deposition and possibly ventricular dilatation (Sutton and Sharpe, 2000). Recent provocative data suggest that stem cells, either resident in the heart or originating from the bone marrow, may play an important role in the repair and regeneration of the injured myocardium (Anversa and Nadal-Ginard, 2002). We and others have shown that intramyocardial transplantation of bone marrow derived stem cells (BMSC) can promote cardiac repair with resulting functional improvement and reduced infarct size (Kocher et al., 2001; Mangi et al., 2003; Amado et al., 2005). In addition to direct transplantation, mobilization of BMSC with cytokines such as granulocyte colony stimulating factor (G-CSF) and stem cell factor has been reported to enhance myocardial repair and improve cardiac function (Anversa and Nadal-Ginard, 2002; Askari et al., 2003). However, in a recent trial, the subcutaneous administration of G-CSF following acute myocardial infarction (MI) did not lead to further improvement in ventricular function than conventional treatment (Ripa et al., 2006). These controversial findings suggest the needs to understand the molecular mechanisms involved with stem cell migration and engraftment into the infarcted myocardium.

It has been reported that hematopoietic stem cells (HSC) migrate in response to stromal derived factor (SDF)-1alpha, the ligand for the CXC chemokine receptor 4 (CXCR4) (Wright et al., 2002), and the upregulation of SDF-1 in the ischemic myocardium mediates homing of HSC via its direct interaction of CXCR4 on the stem cells (Askari et al., 2003; Abbott et al., 2004). However, much controversy exists over the ability of HSC to transdifferentiate into cardiac myocytes (Balsam et al., 2004; Nygren et al., 2004). Recent data suggest that that mesenchymal stem cells (MSC) may be
mobilized from BM, home and generate cardiac myocytes (Mangi et al., 2003; Kawada et al., 2004). The molecular mediators involved with MSC migration and engraftment are unknown. In this study, we developed a functional genomics strategy to identify the mediators of bone marrow derived mesenchymal stem cells (BM-MSC) intramyocardial migration, and engraftment in the infarcted tissue. We focus our investigation on the events that occur within the heart that mediate the movement and engraftment of MSC from the non-ischemic to the ischemic regions. Our approach is based on the hypothesis that specific chemoattractant molecules and adhesion molecules in the ischemic myocardium are up-regulated and interact specifically with corresponding receptors on BM-MSC to induce migration and engraftment. Accordingly, we generated expression profiles of MI heart to identify the chemokines, cytokines and adhesion molecules that are upregulated in myocardial ischemic injury and narrow our study to those whose corresponding receptors and ligands are expressed in BM-MSC (Figure 1A). We then employed a functional approach to define the contribution of selected candidate molecules by evaluating the blocking effect of specific monoclonal antibodies on allogenic BM-MSC transplantation into mouse heart in vivo. Our data showed that distinctly different from that reported for HSC’s, integrin β1, but not integrin α4 or CXCR4, is important for MSC migration and engraftment in the infarcted myocardium.
MATERIALS AND METHODS

Expression Profiling of Acute Ischemic Injury

BalbC mice (female, 8-10 weeks old, Harlan) were used with approval of the Harvard Medical Area Standing Committee on Animals. Myocardial infarctions were created by permanent ligation of left anterior descending (LAD) coronary artery as previously described (Min et al., 2002). Hearts were removed after 1, 8 and 24 hours and examined (n = 3 at each time point). The infarcted zone and bordering regions were carefully dissected away from the normal myocardium and used for RNA extraction with Trizol Reagent (Invitrogen). Corresponding regions from sham-operated littermates were used as controls (n = 3 per time point). Total RNA was used for hybridization to Affymetrix Expression Set MOE430 oligonucleotide arrays according to the manufacturer’s protocol. Affymetrix Microarray Suite v5.0 was used to determine genes that were differentially expressed according to detection, change and signal log ratio (>0.6 SLR) parameters. Genes that met the criteria for such data analysis metrics were further studied using Real-Time PCR to verify differential expression. Primer sets for intercellular adhesion molecule-1 (ICAM-1), interleukin-1B (IL-1B), IL-6, endothelial selectin (Sele), tissue inhibitor of metalloproteinases-1 (TIMP-1), tumor necrosis factor receptor II (TNFRII), and vascular cell adhesion molecule (VCAM-1) were obtained from R&D Systems. The other primer sequences are: SDF-1 forward GTCTCTTTGCTGCTGACTGCT, reverse AGATGCTTGACGTTGGCTCTC; CCL6 forward GGCTGGCCTCATACAAGAAA, reverse TCCCCTCTCTTCTGCTTGATAAAAGA; CCL7 forward GTGTCCCTGGGAAGCTGTTA, reverse AGAAAGAACAGCGGTGAGGA; CXCL2 forward AGTGAACTGCGCTGTCAATG, reverse TCCAGGTCAGTTAGCCTTG.
fibronectin-1 forward AATCCAGTCCACAGCCATT, reverse
TAGTGGCCACCACATGAGTCTC; laminin-1 forward AGTGGAAGGAATGGTTCACG,
reverse TGCCAGTAGCCAGGAAGACT; VEGF-1α forward
AGAGCAACATCACCATGCAG, reverse CAGTGAACGCTCCAGGATTT; CXCR4
forward TGGAAACCGATCAGTGTGAGT, reverse GACCAGGATCACCACCATCCAT;
IL6 receptor-α forward ATGCTCCCTGAATGATCACC, reverse
TTGTCAACCCTCCAGGATCTC; IL6 signal transducer forward
CATGCTTTTCAGGCTTTCTC; reverse CCATACTGAAGTGCCATGC; CCR2
forward TGGCTGTGTTTGCCTCTTA, reverse CGAAACAGGGGTGGAGAAT;
CXCR2 forward TGCCCTCTACCCATCAGAC, reverse
GACCTTTGGAAGAGCAGTCG; E-selectin ligand-1 forward
AGGCGCTTCAGACACTGATT, reverse CAACTTCCAATCCCCGAGAGA; Integrin-
β1 forward CTGATTGGCTGGAGAATGT, reverse
TGAGCAATTGAAGGATAATCATAG; Integrin β2 forward
AGTTTCGACTACCCATCCGTG; reverse GTTGCTGGAGTGCAGACAC; Integrin-α1
forward TTGAGGGCACAACACAGACAG, reverse TCATCCAGGCCACAGTGTA;
Integrin-αL forward TTGAGGGCACAACACAGACAG, reverse
TCATCCAGGCCACAGTGTA; Integrin-αM forward
CTTCTGGTCACAGCCCTAGC, reverse AC ACTGGT GAGGCGACCTG; Integrin-α4
forward TCTATCGTGA CTTTGAGGAGTGCAGAC; Integrin-α5 forward AGCTGGATGTGTATGGGAG; reverse
CAGCTCAGGGCTGGAGAAGTT; Integrin-α6 forward
ATCACGCGCTTCTGAGAGA, reverse GGATGCCTTCTTGAATTTGA; Integrin-α8
forward CTCACCTTGTCAAAACAGCA, reverse CATCATAGGAAGCTGGAGCC;
Integrin-α9 forward AGAGGAACACTGGTGTCATGG, reverse GGATGGATGAGAAGTGGC.

**Bone marrow mesenchymal stem cells**

BM-MSCs were isolated from the bone marrow of Balb/C mice as previously described (Peister et al., 2004). Briefly, nucleated cells were isolated from the bone marrow with a density gradient (Ficoll-Paque; Pharmacia) and cultured in a growth medium consisting of alpha minimal essential medium (α-MEM; GIBCO/BRL) supplemented with 17% fetal bovine serum (FBS) on uncoated polystyrene dishes at 37°C with 5% CO₂ for 24 hours. Then the culture was washed with PBS to remove the unattached cells. The attached cells were maintained in the growth medium to reach 80% confluence. The cells that were lifted by incubating with trypsin/EDTA for 2 minutes at 37°C were collected and the cells that did not detach in 2 minutes were discarded. The collected cells were expanded by seeding into new plates at a density of 50 cells/cm². When reaching 80% confluence, only the cells that were lifted by incubating with trypsin/EDTA for 2 minutes at 37°C were collected. Cells in passage 4-5 were used for the study. FACS analysis of the cells indicated that they were negative for hematopoietic lineage markers CD45, CD19 (see Figure 3A), CD3, CD14 and Flk-1 (data not shown), and positive for Sca-1, CD105 and CD29 (see Figure 3A). When cultured in induction media (Peister et al., 2004), the cells differentiated into adipocytes, osteoblasts and chondrocytes (data not shown).

**Determination of Corresponding Ligands/Receptors on BM-MSC**

Total RNA from cultured murine BM-MSCs was isolated and RT-PCR was used to determine the expression of receptors corresponding to several adhesion molecules/ECM
proteins and chemokines/cytokines identified through profiling. RT-PCR was used to
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proteins and chemokines/cytokines identified through profiling. RNA samples from
murine peripheral blood mononuclear cells (PBMC), juxtaglomerular cells (JGC, from
JG cell line As4.1 (ATCC)) (Klar et al., 2002), vascular smooth muscle cells (VSMC,
isolated from murine thoracic aortae) and skin keratinocytes were used for comparison.

**Flow Cytometric Analysis of Murine BM-MSC**

Cultured BM-MSCs were harvested by trypsinization. Cell aliquots were incubated with
fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)- conjugated mAb (BD
pharmingen) against CD45, CD14, CD29 (integrin β1), CD49d, CD105 (SH2), CXCR4,
Sca-1, or CD126 (IL6 receptor α chain) and analyzed (Becton Dickinson FACScan). For
each analysis, an aliquot of cells was also stained with isotype control IgG conjugated to
FITC or PE as a negative control.

**Characterization of Blocking Antibodies**

The azide-free anti mouse CD49d mAb (IgG, clone PS/2,, Accurate Chemical &
Scientific Corporation) was used previously to block neutrophil migration *in vivo*(Petit *et
al.,* 2002;Bowden *et al.,* 2002). The azide-free anti-CXCR4 IgG (Torrey Pine Biolabs)
was shown to neutralize CXCR4 and block SDF1 mediated leukocyte mobilization in
mice (Petit *et al.,* 2002;Bowden *et al.,* 2002). The azide-free CD29 blocking mAb (IgM,
clone Ha2/5) was purchased from BD Pharmingen. The saturating concentrations of the
blocking antibodies were determined by flow cytometry as described previously (Ridger
*et al.,* 2001b). The antibodies were further verified for their capacity in blocking the
receptor bindings to their ligands. SDF-1 (USBiological) and VCAM-1 (R&D Systems) were FITC conjugated using ProtOn Fluorescein Labeling Kit (Vector Laboratories) following the manufacturer’s instruction. The saturating concentrations of the FITC-conjugated SDF-1 and VCAM-1 to the cells were titrated. Mouse T lymphocytes from EL4 cell line (ATCC) grown in RPMI 1640 medium (GIBCO) containing 10% FBS and L-glutamine were washed, pre-incubated with anti-CXCR4 or control IgG in RPMI 1640 containing 0.5% BSA at a concentration of 10 μg/ml for 20 min at room temperature and then incubation with FITC-labeled SDF-1 for 30 min at 37°C. Mouse bone marrow nucleated cells isolated with a Ficoll density gradient were cultured in α-MEM containing 17% FBS on plastic tissue culture dishes for 10 days. After washes, the adherent cells were detached and used for in vitro tests of anti-CD49d. The cells were resuspended in α-MEM containing 0.5% BSA, stimulated with 100 ng/ml SDF-1 (Ganju et al., 1998) to induce CD49d binding (Glodek et al., 2003), and incubated with IgG or anti-CD49d at a concentration of 10 μg/ml for 20 min at room temperature. The cells were then incubated with FITC-labeled VCAM-1 for 30 min at 37°C. After washes, the cells were fixed with 1% paraformaldehyde in PBS and analyzed on FACS to determine the cells with ligand binding. Cells stained with FITC-conjugated non-immune IgG were used as a negative control.

**Chemotaxis Assay**

EL4 cell chemotaxis assay was performed in 24-well plates containing 5-μm porosity inserts (Costar Corp., Kennebunk, ME) (Ganju et al., 1998). The expression of CXCR4 in these cells was verified by FACS analysis using a FITC-conjugated anti-mouse CXCR4 mAb (BD Pharmingen). The cells were washed twice with serum-free RPMI
1640 and suspended as 1x10⁶/ml in RPMI 1640 and H199 medium (1:1) containing 0.5% bovine serum albumin. 10⁵ cells in 100 μl were loaded onto the top wells. 100 ng/ml SDF-1 was added to the bottom chamber with a total volume of 0.6 ml. Cells migrating to the bottom well were collected after 3 h and counted. Chemotaxis of passage 0 adherent mouse bone marrow nucleated cells was performed in 48 well micro chemotaxis chamber (Neuro Probe) with 8 μm pore fibronectin-coated filter (Ceradini et al., 2004). The cells were suspended in serum-free α-MEM containing 0.5% BSA at a concentration of 0.5x10⁶/ml. 25,000 cells in 50 μl/well were loaded onto the upper chambers. The lower chambers were filled with serum-free α-MEM containing 100 or 500 ng/ml SDF-1. After 4-h incubation, the non-migrating cells were completely wiped from the top surface of the filters and the migrating cells adhering to the undersurface of the filters were stained with Hoeschst and quantified with an imaging software (IPlab). To assess the effect of anti-CXCR4, the cells were pre-incubated with anti-CXCR4 or control IgG at a concentration of 10 μg/ml for 20 min at room temperature prior to chemotaxis test. Each experiment was performed twice in 6 replicate wells.

**Cell Adhesion Assay**

Cell adhesion assays were performed in 48-well plates which were coated with fibronectin (20 ng/ml) or recombinant human VCAM-1 (150 ng/well, R&D Systems) (Glodek et al., 2003). Wells were then washed three times with HBSS containing Hepes and blocked with 2% BSA in PBS for 1 h at 37°C. 2% BSA in PBS alone coated wells were used as negative control. For CD29 blockade, 10⁴ MSCs per well were seeded on fibronectin-coated plates in the presence of isotype control IgM or anti-CD29 blocking
mAb at a concentration of 40 μg/ml and incubated in α-MEM for 3h at 37°C. Cells were photographed for assessment of adhesion and spreading. For CD49d blockade, 10^5 per well of passage 0 adherent mouse bone marrow nucleated cells were stimulated with 100 ng/ml SDF-1 (Ganju et al., 1998) to induce CD49d-mediated cell adhesion (Glodek et al., 2003), incubated with 2.5 or 10 μg/ml of CD49d mAb or 10μg/ml of isotype IgG for 30 min at 37°C, and then placed into VCAM-1–coated wells for 30 min at 37°C. The non-adherent cells were removed by 3 washes with HBSS and the cells adhered were detached and counted. The same experiment was performed twice in quadruplet wells for each variable.

**Intramyocardial Delivery of BM-MSC**

Female BalbC mice (8-10 weeks old, body weight 22-26 g) underwent permanent occlusion of LAD coronary artery. BM-MSCs isolated from male BalbC mice (5-7 weeks old) were transduced with retroviral green fluorescent protein (GFP) as described previously (Mangi et al., 2003). After sorting, over 98% of BM-MSCs were GFP positive. 1 h after ligation, 3x10^5 GFP positive BM-MSCs were intramyocardially injected at a site slightly above the ligature in 20 μl PBS after incubation with blocking antibody or isotype control as described in the results. 72 h later, the hearts were arrested in diastole with KCl and harvested after PBS perfusion. The hearts were transversely dissected at the ligation level. The BM-MSCs in the myocardium below the ligature were assessed by Real-Time PCR and histology.

**Immunohistochemical Staining**
Frozen tissue sections from the heart 48 h post infarct were incubated with rat monoclonal antibody against mouse ICAM-1 (eBioscience), VCAM-1 (Cymbus Biotechnology) or tenascin-C (Chemicon) followed by sequential incubations with anti-rat biotin and FITC-conjugated anti-biotin antibody (Sigma). Myocytes were stained with a mouse monoclonal antibody against sarcomeric α-actin (Sigma) and Cy3-conjugated secondary antibody (Sigma). Nuclei were stained with Hoechst. The samples were visualized under a fluorescence microscope (Nikon Ecliose 80i).

**Histologic Assessment of BM-MSC in the Myocardium**

The apical myocardium bellow the ligation of the heart was sectioned. 10 sections (20 μm in thickness) at 100 μm intervals down to the apex from the ligation were immunostained for GPF positive cells. GFP was detected with an anti-GFP antibody (USBiological) and a FITC-conjugated secondary antibody (Sigma). The area of GFP positive BM-MSCs in each tissue section was measured using an IPLab software (Scanalytics). The volume of BM-MSCs in the myocardium was determined by totalizing the GFP positive cell volumes between each two adjacent sections (average GFP positive area of two adjacent sections times the interval (100 μm)).

**Quantification of BM-MSCs in the Myocardium by Real-Time PCR**

Real-Time PCR was used to quantify BM-MSCs in the myocardium by measuring the amount of Y-chromosome specific sequence derived from the male BM-MSCs. Genomic DNA was extracted from the myocardium bellow the ligation, using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA). Real-Time PCR was carried out using a 7700 Sequence Detection System (PE Applied Biosystem, Foster City, CA). Primers and
probes for murine Y chromosome specific TSPY gene and β-actin (Wang et al., 2002) were synthesized by PE Applied Biosystem. Standard curves were generated by serially diluting genomic DNA prepared from mouse BM-MSCs into samples containing 200 ng genomic DNA from a mouse infarcted heart. PCR was performed for 50 cycles with denaturation at 95 °C for 15 seconds and annealing at 59 °C for 1 minute, using Master Mix (PE Applied Biosystem). β-actin gene was used as an internal control to normalized equal loading of DNA per reaction. Assuming each MSC contains one copy of Y chromosome and 5 pg DNA per diploid nucleus, the numbers of BM-MSCs in the myocardium bellow the ligation were determined (Lee et al., 2006).

Statistical Analysis

All values were expressed as mean ± SD. Student’s paired t test was performed for comparison of data between the control and treated samples.
RESULTS

Expression Profile of Animal Model of Myocardial Infarction

To identify the chemokines, cytokines and adhesion molecules that are upregulated in myocardial ischemic injury, we generated expression profiles of MI heart. Samples from murine myocardial infarcts created by LAD coronary artery was analyzed on Affymetrix Expression Set MOE430 oligonucleotide arrays. Since our goal was to identify cytokines and adhesion receptors involved in trafficking, homing, and engraftment of BM-MSC into ischemic myocardium, we focused on a subset of 461 probes (out of >22,000 probes on this array) related to cell adhesion, chemokines, cytokines and chemotaxis (determined by using the Gene Ontology classification system as well as a thorough evaluation of the current literature). Using Affymetrix MAS software, 175 probes met criteria for “presence” in at least 4 of 6 independent hybridizations, and these were further analyzed for either a mean SLR >0.6 from all nine comparisons at each time point (3 MI x 3 Sham) or a change metrics of increase/marginal increase or decrease/marginal decrease in the majority of the comparisons (>4/9). The results indicated that at 1 hour after LAD occlusion, the number of genes differentially expressed between hearts of MI and sham animals was modest but increased progressively at 24 hours. A composite list of 46 genes is shown in Table 1. Twenty genes were differentially expressed at 8 hours, thirty-two were found at 24 hours, and fourteen were shared at both time points (data not shown). Real-Time PCR was performed for 35 of these apparently upregulated genes. 34 were confirmed to exhibit significant increases in expression. A subset of them that were up-regulated at 24 hours post-MI are shown in Figure 1B. This included several cytokines such as IL-1β, IL-6, SDF-1, TIMP-1 and cell adhesion molecules (such as fibronectin-1 (FN-1)), ICAM-1, E-selectin and VCAM-1).
**Expression Profile of BM-MSC Receptors**

Although some of the adhesion molecules and cytokines identified by the expression profiling are known to be involved in the acute inflammatory response to myocardial ischemia, we postulated that some of these genes might be important for stem cell trafficking and engraftment through interactions with their receptors on BM-MSC. To investigate this, we first determined if their corresponding receptors or ligands are expressed in BM-MSC. Indeed, our BM-MSC expressed 9 counter-receptors to 8 cytokines that are up-regulated in the ischemic myocardium (Figure 2A). To examine the selectivity of gene expression, we studied several different cell types as controls, including PBMC cultured JGC and VSMC. The receptors CXCR4 (for SDF-1), IL6RA and IL6ST (for IL-6), and CC chemokine receptor-2 (CCR2) (for CC chemokine receptor ligand-7 (CCL7)) were expressed by BM-MSC as well as PBMC but not by JGC or VSMC. CXCR2 for CXCL2 was expressed by PBMC but not by BM-MSC. The data support the notion that BM-MSCs express a selective set of membrane proteins that are distinct from hematopoietic, vascular and other cells. We also examined the status of cell adhesion molecules in these cells. E-selectin ligand was universally expressed in all four cell types studied, including BM-MSCs. Several members of the integrin family were also expressed. Very late antigen 4 (VLA-4, integrin $\alpha 4/\beta 1$) and integrin $\alpha 6/\beta 1$ were expressed by both BM-MSC and PBMC, whereas integrin $\alpha 8/\beta 1$ and $\alpha 9/\beta 1$ was expressed in BM-MSC, VSMC and JGC but not in PBMC (Figure 2A). All 4 isoforms (A, B, C and D) of integrin $\beta 1$ were expressed by BM-MSCs at varying levels with $\beta 1A$ the highest, but $\beta 1D$ was not detected in dermal keratinocytes (Figure 2B).
Protein Expression of Receptor/Ligand Pairs

The receptors on BM-MSCs and corresponding ligands in ischemic myocardium were further examined by FACS and immunohistochemistry. Our cultured BM-MSC exhibited differential expression patterns of various receptors as determined by FACS (Figure 3A). Although some of the alpha integrins demonstrated an attenuation of surface expression with successive passages, the integrin β1 (CD29) expression remained unchanged, ~99% through the fifth passage (data not shown). Immunohistochemistry performed on ischemic myocardium validated the up-regulation of extracellular matrix (ECM) proteins, including ICAM-1 (Figure 3B) and VCAM-1 (Figure 3C) at 48 hours and tenascin-C at 72 hours (Figure 3D) after MI.

Functional Validation of Receptor/Ligand Pairs with Antibody Blockade

To prove the functional role of these molecules for BM-MSC attachment to ischemic myocardium and migration within the infarct area, we studied the effect of ex vivo incubation of the cell with blocking monoclonal antibodies directed against potentially important ligands. FACS analysis indicated that incubation with antibody against CD29 blocked 85% of the cell surface receptor in BM-MSCs. Moreover, adhesion assay demonstrated that immuno-blockade of CD29 dramatically reduced BM-MSC attachment to fibronectin-coated plates (Figure 4A and B). To examine whether blockade of CD29 in BM-MSCs caused increased apoptosis, we performed trypan blue exclusion assay and annexin V and caspase 3 analyses. The results showed that blockade of CD29 did not increase cell death and apoptosis (Supplementary Figure 1). To test the in vivo relevance of the interaction between CD29 in the BM-MSCs and its ligands in the ischemic myocardium, female mice underwent permanent occlusion of left anterior descending
coronary artery, and 3x10^5 BM-MSCs, derived from male mice and transduced with green fluorescence protein (GFP) gene, were injected into the left ventricular myocardium at a site above the ligature. To assess the quantity of BM-MSCs that had migrated into the infarcted myocardium, we performed Real-Time PCR assay of the Y-chromosome-specific TSPY genomic sequence that was only present in the male-derived BM-MSCs. In addition, we conducted histologic assessment of GFP positive BM-MSCs. Real-Time PCR analysis indicated that the blockade reduced the amount of BM-MSCs in the ischemic myocardium by 45% compared with control group (i.e. mouse hearts injected with BM-MSCs treated with equal amount of non-immune IgM, Figure 4C, n = 5, P = 0.012).

The amount of BM-MSCs in the infarcted myocardium below the ligation was further assessed by immunohistochemistry analysis of GFP positive BM-MSCs. Injected in a site above the ligation, control BM-MSCs (incubated with non-immune IgM) migrated from the injected site and “homed” to the left ventricular wall infarct (Figure 5A, C and E), whereas a dramatically reduced BM-MSC presence was seen in the infarcted myocardium that was injected with BM-MSCs pre-treated with CD29 blocking antibody (Figure 5B, D and F). The total volume of BM-MSC in the infarcted myocardium (below the ligation) showed a 39% reduction in these cells pre-treated with anti-CD29 antibody compared with cells pre-treated with non-immune IgM (Figure 5G, n = 6, P = 0.004). To examine the possibility that inflammatory cells in the infarcted myocardium contribute to the GFP evaluation by uptaking or cell fusion, we performed co-staining studies of the myocardium 3 days post-MI for GFP and for neutrophils (anti-mouse neutrophil mAb MCA771GA from Serotec), macrophage (anti-mouse Mac-3 mAb) and toxic T lymphocytes (anti-mouse CD8 mAb). We found abundant neutrophils
and macrophages, (but a much lower amount of toxic T cells) in the infarct (Dewald et al., 2004; Vandervelde et al., 2006). None of the inflammatory cells were found GFP-positive (data not shown), indicating that cell fusion or uptake of GFP by inflammatory cells, if existed, was extremely rare.

We applied the same blocking antibodies against CD49d (integrin α4) and CXCR4 as were used in previous studies (Petit et al., 2002; Bowden et al., 2002). We examined the blocking ability of the antibodies. We found that anti-CXCR4 reduced FITC-labeled SDF-1 binding to EL4 T lymphocytes, 90% of them expressed CXCR4 (Figure 6A and B). We tested anti-CD49d on passage 0 adherent cells from culture of mouse bone marrow nucleated cells, and found that anti-CD49d inhibited FITC-labeled VCAM-1 binding to the cells (Figure 6C). Furthermore, anti-CXCR4 reduced SDF-1-induced migration of EL4 T lymphocytes (Fig. 6D, \( P < 0.00001 \)) and passage 0 adherent cells from culture of mouse bone marrow nucleated cells (Figure 6E, \( P < 0.00001 \)), and anti-CD49d inhibited attachment of the passage 0 adherent cells to VCAM-1-coated plates (Figure 6F, \( P < 0.0001 \)). However, when BM-MSCs pre-treated with blocking antibodies specifically against CXCR4 (Figure 6G, \( n = 5 \)) or CD49d (Figure 6H, \( n = 6 \)) were injected into the myocardium, in contrast to our result with CD29 antibody, we observed no statistically significant differences in the quantity of BM-MSC in the infarcted myocardium (below the ligation) as compared to injection of BM-MSC pre-treated with control IgG.

We performed additional experiments with injections of 10 μm microspheres (Vector Laboratories) into the myocardium of infarcted or sham-operated animals and found that very few particles remained in the myocardium after 72 hours in either sham
or MI hearts (data not shown). These data demonstrate that the retention of BM-MSC in
the ischemic myocardium involves specific mediators and cell adhesion.
DISCUSSION

Myocardial infarction is a leading cause of heart failure and death in developed countries. The application of cell based therapy for the treatment of heart disease remains in its preliminary phase but has shown some promise as seen in several early trials (Strauer et al., 2001; Assmus et al., 2002; Perin et al., 2003; Stamm et al., 2003; Britten et al., 2003; Tse et al., 2003). However, cell therapy encounters significant challenges in isolation techniques, scalability, reproducibility, and ease of clinical application. An alternative to cell therapy is to identify the molecules that mediate homing and engraftment of stem cells to the ischemic myocardium and to develop molecular therapies based on these discoveries.

SDF-1 has been shown to be important for the trafficking of BM-HSC and its intramyocardial administration appears to enhance BM-HSC homing to the ischemic myocardium (Askari et al., 2003; Abbott et al., 2004). Recent study indicated that upregulation of SDF-1 by hypoxic endothelial cells was required for the attachment and transendothelial migration of the circulating CXCR4 positive endothelial progenitor cells (Ceradini et al., 2004). However, it has not been shown that this pathway is involved with BM-MSC homing to the ischemic myocardium. Since recent data have demonstrated that MSC mobilized from the bone marrow, rather than HSC, are involved in myocyte regeneration (Mangi et al., 2003; Balsam et al., 2004; Kawada et al., 2004), the elucidation of the pathway mediating MSC homing and trafficking is obviously important.

In this study, we report a functional genomics strategy to determine the signals that mediate intramyocardial migration and engraftment of BM-MSCs to ischemic tissue, and provide “proof of concept” for this approach. We injected BM-MSCs to study the
migration within the heart from the border zone to the infarcted myocardium, and
subsequently engraftment of the cells in the ischemic myocardium. We identified integrin
β1 but not integrin α4 or CXCR4 as a distinctive pathway for BM-MSC intramyocardial
migration and engraftment. Our strategy involves (1) generating gene expression profiles
of murine acute MI hearts to determine the early events involved in stem cell homing and
myocardial repair, (2) narrowing the number of candidates to only those whose counter-
receptors are expressed in BM-MSCs, and (3) proving the functional role of the verified
ligands in vivo by examining the effect of blocking antibodies on allogenic BM-MSC
transplantation in murine acute MI hearts. Using Affymetrix microarrays and Real-Time
PCR, we first found that, compared to hearts from sham-operated animals, MI hearts
showed significantly increased expression of selective chemokines, cytokines and cell
adhesion molecules, including ICAM-1, IL-6, SDF-1, Sele, VCAM-1, FN-1, Lam-1. To
narrow our focus to those that are involved with important cell-cell/cell-matrix
interactions between ischemic myocardium and BM-MSCs, we verified the expression of
corresponding receptor/ligand pairs on BM-MSCs and identified 9 potential targets,
including CXCR4, integrin α4/β1, integrin α5/β1 (Figure 1A and Figure 2A). These
ligand-receptor interactions, which were shown previously to be relevant to stem cell and
cardiac biology, may play an important role in cardiac repair by influencing homing,
migration and engraftment of BM-MSC.

Integrins have been known to play a key role in cell adhesion, migration and chemotaxis
(Gao and Issekutz, 1997; Werr et al., 1998; Ridger et al., 2001a; Lindbom and Werr,
2002; Imhof and Aurrand-Lions, 2004). Localization of leukocytes to extravascular sites
of inflammation is a function of repeated adhesive and de-adhesive events. Following
extravasation, leukocytes migrate toward a source of inflammation in response to locally elaborated chemotaxins and cytokines. Stimulated by a chemotactic gradient, leukocytes traverse the ECM by way of transient interactions between integrin receptors and components of the ECM and that serve as adhesive ligands (Lauffenburger and Horwitz, 1996; Palecek et al., 1997). Integrins have been known to contribute to the process of neutrophil locomotion include members of CD29 and CD18 (Gao and Issekutz, 1997; Werr et al., 1998; Imhof and Aurrand-Lions, 2004). CD29 also involves cell-to-cell adhesion (Behzad et al., 1996; Werr et al., 1998), which may be important for the anchorage of the engrafted cells. We hypothesized that a similar mechanism was employed for the engrafted BM-MSCs homing to the infarct. In this study, we demonstrated that BM-MSCs expressed many integrins on their surface, including CD29 and CD49d, and their binding partners were upregulated in the ischemic myocardium. In agreement with previous findings (Pittenger and Martin, 2004), our BM-MSCs expressed high level of CD29. Theoretically, CD29 has four isoforms which are formed by alternative mRNA splicing and differentially expressed in different cell types (Balzac et al., 1993). In this study, we show that BM-MSCs, as multipotent stem cells, express mRNA of all four isoforms with CD29A the highest level which is the major isoform involved in cell adhesion and migration (Balzac et al., 1993). Correspondingly, the expression CD29 ligands tenascin-C, fibronectin, VCAM-1 and laminin are found increased in the ischemic myocardium in this study. Tenascin-C is highly expressed during embryogenesis (Crossin et al., 1986), whereas its expression is very low after birth. In this study, we show that tenascin-C is expressed in the ischemic border zone of the infarcted myocardium 3 days post-MI. This is consistent with a previous study where tenascin-C was found to reappear in interstitial fibroblasts in the border zone within 24
hours of MI in rats, decrease at day 7 (Imanaka-Yoshida et al., 2001). TN-C possesses
adhesive as well as “de-adhesion” activities, which depend on ECM and cell surface
receptor binding. These special features facilitates cell migration during wound healing
(Murphy-Ullrich, 2001; Tamaoki et al., 2005). Fibronectin has long been known to play
an important role in mediating cell adhesion and migration (Larsen et al., 2006). Rapid
upregulation of fibronectin in the infarcted myocardium has been reported previously
after MI (Knowlton et al., 1992; Kossmehl et al., 2005). The upregulation of fibronectin is
ahead of collagens, suggesting its involvement in the acute phase of MI (Knowlton et al.,
1992). In 5 hours after acute MI in pigs, increased expression of fibronectin was found in
fibroblast-like cells in the infarct (Kossmehl et al., 2005). Based on these findings, we
decided to study whether this particular class of integrins may be responsible for stem
cell homing and engraftment. Indeed, we found that there were significantly lower
numbers of BM-MSC engrafted and migrated into ischemic myocardium if pre-treated
with antibody against CD29, suggesting a crucial role of CD29 in stem cell cardiac
graftment. Similarly, a previous study shows that blockade of CD29 diminished
neutrophil migration to the lung inflammation (Ridger et al., 2001a).

CD49d has been known to be involved in leukocyte transendothelial migration
(Ridger et al., 2001a). Of note, our results did not show a statistically significant
difference after CD49d was blocked with antibodies prior to injection. Consistent with
our finding, a recent study shows that blockade of CD49d in endothelial progenitor cells
does not affect their homing and engraftment into ischemic sites in MI hearts or ischemic
limbs (Qin et al., 2006).

In this study, different from that observed in CD34+ hematopoietic cells (Askari et
al., 2003; Abbott et al., 2004), blockade of CXCR4 in BM-MSCs with a neutralizing
antibody did not reduce their intramyocardial migration and engraftment into the ischemic myocardium. This may be due to the fact the level of CXCR4 expression in BM-MSCs is much lower compared to CD34⁺ hematopoietic cells (Askari et al., 2003; Ceradini et al., 2004; Abbott et al., 2004). A limitation in our study is that our cells are cultivated and their behavior may differ from endogenous BM-MSCs. Notably, a recent study shows that a small subpopulation of bone marrow adherent cells that are small in size, seen in the colonies of the earliest passages, express high levels of CXCR4, exhibit greater engraftment after systemic infusion (Lee et al., 2006). However, these small cells depend on the larger cells for survival and diminish quickly with successive passages and almost disappear in passage 3 (Colter et al., 2001). Nevertheless, it is the larger and uniformly sized MSCs that are being employed for transplantation for cardiac repair (Mangi et al., 2003; Amado et al., 2005). Our data, taken together with those in published literature (Askari et al., 2003; Abbott et al., 2004) would suggest that MSCs and HSCs may utilize distinctive classes of surface adhesion receptors to establish functional interactions with resident cells or the ECM in the ischemic myocardium thereby differentially influencing intramyocardial homing and trafficking.
Acknowledgments

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Reference List


Gao, J.X. and Issekutz, A.C. (1997). The beta 1 integrin, very late activation antigen-4 on human neutrophils can contribute to neutrophil migration through connective tissue fibroblast barriers. Immunology 90, 448-454.


Table 1. Selected differentially-expressed transcripts in MI vs Sham.

**Up-regulated significantly**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
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<tbody>
<tr>
<td>Actb</td>
<td>integrin alpha 6</td>
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<tr>
<td>Adams1</td>
<td>macrophage migration inhibitory factor</td>
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<tr>
<td>Ccl2</td>
<td>matrix metalloproteinase 14</td>
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<tr>
<td>Ccl6</td>
<td>matrix metalloproteinase 8</td>
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<tr>
<td>Ccl7</td>
<td>NFKB lt chn gene enhncr in B-cells inhibr</td>
</tr>
<tr>
<td>Ccl9</td>
<td>platelet factor 4</td>
</tr>
<tr>
<td>Ccr1</td>
<td>plasminogen activator, tissue</td>
</tr>
<tr>
<td>Ccr2</td>
<td>urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>Co11a1</td>
<td>pro-platelet basic protein</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>ribosomal protein L13a</td>
</tr>
<tr>
<td>Cxcl2</td>
<td>selectin, endothelial cell</td>
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<tr>
<td>Cxcr6</td>
<td>secreted acidic cysteine rich glycoprotein</td>
</tr>
<tr>
<td>Fn1</td>
<td>transforming growth factor, beta 1</td>
</tr>
<tr>
<td>Il1ra1</td>
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<td>Ifrd1</td>
<td>thrombospondin 1</td>
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<tr>
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<td>tissue inhibitor of metalloproteinase 1</td>
</tr>
<tr>
<td>Il1rn</td>
<td>tenascin C</td>
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<tr>
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<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>Itga5</td>
<td>vascular endothelial growth factor A</td>
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</table>

**Down-regulated significantly**

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<tr>
<td>Catn1l1</td>
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<tr>
<td>Cst3</td>
<td>tissue inhibitor of metalloproteinase 2</td>
</tr>
<tr>
<td>Il10rb</td>
<td>transcription factor 4</td>
</tr>
<tr>
<td>Kitl</td>
<td>vitronectin</td>
</tr>
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2
Figure Legends

Figure 1

(A) Strategy using genomics to identify potential receptor-ligand pairs involved in stem cell homing and trafficking. (B) Real-Time PCR showing increased expression of numerous cytokines and adhesion molecules in MI vs Sham hearts after 24 hours ($P < 0.05$ except VEGF$\alpha$). Sele, endothelial selectin; TNFRII, Tumor Necrosis Factor Receptor II; CC, chemokine (C-C motif); CXC, chemokine (C-X-C motif); FN, fibronectin; Lam, laminin.

Figure 2

(A) RT-PCR showing expression of receptors/ligands in BM-MSC, PBMC, JGC and VSMC. Items displayed in red were shown to be up-regulated in ischemic myocardium compared to sham at 24 hours by RT-PCR. Items displayed in blue were shown to be counter-receptors/ligands expressed by MSCs. IL6RA, IL6 receptor, alpha; IL6ST, IL6 signal transducer; CCR, CC receptor; Selel, E-selectin-1 ligand; VN, vitronectin; Tnc, tenascin-C; Itg, integrin. The other abbreviations were donated in the legend of Figure 2. (B) RT-PCR (30 cycles) showing expression of integrin $\beta$1 isoforms (A, B, C and D) in BM-MSCs and dermal keratinocytes.

Figure 3

Protein expression of receptor/ligand pairs. (A) Flow cytometric analysis of BM-MSC surface receptors. Aliquots of cultured BM-MSCs were incubated with FITC- or PE-conjugated monoclonal antibodies against CD45, CD14, CD29, CD49d, CD105, CXCR4, IL6 receptor $\alpha$ chain, or Sca-1. Cells stained with isotype control IgG conjugated to FITC
served as a negative controls (grey peak). Representative results from one of three individual experiments were shown. Values represent percentages of positive cells. (B, C and D) Immunohistochemical staining for ICAM-1 (B), VCAM-1 (C) and tenascin-C (D). Murine heart sections, 48h (B and C) and 72h (D) after MI, were stained with anti-ICAM-1, VCAM-1 or tenascin-C (green) monoclonal antibody. Myocytes were stained red except in D and nuclei were stained with blue. Infarcts were indicated with arrows.

**Figure 4**
Effect of CD29 blockade on BM-MSC adhesion, migration and engraftment. (A & B) Blocking mAb against CD29 (B) reduced BM-MSCs attachment and spreading onto the fibronectin-coated plates compared with control IgM (A). (C) Real-Time PCR assessment of BM-MSC migration and engraftment into the infarcted myocardium. BM-MSCs derived from male mice were incubation with anti-CD29 mAb or control IgM and then injected into the myocardium of female mice after MI above the ligation. 72 h later, the BM-MSCs in the apical region of the heart bellow the ligation was assessed by Real-Time PCR assay of the Y chromosome specific DNA sequence. BM-MSCs incubated with antibody against CD29 had reduced accumulation in the apical region as compared with the cells treated with control IgM (n = 5, **P = 0.012).

**Figure 5**
CD29 blockade reduced the accumulation of BM-MSC in the infarcted myocardium. BM-MSCs incubated with control IgM (A, C, E) or anti-CD29 mAb (B, D, F) were injected into the myocardium at one site above the ligation. 72 h later, sections of the heart bellow the ligation were immunostained for GPF positive BM-MSCs (green). BM-
MSCs treated with anti-CD29 blocking mAb (B&D) had reduced accumulation in the heart than BM-MSCs incubated with control IgM (A&C). BM-MSCs incubated with control IgM (E) were found to have migrated from the injection site and “homed” to the entire left ventricular wall infarct while reduced BM-MSC migration and accumulation were seen in the BM-MSCs incubated with anti-CD29 (F). Myocytes (red) were detected by anti-sarcomeric α-actin and nuclei (blue) were stained with Hoeschst. (G) The area of GFP positive BM-MSCs in each section was quantified. Treatment of BM-MSCs with CD29 blocking mAb reduced BM-MSC volume in the apical region of the hearts compared with incubation of the cells with control IgM (n = 6, ** P = 0.004).

**Figure 6**

Effect of CXCR4 or CD49d blockade on BM-MSC intramyocardial homing and engraftment to the infarcted myocardium. (A) FACS analysis indicated that over 90% of EL4 cells expressed CXCR4. (B) EL4 cells were pre-incubated with anti-CXCR4 (peak in middle) or control IgG (peak on right) at a concentration of 10 μg/ml and then incubated with FITC-labeled SDF-1. EL4 cells with SDF-1 binding were determined by FACS. Cell incubated with FITC-labeled non-immune IgG were used as a negative control (grey peak). (C) Passage 0 adherent cells from culture of mouse bone marrow nucleated cells were first incubated with anti-CD49d (peak in middle) or control IgG (peak on right) at a concentration of 10 μg/ml then incubated with FITC-labeled VCAM-1. Cells with VCAM-1 binding were determined by FACS. Cell incubated with FITC-labeled non-immune IgG were used as a negative control (grey peak). (D&E) Anti-CXCR4 (10 μg/ml) reduced SDF-1-mediated migration of EL4 cell (D) and passage 0 adherent mouse bone marrow nucleated cells (E). Each experiment was performed twice.
in 6 replicate wells, $P < 0.00001$ in D & E. (F) Anti-CD49d (2.5 and 10 μg/ml) inhibited attachment of passage 0 adherent mouse bone marrow nucleated cells. The experiment was performed twice in quadruplet wells for each variable ($P < 0.0001$ for both antibody doses). (G&H) A similar procedure as described in figure 5 was used for BM-MSC injection and assessment by Real-Time PCR. Treatment of BM-MSCs with anti-CXCR4 (G, n = 6, $P = 0.83$) or anti-CD49d (H, n = 5, $P = 0.31$) had no significant effect on the amount of BM-MSCs accumulated in the infarcted myocardium as compared with treatment with control IgG.
Affymetrix MOE430 Array (22,690 probes)

Subset of pertinent genes (461)

“Present” on Array (175)

Significant change (46)

Attempt RT-PCR verification of subset (14/15 verified)

Corresponding receptors tested (16)

Potential targets (9)
Figure 1
cytokines/adhesion molecules in ischemic myocardium

counter-receptors/ligands

SDF-1 \rightarrow \text{Cxcr4}

IL-6 \rightarrow \text{IL6RA, IL6ST}

CCL2, CCL7

CCL8, CCL13 \rightarrow \text{CCR2}

CXCL1, CXCL2

CXCL3, CXCL5 \rightarrow \text{CXCR2}

Sele \rightarrow \text{Selel}

VCAM-1, FN, LN, VN, Tnc \rightarrow \text{Itgb1}

VCAM-1, FN \rightarrow \text{Itga4}

LN \rightarrow \text{Itga6}

FN, VN, Tnc \rightarrow \text{Itga8}

Tnc \rightarrow \text{Itga9}

Figure 2
<table>
<thead>
<tr>
<th>ladder</th>
<th>actin</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>H₂O₂</th>
<th>actin</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
</table>

**MSC**

**keratinocyte**

The image shows a gel electrophoresis pattern with lanes labeled A to D, including actin and H₂O₂ treatments.
Figure 3

Intensity of FITC or PE

CD45 0%

CD14 0%

Sca-1 99%

CD105 97%

CD29 100%

CXCR4 27%

CD49d 25%

IL6-R 65%
Figure 4

A, B, and C represent different images and data bars, respectively. The data bars indicate a comparison between IgM and anti-CD29, with a significant difference denoted by two asterisks. The image on the left (A) shows a higher number of MSCs compared to the image on the right (B).
Figure 5

G

Volume (x10^7 um^3)

IgM

anti-CD29

**
Figure 6
Figure 6
Figure 6
Figure 6

G

MSC number (x10^4)

IgG  anti-CXCR4

H

MSC number (x10^4)

IgG  anti-CD49d