Bone marrow molecular alterations after myocardial infarction: Impact on endothelial progenitor cells

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Abstract

Objective: Standard drugs post-myocardial infarction (MI) such as angiotensin converting enzyme (ACE) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) increase levels of endothelial progenitor cells (EPC). However, potential underlying mechanisms have not yet been investigated.

Methods and results: We studied the effects of ACE inhibition or statin treatment on EPC levels and on bone marrow molecular pathways involved in EPC mobilization after MI in rats. Three days post-infarction, acetylated LDL (acLDL)+/Ulex europeus-1 (UEA-1)+/VEGF receptor-2+/eNOS+EPC levels and formation of endothelial colony forming units (CFU) were reduced to 60±12% (p<0.05) and 68±7% (p<0.05). In bone marrow, extracellular signal-regulated kinase (ERK) phosphorylation and matrix metalloproteinase (MMP)-9 activity were repressed. Endothelial nitric oxide synthase (eNOS) activity was unchanged, whereas reactive oxygen species (ROS) were increased two-fold in bone marrow. ACE or HMG-CoA reductase inhibition resulted in significant increases in EPC levels. ACE inhibition increased bone marrow ERK phosphorylation and MMP-9 activity. Statin therapy enhanced bone marrow VEGF protein levels, Akt phosphorylation, eNOS activity and normalized increased ROS levels. Augmented EPC levels in the early post-infarction phase by ACE inhibition or statin treatment were associated with improved cardiac function and increased capillary density in the peri-infarct area 7 days after MI. Moreover, increased EPC levels in response to ACE inhibition or statin treatment were sustained 10 weeks post-infarction.

Conclusions: Increased ROS and impaired MMP-9 activity in bone marrow likely contribute to reduced EPC mobilization in the early post-infarction phase. ACE inhibition or statin treatment increased EPC levels with distinct drug-specific effects on bone marrow molecular alterations.

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1. Introduction

Bone marrow is the major reservoir for adult organ-specific stem cells including endothelial progenitor cells (EPC). Impaired mobilization of EPC to circulation contributes to cardiovascular disease, and patients with reduced numbers of circulating EPC are at increased risk for development of coronary artery disease (CAD) [1–5]. Inadequate coronary collateral development in patients with CAD is likewise associated with reduced numbers of circulating EPC [6].
Certain drugs with well-documented beneficial cardiovascular properties, such as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) and angiotensin converting enzyme (ACE) inhibitors, promote EPC mobilization [7–11]. However, the underlying mechanisms are not known.

EPC are embedded in a microenvironment of bone marrow stromal and endothelial cells, and are translocated to the circulation upon matrix metalloproteinase-9 (MMP-9)-mediated release of soluble c-Kit ligand [12]. Regulation of MMP-9 is complex, but extracellular signal-regulated kinases (ERK) may be involved [13–16]. Furthermore, vascular endothelial growth factor (VEGF)/nitric oxide (NO)-mediated pathways have been previously proposed to be essential for EPC mobilization [9,17].

We tested the hypothesis that acute MI alters bone marrow molecular pathways involved in EPC mobilization. We focused on the effects of ACE inhibition or statin treatment on the VEGF/NO and ERK/MMP-9 pathways in the early post-infarction phase (3 days). EPC levels 3 days, 7 days and 10 weeks after MI were also studied.

2. Materials and methods

The study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Study protocol

Left coronary artery ligations were performed in adult male Wistar rats (250–300 g) as described [18–20]. Starting 3 h after ligature, sham-operated rats received placebo treatment (sham, n = 7) and surviving rats with MI were randomly allocated to 3-day treatment by gavage with placebo (PLA, n = 8), the ACE inhibitor trandolapril (ACE, 0.3 mg/kg/day, n = 8) or the HMG-CoA reductase inhibitor rosvustatin (STA, 20 mg/kg/day, n = 7). In the 7-day study, sham rats (n = 6) received placebo and MI rats were allocated to a 7-day treatment by gavage with placebo (PLA, n = 9), the ACE inhibitor trandolapril (n = 7) or rosvustatin (n = 10). In the chronic study (10 weeks), sham-operated animals (sham, n = 9) received placebo treatment and MI rats received placebo (PLA, n = 9), the ACE inhibitor ramipril (ACE, 1 mg/kg/day, n = 6) or rosvustatin (n = 6). Ramipril was given in drinking water, rosvustatin administered by gavage. Dosage was based on previous studies [20,21].

2.2. Sample collection, infarct size and hemodynamic measurements

The hearts were divided into right and left ventricle (LV), including septum in ice-cold saline. Four longitudinal incisions were made in the LV septum and inferoposterior wall of hearts included in the 3-day study, so that endocardial and epicardial surfaces could be pressed flat on glass plates, as previously described [20]. The endocardial and epicardial borders of the LV and the infarcted area were traced and quantified using computed planimetry [20]. The LV of hearts included in the 7-day and 10-week studies was cut into three transverse sections: apex, middle ring (~3 mm) and base. From the middle ring, 5-µm sections were cut at 100-µm intervals and stained with picrosirius red. Infarct size was calculated as the average of all slices and expressed as a percentage of length. LV end-diastolic pressure (LVEDP) and dP/dt were measured 7 days after MI, under isoflurane anesthesia using micromanometer (Millar Instruments) as described [20].

2.3. Isolation of bone marrow and peripheral blood mononuclear cells (PBMCs)

Blood samples were collected from the right carotid artery into EDTA vials. PBMCs were isolated by Ficoll density centrifugation [1]. Hollow bones of rat legs were prepared by standard surgical procedures and whole bone marrow was harvested by flushing marrow with 500 µl PBS using a syringe with a 20-gauge needle. Bone marrow extracts were shock-frozen prior to further analysis.

2.4. Determination of endothelial progenitor cell numbers and cellular characterization

PBMCs (2 × 10⁶) were cultured on fibronectin-precoated 8-well chamber slides (Lab-Tek, Germany) in EBM-2 culture medium supplemented with EBMB SingleQuots (Clonetics, Germany) and 20% FCS for 3 days. To exclude contamination with mature circulating endothelial cells, we carefully removed non-adherent cells 8 h after initial seeding and placed them on new fibronectin-precoated chamber slides. After dilution of 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled acetylated LDL (dil-acLDL; Molecular Probes, Eugene, USA) and fluorescein isothiocyanate (FITC)-conjugated lectin from Ulex europeus (UEA-1; Sigma, Germany) in serum-free EBM2 media, cells were washed twice and incubated for 4 h at 37 °C in EBM2 medium containing 10 µg/ml dil-acLDL and 20 µg/ml UEA-1. After washing, cells were observed by fluorescence microscopy. Only double positive (dil-acLDL and UEA-1) cells were counted in at least four independent randomly selected high-power fields. For comparison, the number of dil-acLDL and UEA-1 double positive cells was also determined by appropriate flow cytometric analyses. Expression of VEGFR-2 in dil-acLDL+/UEA-1+ cells was additionally determined by flow cytometry (FACScan, Becton Dickenson). In brief, cells were incubated with a monoclonal mouse anti-VEGFR-2 antibody (Abcam, Cambridge, UK; 20 µg/ml) for 15 min on ice, followed by a 10-min incubation with a biotin-labeled goat anti-mouse IgG antibody (Abcam, Cambridge, UK;
1 μg/ml), followed by 10-min incubation with Cy5-labeled streptavidin. Expression of eNOS in adherent dil-acLDL⁺/UEA-1⁺ cells was investigated 3 days post culturing by Western blot analysis (see Methods below). Migratory capacity of EPC was investigated using the modified Boyden chamber assay as described previously [22]. In brief, 1 × 10⁴ EPC were cultured in inlets (Falcon HTS Fluoro Blok insert, 8-μm pore size), which were placed in 24-well culture dishes containing endothelial basal medium (Clonetics, Germany), and 50 ng/ml VEGF and 100 ng/ml stromal cell-derived factor (SDF)-1 or no VEGF/SDF-1 for measuring the migratory capacity of EPC. After 24 h, migrated cells were stained with dil-acLDL, fixed with 2% paraformaldehyde and manually counted by fluorescence-based microscopic evaluation of the bottom side of the membrane (n = 4). Capacity for integration during endothelial tube formation was evaluated 2 h and 12 h post culturing 2 × 10⁴ 3-day-old EPC together with 2 × 10⁴ rat aortic endothelial cells (unstained) on 8-well glass slides precoated with 100 μl Matrigel (BD Bioscience, Germany) in 500 μl EBM-2 medium with supplements (Clonetics, Germany) (n = 4). Rat aortic endothelial cells were isolated and characterized as described previously [23] with the modification that explanted rat aortae were cut into small rings (about 1 mm thick) and then cultured on fibronectin-coated 6-well plates in EBM-2 medium with supplements (Clonetics, Germany). After 2 days, endothelial cells sprouted out from aortic rings and formed sub-confluent cell layers after 7 days.

2.5. Colony forming units (CFU)

PBMCs were isolated by Ficoll® density gradient centrifugation and 1 × 10⁶ cells were plated on fibronectin-coated 12-well plates in EndoCult™ medium (StemCell Technologies, USA). To exclude mature endothelial, non-adherent cells were collected after 48 h and plated in replicate fibronectin-coated 24-well plates. Colonies were evaluated and quantified 12 days later. Medium was changed every third day. A colony was defined as a central core of “round” cells with more elongated “sprouting cells” at the periphery and is referred to as early outgrowth colony forming unit-endothelial cell [1,24]. The endothelial lineage of these cells has been confirmed previously by immunocytochemical staining for von Willebrand factor, vascular endothelial growth factor receptor 2 and CD31 [1]. We additionally tested colonies for binding of UEA-1 by incubation of cells with 20 μg/ml UEA-1 for 2 h prior to fluorescence-microscopic evaluation. Only typical colonies stained positive for UEA-1 were counted.

2.6. Zymography, Western blot analyses and ELISA

Zymography was performed by previously described methods [20]. Briefly, bone marrow extracts (8 μg protein per lane) in cacodylic buffer (10 mM cacodylic acid, 0.15 mM NaCl, 1 μM ZnCl₂, 20 mM CaCl₂, 1.5 mM Na₃, 0.01% Triton, pH 5.0) were mixed with loading buffer and electrophoresed on a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (type A from porcine skin) under non-reducing conditions. The gel was incubated for 1 h in Triton 2.5% followed by incubation in enzyme buffer (50 mM Tris, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij, pH 7.6) for 10 h at 37 °C. Subsequently, the gel was stained with 0.5% Coomassie blue G-250 in 40% methanol and 10% acetic acid for 1 h and destained with four changes of 40% methanol and 10% acetic acid. Areas of digestion were quantified by image software Quantity One (Bio-Rad). Human recombinant purified MMP-9 (OncoGene) was used as a positive control. Protein content was determined by Bradford assay.

For Western blot analyses, bone marrow extracts or cell lysates from EPC cultured for 3 days in the presence of endothelial growth media (EBM-2 supplemented with EBM SingleQuots (Clonetics, Germany) and 20% FCS) were mixed with sample loading buffer and separated under reducing conditions on 12% SDS-polyacrylamide gel. Proteins were electro-transferred onto PVDF membranes (Immun-Blot® 0.2 μm, Bio-Rad), incubated for 2 h in Tris-buffered saline-Tween (TBS-T) with 5% blocking agent (Amersham), followed by overnight incubation at 4 °C with primary antibodies. The bands were detected using a chemiluminescence assay (ECL+Plus, Amersham). Primary antibodies used included rabbit anti-p44/42 MAP kinase (ERK1/2) (Cell Signaling Technology, Germany), rabbit anti-phospho-Akt (Cell Signaling Technology, Germany), rabbit anti-Act and rabbit anti-phospho-Akt (Cell Signaling Technology, Germany), mouse anti-eNOS (Transduction Laboratories, BD Biosciences, Germany) and mouse anti-GAPDH (Abcam, Acris, Germany). Vascular endothelial growth factor (VEGF) was detected in plasma and bone marrow extracts using a standardized ELISA-Kit (DuoSet rat VEGF; R&D Systems, UK) according to the manufacturer’s instructions.

2.7. Detection of eNOS activity in bone marrow

eNOS activity was determined by assessing the conversion of L-[guanidino-¹⁵N₂]arginine to ¹⁵N-nitrate with gas chromatography/mass spectrometry, according to a previously described method [25,26]. Briefly, bone marrow extracts (100 μg protein) were incubated for 60 min at 37 °C with assay buffer (50 mM Tris, pH 7.4; 10 μM BH₄, 1 mM NADPH, 5 μM FAD, 5 μM FMN, 5 mM CaCl₂, 500 nM calmodulin and 5 mM L-[guanidino-¹⁵N₂]arginine). Calcium-independent activity was determined in the presence of 5 mM EGTA and in the absence of calcium/calmodulin.

2.8. Detection of malondialdehyde (MDA) in bone marrow

Malondialdehyde–thiobarbituric acid (MDA–TBA) adducts were measured by high-performance liquid chro-
matography (HPLC) as described [27]. Bone marrow supernatants were separated and fractionation of the protein-free extract was performed using a C18 column (Micro Bondpak, Waters) in an HPLC system (Pharmacia LKB; Germany). We used a flow rate of 1 ml/min at a pressure of 1800 psi at 35 °C. Exact quantification was achieved using 1,1,3,3-tetraethoxy propane standards.

2.9. Immunohistochemistry

Capillary density in the peri-infarct area was determined 7 days after infarction. LV frozen 5-μm sections were immunohistochemically stained using anti-CD31 mAb (PECAM-1, Pharmingen, Germany). Briefly, sections were fixed in cold acetone for 5 min followed by pretreatment with 0.3% hydrogen peroxide for 20 min to inhibit endogenous peroxidase activity. Subsequently, sections were blocked with 2% goat serum for 30 min and incubated with the primary antibody for 1 h at room temperature at a dilution of 1:50. After rinsing with PBS, the sections were incubated for 30 min with a biotinylated secondary antibody (Pharmingen, Germany). Peroxidase activity was detected using the DAB detection system (Pharmingen, Germany). Sections were counterstained with hematoxylin.

2.10. Statistical analysis

Data are expressed as mean±S.E.M. Statistical analysis was performed by one-way ANOVA followed by multiple
comparisons using Fisher’s protected least-significant difference test. Statistical analysis was performed using StatView 5.0 statistic program (Abacus Concepts, Berkley, CA, USA). Statistical significance was assumed at $p < 0.05$.

3. Results

3.1. Infarct sizes

Mean infarct sizes of rats included in the 3-day, 7-day and 10-week studies were similar among the experimental groups (3 days: PLA MI 45±3%, ACE MI 46±4%, STA MI 43±4%; 7 days: PLA MI 46±1%, ACE MI 47±2%, STA MI 46±2%; 10 weeks: PLA MI 49±1%, ACE MI 48±2%, STA MI 50±2%).

3.2. Characterization of rat endothelial progenitor cells

We used a cell isolation protocol identical to that used in many clinical trials, where EPC are transplanted into human myocardium [28]. In brief, after 3 days of culture in the presence of endothelial growth media, >90% of cells were capable of cellular uptake of acLDL and UEA-1 lectin binding (Fig. 1a,b,c). Moreover, >90% of all cells expressed VEGFR-2 and in cell lysates eNOS protein expression was detectable 3 days post culturing (Fig. 1d). Addition of VEGF and SDF-1 to the lower compartment of a modified Boyden chamber significantly enhanced migratory capacity of EPC (Fig. 1e). EPC integrated into forming vascular networks of rat arterial endothelial cells after 2 h and 12 h of co-culturing on Matrigel® (see Fig. 1f). Early after co-culturing (2 h) most EPC adhered to mature endothelial cells, whereas at later time points (12 h), EPC started to integrate between mature endothelial cells. We used the term “EPC” therefore to describe dil-acLDL+/UEA-1+/VEGFR-2+/eNOS+ cells with the potential for migration and incorporation during endothelial tube formation. As a second method for quantification of EPC, we used a modified CFU assay as described previously [1] and endothelial CFU were counted 14 days after initial seeding (Fig. 1g).

3.3. EPC mobilization and bone marrow alterations 3 days post-infarction

Three days after MI, a 40% repression of EPC levels was observed as compared to sham-operated animals (Fig. 1a). Formation of endothelial CFU was also reduced after MI
Bone marrow eNOS protein expression (Fig. 2a) calcium-dependent NOS activity (Fig. 2b) and VEGF expression within plasma or bone marrow extracts (Fig. 2c,d) did not change after MI. In contrast, MDA–TBA adduct formation as an index of ROS formation was two-fold increased in bone marrow of infarcted animals (Fig. 2e). Repression of EPC levels early after MI was accompanied by a significant decrease in bone marrow MMP-9 activity and ERK2 phosphorylation (Fig. 3a,b).

3.4. Effects of angiotensin converting enzyme inhibition

Three days post-MI reduced numbers of dil-acLDL+/UEA-1+/VEGFR-2+/eNOS+EPC and CFUs were markedly increased after treatment with the ACE inhibitor (Fig. 1a). ACE inhibition normalized bone marrow MMP-9 activity and reversed impaired ERK2 phosphorylation (Fig. 3a,b). ACE inhibition increased bone marrow eNOS expression (Fig. 2a). However, eNOS activity, VEGF levels and Akt phosphorylation were not altered (Fig. 2b,d,f). In addition, ACE inhibition did not significantly reduce ROS production in bone marrow (Fig. 2c).

3.5. Effects of HMG-CoA reductase inhibition

HMG-CoA reductase inhibition induced a 4.6-fold increase in circulating EPC levels and a 2.7-fold increase in CFU formation compared with untreated rats 3 days post-MI (Fig. 1a). Statin treatment substantially increased bone marrow eNOS protein expression and eNOS activity (Fig. 2a,b), and normalized increased MDA adducts (Fig. 3e). In addition, bone marrow Akt phosphorylation (Fig. 2f) and VEGF concentration (Fig. 2d) were increased upon statin treatment, whereas plasma VEGF levels were unchanged (Fig. 2c). In contrast to ACE inhibition, statin treatment did not affect ERK phosphorylation and MMP-9 activity in bone marrow (Fig. 3a,b).

3.6. EPC, hemodynamics and capillary density 7 days post-infarction

Seven days post-infarction acLDL+/UEA-1+/VEGFR-2+/eNOS+EPC levels and formation of CFU were reduced in placebo-treated rats, although statistical significance was not achieved (Fig. 4a). Statin treatment and, to a lesser degree,

**Fig. 3.** Altered ERK/MMP-9 signalling in bone marrow 3 days after myocardial infarction: modulation by ACE inhibition or statin treatment. (a) MMP-9 activity as measured by zymography and (b) ERK phosphorylation as revealed by Western blot analysis in bone marrow extracts. Sham, n=7; MI PLA=myocardial infarction placebo treatment, n=8; MI ACE=myocardial infarction ACE inhibition, n=8; MI STA=myocardial infarction HMG-CoA reductase inhibition, n=7; *p<0.05 vs. sham; †p<0.05 vs. MI PLA.
ACE inhibition markedly increased EPC levels (Fig. 4a). Formation of CFU was significantly increased by both drug treatments (Fig. 4a).

MI rats on placebo developed elevated LV end-diastolic pressure (LVEDP) and markedly lower LV $dP/dt_{\text{max}}$ (Fig. 4b). ACE inhibition or statin treatment significantly decreased LVEDP and enhanced $dP/dt_{\text{max}}$. Moreover, ACE inhibition as well as statin treatment increased capillary density in the peri-infarct area at 7 days after experimental infarction (Fig. 4c).

### 3.7. EPC mobilization 10 weeks post-infarction

In rats with chronic heart failure 10 weeks after experimental MI, acLDL$^+$/UEA-1$^+$/VEGFR-2$^+$/eNOS$^+$ EPC levels and formation of CFU were slightly, but not significantly reduced in rats on placebo compared to sham-operated animals (Fig. 5a). ACE inhibition increased EPC levels above those of sham animals and statin treatment led to a 3.1-fold increase compared with placebo-treated rats 10 weeks post-MI (Fig. 5a). Formation of CFU was significantly increased compared to placebo MI animals by both drug treatments (see Fig. 5a). MMP-9 activity remained significantly reduced in bone marrow of chronic infarcted animals and was normalized by ACE inhibition (Fig. 5b). Bone marrow eNOS activity remained significantly enhanced 10 weeks post-MI after statin treatment (Fig. 5c).

### 4. Discussion

Bone marrow molecular alterations early after MI may be linked to insufficient progenitor cell mobilization. Our study shows reduced levels of acLDL$^+$/UEA-1$^+$/VEGFR-2$^+$/eNOS$^+$ EPC and impaired development of endothelial CFU associated with increased ROS production and repressed MMP-9 activity in bone marrow in the early post-infarction phase. ACE and HMG-CoA-reductase inhibition increased...
EPC levels. This was paralleled by distinct drug-specific effects on bone marrow molecular pathways involved in EPC mobilization.

Our observation of decreased EPC levels early after MI is in contrast to the report of Shintani et al. [29]. However, we would like to point out that ACE inhibitors or statins that are administered on a routine basis to patients with acute MI may contribute to the observed effects. This is underlined by a recent article, which indeed could identify use of statins after MI to be the best independent predictor of increased mobilization of stem cells [30,31]. Our results are in agreement with those of Nygren et al. [32], who were unable to detect any increase in peripheral blood progenitor or stem cell activity following MI, when animals were without drug treatment. Of importance, no progenitor cells were detected in the infarcted myocardium or the spleen, excluding trapping of potentially mobilized cells [32]. However, we are aware that acLDL+/UEA-1+/VEGFR-2+/eNOS+ EPC may be different to previous otherwise defined EPC, such as early or late outgrowth EPC, CD34+/VEGFR2+, CD133+/VEGFR2+ or others. Nevertheless, the type of EPC investigated by us (acLDL+/UEA-1+/VEGFR2+/eNOS+ cells) was similar to that already used in clinical trials where EPC transplanted into the heart after MI yielded promising results [28]. Indeed, the adhesion-related selection in culture reveals a proportion of EPC from monocytic origin with phenotypic and functional features of stem cells [33]. Different methodologies for EPC determination may explain the fact that others found increased mobilization of different subtypes of EPC after MI, such as CD34+ cells [29]. However, using the colony forming assay as a second method to quantify EPC, we were able to reproduce the finding of reduced EPC early after MI. Moreover, decreased levels of cardiac-side-population progenitor cells as well as mesenchymal progenitor cells especially after large acute MI [34,35] support our results, as only rats with extensive myocardial infarction (>40%) were included in the present study.

Reduction of circulating EPC was linked to alterations of bone marrow molecular pathways known to be involved in EPC mobilization. MMP-9 was identified as a specific mediator of EPC transit from bone marrow to circulation [12]. Accordingly, we observed a reduction of MMP-9 activity in bone marrow after MI, which may, at least in part, explain the repression of circulating EPC. Regulation of MMP-9 is mediated in part by phosphorylated extracellular signal-regulated kinases [13–16]. Reduction of MMP-9 activity was paralleled by decreased ERK phosphorylation that was significant for ERK2, but only of borderline significance for ERK1. In-depth molecular studies are needed to definitively clarify the role of ERK signalling in EPC mobilization from bone marrow. However, the apparent correlation between ERK phosphorylation and MMP-9 activity is a first hint for a causal relationship.

Our finding that ACE inhibitors increase EPC levels is consistent with previous studies in which treatment with the ACE inhibitor enalapril resulted in an increase of circulating hematopoietic progenitor cells in healthy individuals [36]. Likewise, in patients with stable coronary artery disease, treatment with ramipril correlated with augmentation of circulating EPC [11]. In our study, ACE inhibition did not alter bone marrow eNOS activity and only slightly reduced exaggerated ROS production, although eNOS expression
was increased. As oxidative stress contributes to eNOS uncoupling [37], it is conceivable that enhanced ROS may have led to impairment of eNOS activity. In addition, ACE inhibition did not affect bone marrow levels of VEGF, a major regulator of eNOS activation via Akt phosphorylation [38]. In contrast, the current findings may suggest that the observed normalization of bone marrow MMP-9 by ACE inhibition occurs independently of NO.

Maintenance and mobilization of stem cells in bone marrow is determined by the local microenvironment, which mainly consists of fibroblasts and endothelial cells [39–41]. Besides MMP activation, EPC can additionally be mobilized from bone marrow into the circulation by VEGF [12,42]. In our study, HMG-CoA reductase inhibition significantly enhanced bone marrow concentrations of VEGF. In contrast, plasma concentrations were unchanged, indicating local bone marrow alterations to be independent from systemic changes. VEGF induces phosphorylation of Akt leading to enhanced eNOS activity [43]. Of note, we detected increased levels of phosphorylated Akt, as well as eNOS activity in bone marrow of statin-treated animals. Increased vascular endothelial NO production by rosuvastatin has previously been reported [44]. However, our study demonstrates enhanced eNOS expression and activity specifically in bone marrow by HMG-CoA reductase inhibition. As eNOS-derived NO plays an essential role in mobilization of vascular progenitor cells [9,17], enhanced NO production in bone marrow induced by HMG-CoA reductase inhibition likely contributes to the sustained increase in circulating EPC. In contrast, MMP-9 activity remained reduced and therefore is not likely to play a major role in the mechanism of statin-mediated EPC mobilization. Landmesser et al. demonstrated that the HMG-CoA reductase inhibitor atorvastatin increased EPC mobilization in NOS3+/−, but not NOS3−/− mice, which further underlines the pivotal role of endothelial-derived NO in regulation of the transit of EPC from bone marrow to circulation [9]. Moreover, defective mobilization of stem and progenitor cells contributes to the impairment of ischemia-induced neovascularization in NOS3−/− mice [17]. Local eNOS-derived NO concentrations are further dependent on ROS production as NO can be scavenged by ROS [45,46]. HMG-CoA reductase inhibition attenuated exaggerated ROS production and increased eNOS activity. Thus, we speculate that increased NO bioavailability in bone marrow is strongly correlated with an increase in circulating EPC levels. Our findings are suggestive for HMG-CoA reductase inhibition to lead to a NO-dependent but MMP-9 independent increase of circulating EPC.

After MI, bone marrow-derived EPC incorporated into sites of neovascularization at the border of the infarct [47] and neoangiogenesis after EPC transplantation improved myocardial blood flow, function and remodeling [48]. Therefore, the induction of circulating EPC early after MI by ACE or HMG-CoA reductase inhibition likely contributed to increased capillary density in the peri-infarct area leading to improvement of cardiac remodeling and function 7 days post-infarction. The persistent increases in EPC levels in rats with chronic heart failure after MI especially in statin-treated animals observed in the present study may also contribute to the profound improvement of left ventricular remodeling and function by long-term statin treatment, although a causative relationship has not been definitively proven [9,19].

In conclusion, our data show reduced levels of circulating EPC associated with increased ROS and reduced MMP-9 activity in bone marrow in the early post-infarction phase. ACE inhibition or statin treatment increased EPC levels with distinct drug-specific effects on bone marrow molecular alterations.

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