Comparison of parathyroid hormone and G-CSF treatment after myocardial infarction on perfusion and stem cell homing

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Comparison of parathyroid hormone and G-CSF treatment after myocardial infarction on perfusion and stem cell homing. Am J Physiol Heart Circ Physiol 298: H1466–H1471, 2010. First published March 5, 2010; doi:10.1152/ajpheart.00033.2010.—Mobilization of stem cells by granulocyte colony-stimulating factor (G-CSF) was shown to have protective effects after myocardial infarction (MI); however, clinical trials failed to be effective. In search for alternative cytokines, parathyroid hormone (PTH) was recently shown to promote cardiac repair by enhanced neovascularization and cell survival. To compare the impact of the two cytokines G-CSF and PTH on myocardial perfusion, mice were noninvasively and repetitively investigated by pinhole single-photon emission computed tomography (SPECT) after MI. Mobilization and homing of bone marrow-derived stem cells (BMCs) was analyzed by fluorescence-activated cell sorter (FACS) analysis. Mice (C57BL/6J) were infarcted by left anterior descending artery ligation. PTH (80 μg·kg⁻¹) and G-CSF (100 μg·kg⁻¹) were injected for 5 days. Perfusion defects were determined by 99mTc-sestamibi SPECT at days 6 and 30 after MI. The number of BMCs characterized by Lin⁻/Sca-1⁻/c-kit⁻ cells in peripheral blood and heart was analyzed by FACS. Both G-CSF and PTH treatment resulted in an augmented mobilization of BMCs in the peripheral blood. Contrary to G-CSF and controls, PTH and the combination showed significant migration of BMCs in ischemic myocardium associated with a significant reduction of perfusion defects from day 6 to day 30. A combination of both cytokines had no additional effects on migration and perfusion. In our preclinical model, SPECT analyses revealed the functional potential of PTH reducing size of infarction together with an enhanced homing of BMCs to the myocardium in contrast to G-CSF. A combination of both cytokines did not improve the functional outcome, suggesting clinical applications of PTH in ischemic heart diseases.

Regenerative medicine; myocardial infarction; remodeling; cytokines; parathyroid hormone

In recent years, regeneration of damaged myocardial tissue has been attempted using stem and progenitor cells (1, 9, 17). Mobilization of bone marrow-derived stem cells (BMCs) using growth factors such as granulocyte macrophage colony-stimulating factor (G-CSF) offers an alternative to the direct injection of stem cells (10). Circulating mobilized stem cells can be recruited from the blood pool in the damaged myocardial tissue, and this homing behavior is mentioned to play a pivotal role for myocardial regeneration (3). In contrast to preclinical studies showing beneficial effects of G-CSF on myocardial function (8, 16, 18), larger clinical trials failed to demonstrate superiority of G-CSF over placebo treatment when given additionally after myocardial infarction (MI) (11, 28).

In search of alternative cytokines to G-CSF, a promising agent promoting cardiac repair is parathyroid hormone (PTH). PTH is a peptide hormone secreted from the parathyroid glands that mainly acts on bone and kidney cells and is involved in systemic calcium regulation (22). Besides its classical functions, PTH was also shown to act on cardiovascular cells. Upon PTH/PTH-related peptide receptor type I activation, PTH effects an increase of cAMP production leading to a decreased calcium influx resulting in vasodilatation (20). Moreover, it was recently shown that PTH treatment increased proliferation of bone marrow stem cells and facilitated homing to lethally irradiated recipients (7). In patients with primary hyperparathyroidism, we were able to show an increased number of circulating hematopoietic stem cells in the peripheral blood (5). In addition, PTH administration effectively induced stem cell mobilization related to an endogenous release of G-CSF without depletion of the bone marrow (6). Moreover, PTH treatment after MI in mice improved cardiac function associated with increased mobilization and homing of CD45⁺/CD34⁺ stem cells (21, 26). One problem of initially conducted small-animal trials was the lack of noninvasive, repetitive analyses because mice had to be killed for hemodynamic measurements by catheterization and histological analyses (8). Therefore, in the present study, we used the innovative pinhole single-photon emission computed tomography (SPECT) technique, which has recently been validated for myocardial defect size measurement in mice by comparison with histology and allows noninvasive, repetitive, quantitative, and especially intraindividual evaluations of infarct size (25). Because PTH-mediated stem cell mobilization is partly dependent on G-CSF (6), we focused our work on the impact of the two cytokines G-CSF and PTH alone and in combination on change of perfusion defect size after MI and analyzed effects of G-CSF, PTH, and the combination of both on mobilization and homing of BMCs.

Materials and Methods

Animal model and administration of PTH/G-CSF. MI was induced in 8- to 12-wk-old male C57BL/6 mice by surgical occlusion of the left anterior descending artery (LAD) through a left anterolateral approach as described previously (26). As shown in Fig. 1, mice were divided into the following groups: 1) intraperitoneal administration of saline (0.9% NaCl) daily for 5 days, 2) administration of G-CSF daily for 5 days [100 μg·kg⁻¹·day⁻¹ ip; Amgen Biologicals (8)], 3) administration of PTH daily for 5 days [80 μg·kg⁻¹·day⁻¹ ip; Bachem (6, 26)], and 4) administration of G-CSF (100 μg·kg⁻¹·day⁻¹ ip) and PTH (80 μg·kg⁻¹·day⁻¹ ip) daily for 5 days. Because
stimulation with PTH for 5 days showed no differences regarding stem cell mobilization compared with a 14-day administration, we used the short PTH treatment protocol (6). G-CSF and PTH treatment was initiated immediately after the surgical procedure. Animal care and all experimental procedures were performed in strict accordance to the German and National Institutes of Health animal legislation and were approved by the local animal care and use committees.

Perfusion measurement by pinhole SPECT. Animals with either G-CSF or PTH treatment or animals with a combination of G-CSF and PTH treatment as well as infarcted control animals (each n = 7) were scanned. Imaging was performed 6 days and 30 days after LAD ligation. After induction of anesthesia by intraperitoneal injection of a mixture containing medetomidine (0.714 mg/kg), midazolam (7.14 mg/kg), and fentanyl (0.07 mg/kg), each mouse was injected with ~370 MBq 99mTc-sestamibi (Cardiolite; Bristol-Myers Squibb Medical Imaging) in the tail vein. After injection (45 min), the mouse was positioned in the scanner. Left ventricular perfusion was measured using a clinically used triple-headed SPECT system (Prism 3000XP; Philips Medical Systems), with each detector head equipped with a 0.5-mm-diameter custom-made tungsten knife-edge pinhole collimator (Nuclear Fields). The radius of rotation was set to 4 cm (magnification 4), and data were acquired over 20 projection angles (120° for each head), 90 s/projection, giving a total acquisition time of 30 min. Zoom factor was set as 2.0. Center of rotation error was corrected by scanning a multiple-point phantom and iteratively adjusting the center-of-rotation offsets. The same source was used to measure the spatial resolution of the system for 99mTc. Images consisted of a matrix of 128 × 128 × 128 with an isotropic voxel size of 0.445 mm. All of the images were reconstructed using six iterations. Dedicated software was used to generate transverse slices (Fig. 2). A volumetric sampling tool was applied to create polar maps of the relative distribution of activity throughout the left ventricle (15). Each polar map was adjusted for its own maximal value. The size of the defect was calculated with the use of a threshold of 60%, which was derived from the histological infarct sizes as described previously (25). Defect size was indicated as percent of left ventricular myocardium. To investigate the effectiveness of different treatment protocols, the primary endpoint was chosen as change of defect size from 6 days to 30 days after MI.

Flow cytometry. Peripheral blood and heart of the mice were analyzed by flow cytometry [fluorescence-activated cell sorter analysis (FACS)] as specified (6). Briefly, at day 6, 1 ml of peripheral blood was harvested from each mouse by aspirating the carotid artery. Mononuclear cells were separated by density-gradient centrifugation using Histopaque solution (1.077 g/ml; Sigma Chemicals), purified, and resuspended in PBS containing 1% BSA. For cardiac FACS analyses, infarcted hearts of the mice were explanted at day 6 and retrogradely perfused with saline (0.9% NaCl) to wash out circulating blood. Thereafter, a “myocyte-depleted” cardiac cell population (27) was prepared, incubating minced myocardium in 0.1% collagenase IV (GIBCO-BRL) for 30 min at 37°C, lethal to most adult mouse cardiomyocytes. Cells were then filtered through a 70-μm mesh to exclude spurious effects of enzymatic digestion. Cells from peripheral blood and heart were incubated in the dark at 4°C with biotinylated lineage antibodies (CD3, CD4, CD8, Ter119, Gr-1, Mac-1, B220) and a FITC-conjugated Sca-1 antibody and a phycoerythrin-conjugated c-kit antibody (BD Pharmingen). After incubation for 40 min, cells were labeled with a secondary peridinin-chlorophyll protein-conjugated streptavidin (BD Pharmingen). Isotype identical antibodies (BD Pharmingen) served as controls. Cells were analyzed by three-color flow cytometry using a Coulter Epics XL-MCLTM flow cytometer (Beckman Coulter). Each analysis included 50000 events.
RESULTS

Perfusion defects in G-CSF/PTH-treated mice. After showing myocardial perfusion imaging by a human SPECT system is feasible for noninvasive, repetitive, and quantitative, and quantitative infarct size quantification in mice, we evaluated the impact of different cytokine treatment protocols on myocardial perfusion. At day 6 after MI perfusion defects were similar in all groups [Supplemental Fig. S. 1A (Supplemental data for this article may be found on the American Journal of Physiology: Heart and Circulatory Physiology website.)]. In contrast to G-CSF-treated and control mice, administration of PTH and the combination of G-CSF and PTH resulted in a significant decrease in absolute infarct size after 30 days (Fig. 3, A and C, and Supplemental Fig. S. 1B). Evaluating change of defect size from day 6 (baseline) to day 30 after MI, we found a relative reduction of infarct sizes after cytokine therapy, whereas an increase in perfusion defects (+1.36%; Fig. 3B) was only found in controls. PTH treatment (−2.64%) and the combination of G-CSF + PTH (−2.80%) showed more pronounced effects compared with single G-CSF treatment (−1.41%). Compared with single PTH administration, a combination of both cytokines had no additional effects on myocardial perfusion after MI.

Mobilization of BMCs in the peripheral blood and the myocardium. To measure the mobilizing efficiency of G-CSF and/or PTH treatment, BMCs characterized by Lin−/Sca-1+/c-kit+ cells were detected by flow cytometry (FACS) out of the peripheral blood (see Fig. 4). A significant increase of BMCs by 3.8-fold (P < 0.05) after G-CSF, 3.3-fold (P < 0.05) after PTH, and 2.8-fold (P < 0.05) after dual stimulation was detected compared with saline-treated controls. However, there were no significant changes among the different cytokine regimes (Fig. 4, A and B).

Homing of BMCs in the myocardium. To compare the impact of different cytokine treatments on migrated BMCs, we isolated a myocyte-depleted fraction of cardiac cells for FACS analysis. In contrast to G-CSF and control animals, PTH and the combination of both cytokines significantly increased migration of BMCs in the ischemic myocardium by 6.2- and 7.5-fold, respectively (Fig. 5). However, the combination of both cytokines had no additional effect on homing of BMCs to the infarcted myocardium.

DISCUSSION

In our preclinical infarction model, we examined the effect of G-CSF, PTH, and the combination of both on myocardial perfusion and stem cell migration after MI. Our main findings were the following: 1) PTH administration significantly reduced LV perfusion defects after MI, whereas G-CSF alone had no significant beneficial effect on infarct size, 2) G-CSF and PTH treatment enhanced mobilization of BMCs in the peripheral blood, but only PTH and the combination of both were able to augment homing of these cells in the ischemic myocardium, and 3) a combination of G-CSF and PTH was not sufficient to boost PTH effects on myocardial perfusion and homing of BMCs to the ischemic tissue.

In the last years, the concept of cardiac regeneration using BMCs has been developed in many promising preclinical studies. Alternative to the direct delivery (24), BMCs can be mobilized by cytokines such as G-CSF (8) or PTH (26). However, the biological effectiveness and the mechanism of action has not been studied systematically (21). One way to explain the favorable influence on myocardial regeneration is the reduction of ischemic heart tissue (10). To answer this question, a repetitive online measurement of myocardial perfusion is necessary (26). Therefore, we established the SPECT bioimaging technique, which also allows noninvasive, repetitive, quantitative, and especially intra-individual evaluations of infarct size in mice (25). To exclude long-term effects of PTH, we applied the short PTH treatment protocol (6). Our follow-up data of individual mice demonstrated for the first time that treatment with PTH significantly reduced left ventricular perfusion defects after MI. This is in accordance with our recent histological data demonstrating a PTH-mediated decrease in infarct size 30 days after MI associated with a diminished postinfarct remodeling reflected by a reduced wall thinning without detectable signs of hyper trophy (26). Furthermore, PTH treatment was shown to increase myocardial blood flow by a reduction of coronary artery resistance in stunned myocardium of pigs and rats (13). Moreover, PTH treatment after coronary artery ligation in dogs revealed a tissue-protecting effect on the myocardium, ameliorating LV function and preventing the development of cardiogenic shock (12). Recently, our group was able to demonstrate that PTH-treated mice had an improved myocardial function associated with enhanced neovascularization and cell survival (26). These effects could be explained by a PTH-mediated influence on the bone marrow stem cell niche promoting stem cell mobilization and homing (7). Our data are supported by a recently published clinical trial showing the potency of PTH to facilitate stem cell mobilization in humans (2). Moreover, data from our laboratory revealed a positive correlation of PTH levels with the number of hematopoietic stem cells in patients with primary hyperparathyroidism (5). Possible mechanisms for the increased mobilization and homing of stem cells may be a PTH-mediated increase of expression and secretion of growth factors, such as vascular endothelial growth factor, a known inducer of stem cell mobilization and migration (19, 26). In contrast to the enhanced migration of BMCs to the ischemic myocardium after PTH treatment, our data showed an impaired homing of stem cells after G-CSF administration. Recently, G-CSF was shown in bone marrow chimeric mice to reduce migration capacity after MI (4). Furthermore, Honold et al. (3) demonstrated a reduced functional activity of endothelial progenitor cells leading to diminished stem cell homing after G-CSF treatment in humans, which may explain the poor clinical outcome of G-CSF treatment trials after acute MI (11, 28). Therefore, new treatment regimes improving mobilization and homing of BMCs have to be developed (23). In this regard, the application of G-CSF in combination with a dipeptidylpeptidase (DPP-IV) inhibitor was recently shown to increase myocardial homing of circulating stem cells associated with reduced cardiac remodeling and improved heart function after
Fig. 3. Quantification of myocardial perfusion defects by pinhole SPECT. A: bar graph representing defect size measured by pinhole SPECT of control, G-CSF-treated, PTH-treated, and G-CSF + PTH-treated animals at day 6 and day 30 after MI. NS, not significant. B: bar graph representing absolute change of perfusion defect size between baseline (day 6) and day 30 of control, G-CSF, PTH, and G-CSF + PTH mice (each n = 7); all values are means ± SE. C: representative repetitive pinhole SPECT bull’s eye polar maps of control, G-CSF-treated, PTH-treated, and G-CSF + PTH-treated mice at day 6 (top) and day 30 (bottom) after MI. White area represents left ventricular (LV) perfusion defects with signal intensities below the threshold.
MI (27). Recently, it was demonstrated that the beneficial effect of G-CSF can be amplified by the addition of PTH in a model of peripheral ischemia (14), and the combination of PTH and G-CSF improved stem cell mobilization and reduced the number of nonresponders in humans (2). Therefore, we addressed the question whether treatment with G-CSF and PTH may be beneficial for the regeneration of the ischemic myocardium. Interestingly, the combination of both cytokines had no significant effect on the size of infarction. Moreover, our FACS analyses revealed that stem cell mobilization by PTH cannot be further boosted by G-CSF. This may be explained by the data of our group (6). Brunner et al. (6) showed an indirect mobilizing effect of PTH via stimulation of osteoblasts producing G-CSF. PTH administration enhanced endogenous G-CSF protein levels by threefold, and pretreatment of PTH-stimulated mice with a G-CSF antibody inhibited significantly the mobilizing effect of PTH (6). Hence, endogenous G-CSF plays an important role for the mobilizing effect of PTH and cannot be further enhanced by treatment with exogenous G-CSF. Therefore, it will be important to know whether the complete blockage of G-CSF receptors in knockout mice inhibits most of the mobilizing effects of PTH. These data suggest that the reduced perfusion defects after PTH administration cannot completely explain the beneficial outcome. Therefore, additional mechanisms such as reduced apoptosis, direct activation of PTH-receptor positive cardiomyocytes, and proliferation of resident cardiac stem cells sum up to the observed improved myocardial function and diminished remodeling. It will be the goal of future studies to further unravel this mechanistic puzzle by using genetic mouse models and cell lineage tracking. Moreover, the beneficial effects after cytokine treatment have been seen in the absence of reperfusion strategies; therefore, the impact of PTH and G-CSF on infarct size and perfusion defects needs an additional evaluation in an ischemia-reperfusion model.

In summary, our study on cardiac regeneration shows that PTH treatment after MI significantly reduced LV perfusion defects associated with enhanced migration of BMCs in ischemic tissue, whereas G-CSF alone had no significant beneficial effects on perfusion and stem cell homing.
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DISCLOSURES

The Ludwig Maximilians University is the holder of pending patents (“Uses and Methods for Treating Ischemia,” EP 03 02 4526.0 and US 60/514,474 and “Remedies for ischemia,” EP 2007/003272 and US 60/792,943) claiming a second medical use of G-CSF/PTH to treat ischemic organ failure.

REFERENCES