Parathyroid hormone treatment after myocardial infarction promotes cardiac repair by enhanced neovascularization and cell survival

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Aims An ongoing concept is that stem cells have the potential to regenerate the injured myocardium. In addition to direct vasorelaxing effects on the vasculature, which are mediated by an increased cAMP production leading to a decreased calcium influx in smooth muscle cells, parathyroid hormone (PTH) was recently shown to facilitate stem cell mobilization. Therefore, we analysed in a murine model of experimental myocardial infarction (MI) the influence of PTH treatment on survival, functional parameters, stem cell migration, and expression of vascular endothelial growth factor A (VEGF-A).

Methods and results Mice (C57BL/6) were treated with PTH (80 μg/kg/d) for up to 14 days after coronary artery ligation. Functional and immunohistochemical analyses were performed at days 6 and 30 after MI. Stem cells and VEGF expression in the myocardium were analysed by FACS and qRT-PCR at day 2 after MI. PTH-treated animals revealed a significant improvement of post-MI survival and myocardial function that was related to a subsequent reduction of left ventricular wall thinning and scar extension. Infarcted hearts of PTH-treated mice revealed increased numbers of CD45+/CD34+ progenitor cells as well as an upregulation of VEGF-A mRNA associated with increased neovascularization and cell survival.

Conclusions PTH application after MI increases migration of angiogenic CD45+/CD34+ progenitor cells to the ischaemic heart, which may attenuate ischaemic cardiomyopathy. As PTH is already used in patients with osteoporosis, our findings may have a direct impact on the initiation of clinical studies in patients with ischaemic heart disease.

1. Introduction

Parathyroid hormone (PTH) is known to induce arterial vasodilation, which is based on the activation of PTH/PTHrP receptor type I. Upon receptor activation, PTH causes an increase of cAMP production leading to a decreased calcium influx resulting in vasodilation.1,2 In patients with primary hyperparathyroidism, we were recently able to show an increased number of circulating CD45+/CD34+ stem cells in the peripheral blood.3 Moreover, PTH treatment increased proliferation of bone marrow (BM) stem cells and facilitated homing to lethally irradiated recipients.4 In addition, PTH was recently shown in a phase I trial to facilitate stem cell mobilization.5

In several approaches, regeneration of damaged myocardial tissue has been achieved by stem and progenitor cells. Preclinical data of animal models and clinical studies revealed positive effects related to transplanted BM-derived stem cells and endothelial progenitors ameliorating pump function after myocardial infarction (MI).6–11 The original concept of cardiac regeneration by transdifferentiation of BM-derived stem cells to functionally active cardiomyocytes was questioned by the identification of paracrine repair mechanisms leading to neovascularization and prevention of apoptosis.12–16

KEYWORDS
Parathyroid hormone; Myocardial infarction; Neovascularization; VEGF; Stem cells
An elegant alternative to transplantation is the delivery of cytokines like G-CSF or erythropoietin, both known to mobilize stem cells and reduce myocardial damage after MI.\(^1\)\(^7\)\(^-\)\(^20\) We have shown an improved survival and myocardial function after G-CSF administration by ICAM-1-mediated effects on arterial vessel growth.\(^1\)\(^7\) However, randomized clinical trials including our own study revealed no improvement of ejection fraction compared with a placebo-treated control. However, myocardial perfusion was significantly increased in the G-CSF treated group.\(^2\)\(^1\)\(^-\)\(^2\)\(^3\) In addition to direct effects on the vasculature, PTH treatment also affects the BM stem cell niche. Therefore, we investigated the role of PTH treatment after MI in mice. We aimed to define the impact of PTH on post-MI survival and functional parameters, as well as mobilization and migration of BM-derived stem cells. In order to maximize the potential impact of PTH after MI, we decided to inject the biological active fragment of PTH, PTH1–34, for up to 14 consecutive days subcutaneously (s.c.), (Bachem) as described previously\(^4\) for PTH treatment was initiated the day after the surgical procedure (Figure 1A).

2. Methods

2.1 Animal model

MI was induced in 8-12 weeks old male C57BL/6 mice by surgical occlusion of the left anterior descending artery (LAD) through a left anterolateral approach as described previously.\(^1\)\(^7\) Animal care and all experimental procedures were performed in strict accordance 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).

2.2 Administration of PTH (1–34)

Mice were divided into the following groups: Sham-operated animals receiving saline (0.9% NaCl) (1) or PTH1–34 (80 μg/kg/d) for up to 14 days subcutaneously (s.c.), (Bachem) as described previously\(^4\) \(^2\) and infarcted animals receiving saline (3) or PTH (4) accordingly. PTH treatment was initiated the day after the surgical procedure (Figure 1A).

2.3 Functional parameters

For evaluation of pressure-volume relationships in vivo, surviving mice of the previously described groups were anesthetized with thiopental (100 mg/kg, i.p.), intubated, and ventilated (MiniVent, HUGO SACHS, Freiburg, Germany). After catheterization via the right carotid artery, a 1.4 French impedance-micromanometer catheter (Millar Instruments, Houston, TX) was introduced into the left ventricle and pressure-volume loops were recorded. The method was based on measuring the time-varying electrical conductance signal of two segments of blood in the left ventricle from which total volume is calculated. Raw conductance volumes were corrected for parallel conductance by the hypertonic saline dilution method. Therefore, a bolus of 10 μL of 7.5% hypertonic saline was injected via the jugular vein. For absolute volume measurements, the catheter was calibrated with known volumes of heparin-treated mouse blood.\(^2\)\(^5\) Data analyses were performed according to previous literature.\(^2\)\(^5\)

2.4 Histology and immunohistochemistry

At day 6 \((n = 10)\) and day 30 \((n = 6)\), hearts were excised. After fixation in 4% phosphate buffered formalin, the hearts were cut transversally into 2 mm thick slices and embedded in paraffin. Four-micron-thick sections were cut and mounted on positively charged glass slides. Standard histological procedures (haematoxylin/eosin and Masson trichrome) and immunostaining were performed. Infarct size and LV wall thickness was determined as previously described.\(^1\)\(^7\) For immunostaining the following primary antibodies were used: CD31 (goat anti-mouse, Santa Cruz), Ki67 (goat anti-mouse, Santa Cruz), and VEGF-A (rabbit anti-mouse, Zytomed). AEC was used as chromogen. Double staining was performed for CD31 and Ki67 using DAB as chromogen and the APAAP-Rat system and chromogen red ACE (all from Dako), respectively. Apoptotic cells were detected using the TUNEL assay (Apoptag, MP Biomedicals). Quantitative assessments of capillaries: Blood vessel density was assessed by CD31\(^+\) immunohistochemistry in the granulation tissue at the border zone. The numerical density of CD31\(^+\) capillary structures were quantified from 10 random \(\times 400\) fields and was converted to mm\(^2\).

Figure 1

Experimental design and cumulative survival. (A) Animals were divided into four groups and sacrificed at two time points (day 6 and 30) to evaluate the impact of parathyroid hormone on survival, haemodynamic function as well as post-infarct remodelling. After left anterior descending artery ligation, parathyroid hormone was applied (80 μg/kg/d) for up to 14 days. At day 2 after myocardial infarction cardiac FACS-analyses, PCR and TUNEL-staining were performed. (B) Kaplan-Meier curve showing survival rates after myocardial infarction in the parathyroid hormone-treated \((n = 15)\) group compared with saline-treated mice \((n = 25)\). All mice revealed histologically confirmed myocardial infarctions.
2.5 Flow cytometry of peripheral blood and non-myocyte cardiac cells

Eight-to-twelve weeks old C57BL/6 mice (n = 5) were treated with PTH (80 μg/kg/d) or saline daily for 6 days. 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 phosphate-buffered saline containing 1% bovine serum albumin. Cells were incubated for 40 min at 4°C with the following monoclonal antibodies: CD45-PerCP and CD34-FITC (all from BD Pharmingen). Matching isotype antibodies (BD Pharmingen) served as controls. Cells were analysed by two-colour flow cytometry using a Coulter® XL-MCLTM flow cytometer (Beckman Coulter). Each analysis included 20,000 events.

Flow cytometry of cardiac cells was performed from sham-operated (n = 6), infarcted hearts of saline (n = 6) or PTH-treated (n = 8) C57Bl/6 mice at day 2 after MI. Therefore, a ‘myocyte-depleted’ cardiac cell population was prepared, incubating minced myocardium in 0.1% collagenase IV (Gibco BrL) 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, BM cells with or without collagenase treatment were stained revealing no significantly changed staining of labelled cell antigens (data not shown). Cells were labelled with CD45-PerCP and CD34-FITC and subjected to flow cytometry using EPICS XL-MCL flow cytometer and Expo32 ADC Xa software (Beckman Coulter). Each analysis included 50,000 events.

2.6 Quantitative reverse transcriptase–polymerase chain reaction

Infarcted hearts of C57BL/6 mice 48 h after MI (n = 3-5) were explanted and the area of infarction, including its border zone, were separated from the non-infarcted myocardial tissue. Ventricular tissue of explanted hearts of untreated control mice served as controls. Isolation of total RNA from mouse heart tissue was performed using TRI REAGENT (Molecular Research Center, Inc.) according to the manufacturer’s protocol. Reverse transcription was performed using the ImProm-ITM Reverse Transcription System (Promega) according to the manufacturer’s protocol. cDNA samples were analysed by quantitative RT–PCR using the following murine primers purchased from MWG-BIOTECH AG: 18S rRNA (sense, 5’-GGA CAG GAT TGA CAG ATT GAT AG-3’; antisense, 5’-CTC GTT CGT TAT CCG AAT TAA C-3’); VEGF-A (sense, 5’-GAA CTT TCT GCT TCT TTG GG-3’; antisense, 5’-GAC GGC TTG AAG ATG TAC TC -3’). Quantitative RT–PCR was performed using SYBR Green Reaction Mix (Eurogentec) on an ABI PRISM 7900HT Detection System (Applied Biosystems). Each sample was run in duplicate. The expression of each gene within the different tissue samples was quantified relative to 18S RNA expression levels according to the Sequence Detector User Bulletin (Applied Biosystems). Relative mRNA expression of the target genes was related to untreated control hearts.

2.7 Statistical analyses

Results were expressed as mean ± SEM. Multiple group comparisons were performed by one-way analyses of variance (ANOVA) followed by the Bonferroni procedure for comparison of means. Comparisons between two groups were performed using the unpaired Student’s t-test. Statistical analyses were performed according to Table 1. Data were considered statistically significant at a value of P ≤ 0.05. Mortality was analysed by the Kaplan–Meier method.

3. Results

3.1 Improved survival after PTH treatment

According to the experimental design (Figure 1A), cumulative survival of PTH (n = 15) as well as saline (n = 25) treated mice was recorded for 4 weeks after induction of MI (Figure 1B). Mortality was very high within the first 6 days, in particular in the saline-treated group, but decreased afterwards in both groups. Thirty days after MI, PTH-treated animals showed a significantly improved survival (60 vs. 40%).

3.2 Beneficial effects of PTH treatment on myocardial function after MI

Using conductance catheters, pressure–volume relations were measured from sham-operated, saline- and PTH-treated mice at day 6 and 30 after MI in vivo. Six and 30 days after LAD ligation, saline- and PTH-treated animals showed a significantly decreased systolic and diastolic function (Figure 2A). At day 6, PTH treatment resulted in an improved systolic function reflected by an increased ejection fraction (Figure 2B), cardiac output, and contractility (Table 1). Effective arterial elastance, Ea, was calculated from the ratio of endystolic pressure and stroke volume, and is known to be related to peripheral resistance.26,27 Our data showed that Ea, and therefore peripheral resistance, were significantly reduced in PTH-treated animals (Figure 2C). Diastolic heart function reflected by the isovolumetric relaxation parameter Tau weiss was also improved after PTH treatment (Table 1). At day 30, ejection fraction (Figure 2B) and cardiac output were significantly improved in the PTH-treated group. Similar to day 6, arterial elastance was significantly reduced at day 30 after PTH treatment (Figure 2C). Moreover, diastolic relaxation was improved after PTH treatment reflected by an accelerated diastolic relaxation constant Tau weiss (Table 1).

3.3 Attenuated infarct remodelling after PTH treatment

Histological analyses revealed no difference in LV-infarct size between saline and PTH-treated mice at day 6 (37.3 ± 4.9 vs. 39.8 ± 3.1% of total LV area, P=n.s.), but 30 days after MI infarct size was significantly smaller in PTH-treated hearts (23.3 ± 4.6 vs. 33.6 ± 2.6%, P < 0.05; Figure 3A). At day 6 and at day 30, the anterior wall of the PTH-treated group was thicker compared with saline-treated animals (day 6: 0.58 vs. 0.42 mm, day 30: 0.22 vs. 0.13 mm). The anterior wall thickness of the myocardium declined over time in both groups, however, to a smaller extent in hearts of PTH-treated animals (Figure 3B and C). At day 30, the hearts of saline-treated animals revealed a distinct post-infarct remodelling reflected by a pronounced thinning and formation of large apical aneurysms. In contrast, hearts of PTH-treated mice showed a lower frequency of aneurysms (Figure 3C). Furthermore, the hearts of PTH- and saline-treated infarcted animals were evaluated at day 30 for signs of cardiac hypertrophy by measuring the thickness of the septal (1.00 ± 0.19 vs. 0.86 ± 0.13, P ≤ 0.18) and the right ventricular wall (0.43 ± 0.04 vs. 0.37 ± 0.05 mm, P ≤ 0.36) revealing no significant differences.
Table 1  Haemodynamic data

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<th>Parameters</th>
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<th>MI + saline, d6 (n = 6)</th>
<th>MI + PTH, d6 (n = 6)</th>
<th>MI + saline, d30 (n = 10)</th>
<th>MI + PTH, d30 (n = 6)</th>
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<tr>
<td>HR (b.p.m.)</td>
<td>460 ± 4.6</td>
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<td>MAP (mmHg)</td>
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<td>Pmax (mmHg)</td>
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<td>62.6 ± 4.0***</td>
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<td>EDV (μL)</td>
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<td>43.9 ± 7.4</td>
<td>38.3 ± 3.6***</td>
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<td>EF (%)</td>
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<td>14.5 ± 1.3***</td>
<td>30.5 ± 6.0**</td>
<td>16.8 ± 0.9***</td>
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<td>CO (μL/min)</td>
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<td>7319 ± 563</td>
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<td>5537 ± 477**</td>
<td>2678 ± 360***</td>
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<td>dp/dt max (mmHg/s)</td>
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<td>Tau weiss (ms)</td>
<td>8.3 ± 0.5</td>
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<td>7.8 ± 0.3***</td>
<td>6.8 ± 0.3**</td>
<td>9.9 ± 0.4*,***</td>
<td>8.2 ± 0.4**</td>
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<tr>
<td>Arterial elastance Ea (mmHg/μL)</td>
<td>6.1 ± 0.7</td>
<td>5.5 ± 0.4</td>
<td>8.9 ± 0.9***</td>
<td>6.0 ± 0.5**</td>
<td>11.5 ± 1.6***</td>
<td>7.5 ± 0.6**</td>
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Pressure–volume relations were gained from sham-operated mice with and without PTH treatment, from saline as well as PTH-treated mice at day 6 and 30 after MI in vivo using Millar-tip conductance catheters. PTH, parathyroid hormone; MI, myocardial infarction; HR, heart rate; Pmax, maximal pressure of LV; MAP, mean arterial pressure; EDV, end-diastolic volume; EF, ejection fraction; CO, cardiac output (HR × stroke volume); dp/dt max, derivative of maximum rate of change in left ventricular pressure; SW, stroke work (area enclosed by pressure–volume loops); dp/dt min, derivative of minimum rate of change in left ventricular pressure; Tau weiss, time constant of the left ventricular isovolumic relaxation; arterial elastance (Ea), endsystolic pressure/stroke volume.

*P < 0.05: MI + saline d6 vs. MI + saline d30 and MI + PTH d6 vs. MI + PTH d30.

**P < 0.05: MI + saline vs. MI + PTH d6 and MI + saline vs. MI + PTH(1-34) d30.

***P < 0.05: Sham vs. MI + saline d6 and MI + saline d30.

Values are mean ± SEM.
3.4 Enhanced mobilization of CD45+/CD34+ stem cells after PTH treatment

To show the extent of stem cell mobilization after PTH application, FACS analyses from peripheral blood samples drawn at day 6 with or without ischaemia were performed. Compared with controls, the amount of CD45+/CD34+ stem cells increased 4.4-fold at day 6 after PTH treatment. Although there was a tendency towards higher values, myocardial ischaemia did not significantly increase the amount of PTH-induced stem cell mobilization at day 6 (Figure 4A and B).

3.5 Paracrine supply of VEGF-A associated with increased neovascularization and reduced apoptosis

Since mobilized CD34+ BM stem cells are known carriers of growth factors, we investigated the expression level of the pro-angiogenic factor VEGF-A. PTH treatment significantly increased the expression level of VEGF-A (10.8 ± 1.9 vs. 4.7 ± 0.6; P < 0.05) in the infarct area (Figure 5A). The expression pattern in the remote area was not significantly changed. To further elucidate the possible source of...
VEGF-A in the ischaemic heart, immunohistochemical stainings were performed. Our data showed an increased level of VEGF-A protein in infiltrated cells exclusively in the granulation tissue (Fig 5B). The upregulated level of VEGF-A was associated with a significantly increased numerical density of CD31\(^+\) capillary profiles at day 6 and at day 30 after PTH application (Figure 5C and D). Co-staining using CD31 and Ki67 antibodies demonstrated a proliferation of CD31\(^+\) endothelial cells at the border zone (see Supplementary material online, Figure S1). Since VEGF exerts anti-apoptotic effects, TUNEL-stainings of infarcted hearts were performed. In the hearts of sham-operated animals, almost no apoptotic cardiomyocytes were found (data not shown). In contrast, 48 h after MI, a high number of cardiomyocytes (36 \(\pm\) 0.06\%) at the border zone stained TUNEL positive, whereas PTH treatment significantly reduced the amount of apoptotic cells (19 \(\pm\) 0.02\%) (Figure 5E and F).

### 3.6 Enhanced homing of BM-derived CD45\(^-\)/CD34\(^+\) stem cells after PTH treatment

Based on an increased stem cell mobilization and neovascularization after PTH treatment, we addressed the question whether BM-derived progenitors contribute to myocardial regeneration. Sham-operated animals revealed a very small population of cardiac cells expressing the surface markers CD45 and CD34 (0.13\%). Forty-eight hours after myocardial ischaemia, the amount of migrated CD45\(^-\)/CD34\(^+\) progenitor cells (0.28\%) did not increase. PTH treatment significantly enhanced the migration of CD45\(^-\)/CD34\(^+\) cells.
stem cells (0.78%) when compared with sham-operated and saline-treated infarcted mice (Figure 6A and B).

4. Discussion

In this study, we examined the effect of PTH1-34 on survival, cardiac function, histopathological changes, and stem cell migration after MI. Our main findings suggest a novel role of PTH on myocardial remodelling and stem cell homing: PTH treatment after MI exerts beneficial effects on survival and myocardial function which can be explained by (i) vasodilating effects leading to a reduced peripheral resistance, (ii) an altered remodelling reflected by a reduced LV wall thickness and a smaller infarct size, and (iii) an augmented mobilization and homing of angiogenic CD45\(^+\)/CD34\(^+\) stem cells. The presence of these cells may lead to paracrine supply of VEGF-A and improved neovascularization.

Administration of PTH significantly increased survival 30 days after MI. Recently, we found comparable survival rates after treatment with G-CSF, which are in accordance to other groups and were related to an increased arterial vessel growth by activation of ICAM-1.\(^1\)\(^,\)\(^17\)\(^,\)\(^28\) However, haemodynamic data showed differences in LV ejection fraction between G-CSF and PTH-treated animals 6 days after MI.\(^17\) The early effects of PTH on myocardial pump function can be related to direct effects on smooth muscles cells leading to vasodilation.\(^1\)\(^,\)\(^2\)\(^,\)\(^29\)\(^,\)\(^30\) Arterial vasodilation is based on the activation of PTH/PTHrP receptor type I, which is known to be expressed on smooth muscle cells.\(^29\) Receptor activation results in an increased cAMP production leading to a decrease of Calcium influx, which results in vasodilation.\(^1\)\(^,\)\(^2\) At day 6 and day 30, our data suggested sustainable vasodilation reflected by a reduced effective arterial elastance, a known parameter to be related to peripheral resistance.\(^27\) Since the early effects of PTH on peripheral resistance can be explained by a direct impact on smooth muscle cells, the late effects may reflect an attenuated myocardial remodelling. In this context, PTH treatment was shown to increase myocardial blood flow by reduction of coronary artery resistance in stunned myocardium of pigs and rats.\(^31\) Moreover, PTH administration intravenously starting 30 min after coronary occlusion in dogs exerted a tissue-sparing effect on the myocardium, restored LV function, and prevented the development of cardiogenic shocks in a short-term manner.\(^32\) Since clinical trials with the cytokine G-CSF evidenced no improvement in EF,\(^22\) although experimental data showed increased neovascularization and collateral vessel growth,\(^17\)\(^,\)\(^19\) effects like early vasorelaxation may favour PTH for the treatment after MI.

In addition, PTH treatment revealed a stable mobilization of CD45\(^+\)/CD34\(^+\) BM stem cells into the peripheral blood. A recently published phase I trial supports our data showing the potency of PTH to facilitate stem cell mobilization.\(^5\) Moreover, data from our laboratory show a positive correlation of PTH levels with the number of CD34\(^+\) cells in patients with primary hyperparathyroidism.\(^3\) Possible mechanisms for the increased mobilization of stem cells may be a PTH-mediated enforced expression and secretion of SDF-1\(\alpha\), MMP-9, and G-CSF in the BM, which are known inducers of stem-cell mobilization and homing.\(^33\)\(^,\)\(^34\) Since we found an increased mobilization and homing of CD45\(^+\)/CD34\(^+\) progenitor cells, we addressed the question if these cells may have induced neovascularization in the ischaemic myocardium. Our data showed an improved angiogenesis reflected by a high number of CD31\(^+\) vessels at the infarct border zone, which was associated with an increased expression
and supply of VEGF-A. Our data are supported by previous reports showing that transplantation and mobilization of CD34⁺ stem cells from peripheral blood contribute to enhanced neovascularization and reduced apoptosis after MI. However, it remains to be elucidated, if the increased expression of proangiogenic factors like VEGF-A is a direct effect of PTH or results from migrated blood cells secreting angiogenic growth factors. In this regard, Majka et al. showed that CD34⁺ stem cells isolated from BM and peripheral blood express high levels of numerous growth factors and cytokines, which may contribute to neovascularization. Moreover, Norol et al. detected after transplantation of CD34⁺ cells in the area of infarction a two-fold increased expression of VEGF-A. In our own study, immunostaining of hearts after MI revealed a high number of cells in the granulation tissue, which stained positive for VEGF-A protein supporting the hypothesis that angiogenic factors have been carried to the site of ischaemia. However, additional paracrine factors may also contribute to improved angiogenesis and reduced apoptosis. Yet,
the direct influence of PTH on neovascularization is not well understood so far. Direct effects of PTH on the expression of VEGF are suggested in a nude mouse model, where serum levels of intact human PTH correlated with VEGF protein in the transplants of human parathyroid glands. It will be the goal of further studies to elucidate the contribution of the suggested mechanisms. In this regard, the use of tracked BM cells could be of interest. Furthermore, the impact of PTH on resident cardiac stem cells should also be addressed.

In summary, our results show that PTH application after MI ameliorates myocardial function, which apart from direct vasodilating effects may be explained by the mobilization and homing of CD45⁺/CD34⁺ stem cells to the ischaemic myocardium. This may lead to improved neovascularization and cell survival via supply of VEGF-A. Since PTH is already used in patients with osteoporosis, our findings may have direct impact on the initiation of clinical studies in patients with ischaemic heart disease.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: The Ludwig Maximilians University is the holder of a pending patent (‘Remedies for ischemia’ EP 2007/003272 and US 60/792 943) claiming a second medical use of PTH to treat ischaemic organ failure.

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