Pressure-induced cardiac overload induces upregulation of endothelial and myocardial progenitor cells

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Aim The regulation of angiogenesis in the hypertrophied overloaded heart is incompletely understood. Bone-marrow-derived progenitor cells have been shown to contribute to endothelial homeostasis, repair, and new blood vessel formation. We therefore studied the effects of pressure overload on angiogenesis and progenitor cells.

Methods and results Pressure overload induced by transaortic constriction (TAC, C57/Bl6 mice, 360 μm for 35 days) increased left ventricular (LV) systolic pressure, the ratio of heart weight to tibia length, cardiomyocyte diameters, and cardiac apoptosis and fibrosis compared to sham-operated mice. In the TAC group, the number of cycling Ki67pos cells increased from none to 0.1 ± 0.02% in cardiomyocytes and from 0.17 ± 0.02% to 0.65 ± 0.1% in non-cardiomyocytes, P < 0.001. Stem cell antigen 1pos/vascular endothelial growth factor receptor 2pos endothelial progenitor cells (EPC) increased to 210 ± 25% in the blood and to 196 ± 21% in the bone marrow (P < 0.01). TAC upregulated cultured spleen-derived DiLDLpos/lectinpos EPC to 221 ± 37%, P < 0.001. Cardiac hypertrophy and upregulation of EPC secondary to cardiac pressure overload were associated with increased extra-cardiac neoangiogenesis (54 ± 12% increase, P < 0.05). In endothelial nitric oxide synthase double knockout mice, the upregulation of EPC by TAC was abolished. Maladaptive myocardial remodelling in TAC mice was characterized by a reduction of CD31pos cells. In mice transplanted with green fluorescent proteinpos bone marrow, TAC markedly increased myocardial bone marrow-derived CD31pos cells from 2.37 ± 0.4% to 7.76 ± 1.5% and MEF2pos cells from 1.8 ± 0.4/mm² to 20.5 ± 5.3/mm², P < 0.05.

Conclusion Pressure-induced myocardial hypertrophy leads to upregulation of systemic EPCs, increased extra-cardiac angiogenesis, and upregulation of intra-myocardial bone marrow-derived endothelial and myocyte precursor cells. The data show that afterload-dependent regulation of bone marrow-derived progenitor cells contributes to angiogenesis in myocardial hypertrophy.

KEYWORDS myocardial hypertrophy; endothelial progenitor cells; angiogenesis; transgenic animal models; nitric oxide

1. Introduction

Hypertrophy and remodelling of cardiac muscle occur in pathological load situations such as hypertension or aortic valve stenosis. Sustained pressure load leads to contractile dysfunction and heart failure,¹ which is associated with high morbidity and mortality.² During the development of myocardial hypertrophy, structural changes of the microvasculature occur.³,⁴ The diffusion distance from capillaries to cardiomyocytes increases, and the contractile reserve is diminished.⁵ It has been suggested that a mismatch between the ratio of capillaries and cardiomyocytes develops, leading to myocardial ischaemia.⁶–⁹ These observations indicate that the regulation of angiogenesis may play an important role in cardiac hypertrophy.

Angiogenesis is not only determined by preformed vascular structures. Circulating cells derived from the bone marrow significantly contribute to vascular function and growth. Specifically, endothelial progenitor cells (EPC) play an important role during angiogenesis and vascular repair.¹⁰,¹¹ In patients with coronary artery disease the level of circulating CD34posKDRpos EPC predicts cardiovascular events and death from cardiovascular causes.¹² The number of EPC is regulated by interventions such as physical activity or lipid-lowering with statins increasing the number and migratory function of EPCs.¹³,¹⁴ Vascular risk factors such as hypertension, hyperlipidemia or diabetes may reduce their number and function.¹⁵–¹⁷ The effects of myocardial hypertrophy on EPC numbers and their function in remodelling are not known.

In addition to their contribution to vascular function, recent studies have presented evidence for the potential role of bone-marrow-derived progenitor cells in myocyte...
regeneration in the human heart. Hsieh et al. provided evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after TAC. Cardiomyocytes undergoing cell cycle progression have been identified after myocardial infarction as well as in cardiac hypertrophy. Whether these cycling cells are derived from progenitor cells of bone marrow origin or from resident cardiac precursor cells is still fervently debated. The aim of this study was to examine the effect of pressure-induced myocardial hypertrophy on bone-marrow-derived progenitor cells and angiogenesis.

2. Methods

2.1 Animals and aortic constriction

The study was approved by the animal ethics committee of the Universität des Saarlandes and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Pub. No. 85-23, revised 1996). Ten-week-old male C57Bl/6j (Charles River Laboratories, Sulzfeld, Germany) and endothelial nitric oxide synthase (eNOS) mice (B6129/P2-Nos3, Charles River Laboratories, Sulzfeld, Germany) were housed under standard conditions. Animals were anaesthetized with ketamine (100 mg/kg body weight, i.p.) and xylazine (10 mg/kg, i.p.) for transaortic constriction (TAC). Once orotracheal intubation using a 20 gauge catheter, the tube was connected to a volume cycled rodent ventilator (Harvard Apparatus, Holliston, MA) on supplemental oxygen with a tidal volume of 0.2 ml and respiratory rate of 110/min. The chest cavity was entered in the second intercostal space at the left upper sternal border through a small incision and aortic concretion was performed by tying a 7-0 nylon suture ligature against a 27 gauge needle to yield a narrowing 360 μm in diameter and a transverse aortic concretion of 65–70%. Control mice underwent a sham operation. After 5 weeks, animals were anaesthetized with ketamine (100 mg/kg body weight, i.p.) and xylazine (10 mg/kg, i.p.) and a 1.4 Fr pressure-transducing catheter (Milnor Instruments) was used for LV-pressure measurements. After the LV-pressure measurements hearts were rapidly excised and embedded in paraffin after fixation in PBS-buffered formalin (4%). Blood and bone marrow were sampled, as well as the spleen for further preparation.

2.2 Bone marrow transplantation

Six-week-old C57Bl/6-Tg(ACTbEGFP)1Ob mice (Jackson laboratory) were killed and marrow was obtained from the long bones. Unfractionated green fluorescent protein (GFP)-expressing bone marrow cells (1–2 × 107) were transplanted into 10-week-old male, lethally irradiated (total dose 9 Gy) wild-type C57Bl/6 recipient mice by injection into the tail vein 5 h after irradiation. Four weeks after bone marrow transplantation, transaortic constriction or sham operation was performed as described above.

2.3 Fluorescence-activated cell sorter analysis

Blood and bone marrow from at least 13 respectively 8 animals per group were analysed as described previously. The viable lymphocyte population was analysed for stem cell antigen 1 (Sca-1)-FITC (E13-161.7, Pharmingen, Germany) and vascular endothelial growth factor receptor 2 (VEGFR-2) (Flk-1; Avas12x1, Pharmingen, Germany) conjugated with the corresponding phycoerythrin-labelled secondary antibody (Sigma, Germany). Isotype-identical antibodies served as controls in every experiment (Becton Dickinson, Germany).

2.4 Culture of spleen-derived endothelial progenitor cells

In mice, the spleen functions as a haematopoietic organ. Spleen mononuclear cells from at least six animals per group were isolated and cultured in fibronectin (Sigma, Germany) as described previously. Antibiotics, calf serum, and cell culture medium were obtained from Invitrogen. After 7 days in culture, EPCs were identified by uptake of 1,1′-dioctadecyl-3,3,3′,3′ tetramethylindo-carbocyanine-labelled acetylated low-density lipoprotein (DilDL; 2.4 μg/ml; CellSystems, Germany) and staining with FITC-labelled Giffonia (bandeiraea) simplicifolia lectin I (lectin, 10 μg/ml; Vector Laboratories, Burlingame, CA). At least three random high-power fields of each well were analysed using a Nikon E600 epifluorescence microscope (Nikon, Germany) with appropriate filters. Cells double positive (yellow) for red Dil-AC-MLD and green lectin staining were judged to be EPCs and were counted.

2.5 Disc angiogenesis model

A disc of polyvinyl alcohol sponge (Rippey, Germany), covered with nitrocellulose cell-impermeable filters (Millipore, Germany), allowed capillaries to grow only through the rim of the disc. The discs were implanted subcutaneously. After 14 days, space-filling fluorescent microspheres (0.2 μm; Invitrogen, Germany) were injected into the LV to deliver them to the systemic microvasculature. The area of the disc invested by fibrovascular growth was assessed with Lucia Measurement version 4.6 software.

2.6 Immunofluorescence analysis

For pretreatment slices with 3 μm thick sections were placed in Coplin jars with 0.05% citraconic anhydride solution, pH 7.4 for 1 h at +98°C, and then incubated overnight at 4°C with the first antibody, followed by the appropriate secondary antibody at 37°C for 1 h. Immunofluorescence studies were performed by applying monoclonal antibodies against α-sarcomeric actin (clone5c5, Sigma-Aldrich, Germany) to detect cardiomyocytes. Serial sections were also counterstained for endothelial cells (von Willebrand factor, clone FB/68, DAKO, Germany; CD 31, Santa Cruz, Germany) and smooth muscle cells (α-smooth muscle actin, clone 1A4, Sigma-Aldrich, Germany). By using the citraconic anhydride pretreatment, even staining for CD31 of the formalin-fixed tissue was possible, which was before only reported in tissue fixed in zinc–formalin or in cryosections. Co-immunostaining for the antigen Ki67 (Novocastra Laboratories LTD, UK) was used to detect cycling cells. In co-immunostainings of bone-marrow-transplanted mice for CD31, for the myocyte enhancer factor 2 (MEF2, Santa Cruz, Germany) and for α-smooth muscle actin a GFP-specific antibody (abcam, UK) was used to enhance GFP-fluorescence. FITC-, TRITC-, Biotin-, Peroxidase- (all Dianova, Germany), and AlexaFluor 635-conjugated (Invitrogen, Germany) anti-mouse IgM, anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG were used as secondary antibodies for amplification if necessary. Sections were counterstained with DAPI (Calbiochem, Germany), washed and mounted with fluorescent-mounting medium (Vectashield, Vector Laboratories, Burlingame, CA) for fluorescence microscopic analysis. All sections were evaluated using a Nikon E600 epifluorescence microscope (Nikon, Germany) with appropriate filters. In addition colocalization of stainings (e.g. Ki67 and α-sarcomeric actin) were controlled using a confocal microscopy unit (QLC100, Vistech, UK, on a Nikon E600 microscope).

2.7 Apoptosis detection

To detect apoptosis 3 μm thick paraffin sections of formalin-fixed mice heart tissue were examined using the ApopTag Peroxidase In Situ Oligo Ligation Kit from Chemicon according to manufacturer’s instructions. The in situ oligo ligation assay specifically detects apoptosis by staining only cells that contain double-stranded breaks
that are blunt-ended or have a one base 3′ overhang (cells containing nicked, gapped, 3′-recessed, 3′-overhanging ends longer than one base and single-stranded ends are not detected). Unlike conventional terminal transferase-based labelling (TUNEL), the assay stains apoptotic but not necrotic or transiently damaged cells.25

To distinguish if the apoptotic cells were cardiomyocytes, endothelial cells or other cardiac cells and if they were derived from the bone-marrow and therefore expressed GFP, specific immunostaining was performed after the in situ oligo ligation assay. Apoptosis was detected using light field microscopy for the brown 3′-3,3′-diaminobenzidine staining of the apoptosis kit; the co-immunostaining for the respective marker was evaluated by switching to the fluorescence unit of the same Nikon E 600 microscope.

2.8 Tissue morphometry
The procedures used for morphometric analyses are provided in detail in the Supplementary material online.

2.9 Statistical analysis
Results are presented as mean ± standard error of the mean (SEM). Unpaired t test, Mann–Whitney test or two-way ANOVA with a Bonferroni post hoc test were used where applicable. Values of P < 0.05 were considered significant.

3. Results
3.1 Myocardial hypertrophy and haemodynamic data
Transaortic constriction (360 μm, 35 days, n = 23; sham group n = 19) increased the ratio of heart to body weight (%) from 5.18 ± 0.19 in sham-operated mice [bone-marrow-transplanted sham mice (BM-SHAM), n = 15, 5.7 ± 0.2] to 8.0 ± 0.5 after transaortic constriction (P < 0.0001 for both groups) [BM-SHAM after transaortic constriction (BM-TAC), n = 26, 7.8 ± 0.3] and the ratio of heart weight to tibia length (milligrams/millimetres), respectively, from 8.4 ± 0.3 (BM-SHAM 6.9 ± 0.3) to 12.3 ± 1.1 (BM-TAC 9.2 ± 0.4), (P < 0.01 and P < 0.001, Figure 1A).

Animals with aortic constriction exhibited increased LV systolic pressure from 121 ± 5 mm Hg in TAC (BM-TAC 97 ± 6 mm Hg) vs. 79 ± 5 mm Hg in BM-SHAM (67 ± 3 mm Hg) (P < 0.001 and P < 0.001, Figure 1B). These data correlate to those obtained in a previous study.26

3.2 Histopathology data
Cardiomyocyte short axis diameter as measured microscopically in cross-sectioned LV tissue sections was used to determine cardiomyocyte size (n ≥ 7/group). Transaortic constriction led to an increase in cardiomyocyte short axis diameter (TAC 15.5 ± 0.5 μm; BM-TAC 12.3 ± 0.3 μm) compared with sham-operated animals (SHAM 10.5 ± 0.4 μm, BM-SHAM 9.2 ± 0.3 μm; P < 0.000001 and P < 0.00001, Figures 1C–E). Measurement of cardiomyocytes per high-power field showed that the number of cardiomyocytes per millimetre² was lower in animals after transaortic constriction (TAC 480 ± 25; BM-TAC 584 ± 24) in comparison with the sham group (SHAM 648 ± 29, BM-SHAM 694 ± 34; P < 0.001 and P < 0.05).

Figure 1 Effect of transaortic constriction to 360 μm on the ratio of heart weight to tibia length (A), on left ventricular (LV) systolic pressure (B), and on cardiomyocyte short axis diameter (C) after 35 days. n.s. not significant, * P < 0.05, # P < 0.01, $ P < 0.001, ** P < 0.0001, ### P < 0.00001, #### P < 0.000001. Representative cardiac sections from mice, which underwent sham procedure (D) or transaortic constriction (E) after immunostaining for the myocyte marker α-sarcomeric actin (red). Nuclei are stained blue by DAPI. Bars = 10 μm.
A significant increase of cardiac fibrosis in mice after transaortic constriction was measured morphometrically (n ≥ 7/group) using Sirius red staining compared with sham [3.9 ± 0.7 fold increase (see also Supplementary material online, Figure S7A and B), respectively 3.4 ± 0.8 fold increase in BM-transplanted animals; P < 0.05 for both groups].

Transaortic constriction led to significantly enhanced apoptosis of cardiomyocytes (0.7 ± 0.2%) and non-cardiomyocytes (1.0 ± 0.3%) in the heart compared with sham-operated mice (0.02 ± 0.01% respectively 0.13 ± 0.05%; P < 0.05 for both) (see Supplementary material online, Figure S8).

3.3 Transaortic constriction increases markers of cycling cardiac cells

The nuclear protein Ki67, which is expressed during all stages of the cell cycle excluding G₀, was used to measure the number of cardiac cells undergoing cell division (n ≥ 9 in each group). Numbers of Ki67 expressing non-cardiomyocytes increased significantly from 3.7 ± 0.7/mm² (0.17 ± 0.02% Ki67⁺⁺ non-cardiomyocytes) in the sham group to 17.5 ± 2.4/mm² (0.65 ± 0.1% Ki67⁺⁺ non-cardiomyocytes) after aortic constriction (P < 0.001, Figures 2A and C). Although there were no cycling cardiomyocytes in sham-operated mice, 0.5 ± 0.1 cardiomyocytes/mm² (0.1 ± 0.02% Ki67⁺⁺ cardiomyocytes) expressed Ki67 in mice after aortic constriction (P < 0.001, Figures 2B and D and Supplementary material online Figures S7C–E). Two of these Ki67-positive cells were binucleated (Supplementary material online, Figure S7E). The histological feature of ongoing mitosis was not observed.

In the BM-SHAM the results were similar: Numbers of Ki67 expressing non-cardiomyocytes increased significantly from BM-SHAM (11.4 ± 4.4/mm², 0.49 ± 0.17%) to BM-TAC (24 ± 5.1/mm², 1.1 ± 0.23%; P < 0.05) as well as the numbers of Ki67-expressing cardiomyocytes from BM-SHAM (0.1 ± 0.07/mm², 0.02 ± 0.01%) to BM-TAC (0.5 ± 0.1/mm², 0.09 ± 0.03%; P < 0.05).

3.4 Myocardial hypertrophy is associated with endothelial nitric oxide synthase dependent upregulation of endothelial progenitor cells

The effect of increased cardiac afterload induced by transaortic constriction on the numbers of EPC in peripheral blood, bone marrow, and spleen is depicted in Figure 3 and Supplementary material online Figure S9 (n ≥ 6/group). Aortic constriction for 35 days increased EPC numbers to 210 ± 25% (P < 0.001), 196 ± 21% (P < 0.01), and 221 ± 37% (P < 0.001) of baseline in blood, bone marrow, and spleen, respectively. In eNOS⁻/⁻ mice there was no difference in EPC numbers in peripheral blood or bone marrow between sham-operated (n = 8) or transaortic-constricted mice (n = 12).

3.5 Pressure-induced myocardial hypertrophy leads to increased angiogenesis

EPCs have been reported to significantly contribute to new blood vessel formation. Therefore, we determined whether pressure-derived myocardial hypertrophy modulates the angiogenic response in vivo in a model of inflammation-induced neoangiogenesis in mice (Figure 4). Subcutaneous implantation of a polyvinyl sponge for 14 days resulted in an in-growth of new vessels. Transaortic constriction, initiated after implantation of the disc, increased the area of neoangiogenesis by 54 ± 12% compared with sham-operated mice (n = 7/group).

CD31 staining was performed in sections of mouse hearts (Figure 5). The number of CD31-expressing cells decreased significantly from 475 ± 28/mm² in sham to 376 ± 27/mm² after transaortic constriction (P < 0.05), whereas the ratio...
of CD31\(^{\text{pos}}\) cells to total cells (0.16 ± 0.01 in sham vs. 0.13 ± 0.01 in transaortic constriction) as well as the ratio of CD31\(^{\text{pos}}\) cells to cardiomyocytes did not change (detected by staining for \(\alpha\)-sarcomeric actin) (0.75 ± 0.04 in sham vs. 0.78 ± 0.08 in TAC). Stainings for von Willebrand factor (see Supplementary material online, Figure S10) showed similar results as the CD31 staining confirmed the data. The percentages of apoptotic CD31 cells increased significantly with elevated afterload (0.04 ± 0.01% in sham vs. 0.24 ± 0.07% in TAC) (see Supplementary material online, Figure S11). In eNOS\(^{-/-}\) mice, numbers and ratios of CD31\(^{\text{pos}}\) cells in SHAM and TAC animals did not significantly differ from those of the wild-type animals.

### 3.6 Identification of bone-marrow-derived cardiac cells after transaortic constriction

Transplantation of GFP-positive bone marrow cells in lethally radiated wild-type mice was used to detect, if pressure-induced myocardial hypertrophy leads to integration and transdifferentiation of bone-marrow-derived cells (\(n = 10\) for sham respectively transaortic constriction) in the heart. To examine to what degree bone-marrow-derived cells contribute to cardiac angiogenesis, co-staining of heart sections for CD31 and GFP was performed (see Figures 6A and C). The number of CD31/GFP double-positive cells increased significantly from 9.2 ± 1.7/mm\(^2\) in sham to 35.4 ± 6/mm\(^2\) in mice after transaortic constriction \((P < 0.01)\). In sham mice 2.4 ± 0.4% of CD31\(^{\text{pos}}\) cells were positive for GFP, after transaortic constriction 7.8 ± 1.5% \((P < 0.05)\).

We did not detect any large cardiomyocyte derived from bone marrow by co-staining with \(\alpha\)-sarcomeric actin and GFP. By performing simultaneous staining for GFP and MEF2, a transcription factor expressed in cardiomyocytes and in cardiac progenitor cells,\(^{29}\) we observed an increase in GFP\(^{\text{pos}}\)/MEF2\(^{\text{pos}}\) cells/mm\(^2\) in transaortic constriction group (20.5 ± 5.3) vs. sham group (1.8 ± 0.4; \(P < 0.05\)) (see Figures 6B and D, Supplementary material online Figure S12 and S13). These cells were negative for smooth muscle actin in co-immunostaining in which staining for GFP, MEF2 and smooth muscle actin was simultaneously performed.

Percentages of GFP\(^{\text{pos}}\) apoptotic non-cardiomyocytes increased significantly after aortic constriction in BM-SHAM (3.8 ± 1%, BM-TAC 20 ± 6%) (see Supplementary material online, Figure S14). The percentage of K\(i\)67\(^{\text{pos}}\) non-cardiomyocytes, which were positive for GFP, did not change significantly (BM-SHAM 24 ± 3.6%, BM-TAC 24.3 ±

![Figure 3](image-url)  
**Figure 3** Effect of transaortic constriction on EPC numbers in spleen (A-C) of wild-type mice as well as in peripheral blood and bone marrow of wild-type (D, E) and of eNOS\(^{-/-}\) animals (F, G). In (A) EPC were measured as co-expression of DLDL-labelling and lectin, in (D-G) by double labelling for sca-1 and VEGF-receptor-2 by FACS-analysis. Numbers are expressed as percentage of the EPC number measured in the relevant sham-group. n.s. not significant, \# \(P < 0.01, \$ P < 0.001\). (B) and (C) show spleen-derived cells from mice after sham procedure (B) and after transaortic constriction (C). DLDL-staining is depicted in red, Lectin in green. Double-stained cells in yellow are regarded as EPCs. Bars = 20 \(\mu\)m.

![Figure 4](image-url)  
**Figure 4** Transaortic constriction enhances neoangiogenesis in a disc angiogenesis model. Subcutaneous implantation of polyvinyl sponge for 3 weeks resulted in an in-growth of new vessels. (A) Quantitative histomorphometric measurements. \(\ast P < 0.05\). (B) and (C) show representative examples of vascularized areas around borders of discs in animals after sham-surgery (B) and transaortic constriction (C). Bars = 100 \(\mu\)m.
2.0%). We did not observe any GFP-pos apoptotic or GFP-pos/Ki67-pos cardiomyocytes.

4. Discussion

The main novel finding of the study is the upregulation of EPCs in the bone marrow as well as the spleen and circulating EPC in the peripheral blood following induction of pressure overload. Systemic upregulation of EPC during cardiac hypertrophy was associated with marked increase of extra-cardiac angiogenesis. Bone marrow chimera revealed upregulation of intramyocardial bone-marrow-derived endothelial and myocyte precursor cells.

Five weeks of transaortic constriction decreased the number of capillaries per surface area, whereas the ratio of CD31-pos cells to the number of total cells and cardiomyocytes remained unaltered. The number of CD31-positive cells undergoing apoptosis increased as well as the number of apoptotic cardiomyocytes. The experiments indicate that transaortic constriction stimulates angiogenesis in the heart and bone-marrow-derived cells contribute to this process. However, the extent of angiogenesis is not sufficient to reverse the capillary–myocyte mismatch following cardiac apoptosis of both cell lineages. During the development of myocardial hypertrophy, cardiomyocytes enlarged without concomitant, adaptive growth of capillaries. The area of myocardial tissue supplied by one capillary increases. The increase of diffusion distance limits the supply of oxygen and nutrient substrates. During acute pressure overload a hypoxia-inducible factor-1 (Hif-1)-dependent induction of
angiogenic factors such as VEGF is observed, but sustained pressure overload leads to an accumulation of p53 that contributes to impaired cardiac angiogenesis and systolic function.8

Endothelial nitric oxide synthase has been shown to contribute to LV remodelling under chronic pressure overload: in eNOS−/− mice, increases in fibrosis index as well as depression of contractility and an increase in relative wall thickness were observed after aortic constriction.9 Since endothelial NO is a central mediator of EPC numbers,11,31 aortic constriction was performed in eNOS−/− mice. In contrast to wild-type animals, eNOS−/− mice showed no increase of EPC numbers in the peripheral blood or the bone marrow during hypertrophy. These findings show that eNOS plays an important role for the regulation of EPC production and mobilization during increased cardiac afterload. However, the capillary density in eNOS−/− mice did not differ from those of wild-type mice. Further studies are needed to characterize this effect that may relate to an impaired homing of EPCs in the myocardial capillaries during pressure overload.

Transaortic constriction caused cardiac hypertrophy, fibrosis, and increased cardiomyocyte diameters as expected.8 Very similar relative changes of cardiomyocytes and fibrosis were observed in the bone-marrow-transplanted mice. Apoptosis of cardiomyocytes, endothelial cells and non-cardiomyocytes increased significantly in mice after transaortic constriction. In bone-marrow-transplanted animals the percentage of bone-marrow-derived apoptotic cells was significantly higher in the mice receiving aortic constriction compared with the respective sham group. Increased cell death is therefore likely to contribute to the failure of adaptive mechanisms to compensate for the shortage of capillaries; in addition, further studies need to show how impaired recruitment and homing of progenitor cells contribute to the pathology.

In addition to hypertrophy and apoptosis, we observed a profound increase of myocardial cells—both cardiomyocytes and non-myocytes—expressing the marker Ki67, which indicates cell cycle progression. However, Ki67 can indicate nuclear division as well as cell division. We did not directly document mitosis in the cardiac sections examined and mice have substantial numbers of binucleated nuclei.32 But our findings are in agreement with previous findings of increased Ki67-expressing cardiomyocytes in the border zone of the myocardial infarction area18 and in patients with aortic valve stenosis19 in which also mitosis was found—although in much lower numbers than Ki67 expression—and demonstrate that adaptive mechanisms in addition to cardiomyocyte hypertrophy contribute to the remodelling after pressure overload. Since the extent of myocyte apoptosis is also raised in increased afterload, cycling of cardiac myocytes is not sufficient to restore lost myocardium. The experiments show that the Ki67pos cardiomyocytes induced by aortic constriction were not derived from the bone marrow. The detected cycling cardiomyocytes therefore may stem either from cardiomyocyte division or from resident cardiac stem cells.21,33,34 These findings are in agreement with data showing no or only very low numbers of bone-marrow-derived cardiomyocytes after direct application of bone-marrow-derived stem cells into murine hearts after myocardial infarction.23–37 However, Kucia et al.38 demonstrated that cells from the bone marrow expressing early cardiac markers, including myocyte enhancer factor 2 (MEF2) are mobilized to the peripheral blood and chemo-attracted to the infarcted myocardium in mice. Recent evidence from a genetic fate-mapping study shows that stem cells refresh adult mammalian cardiomyocytes after TAC.20 Here we demonstrated that GFPpos MEF2pos cells were upregulated in the myocardium of mice with increased cardiac afterload. These cells were not transdifferentiated into smooth muscle cells. The MEF2pos myocyte precursor cells were increased but did not undergo a detectable full differentiation to large cardiomyocytes, at least within the 5 week period observed. It remains speculation whether impairment of further differentiation contributes to maladaptive remodelling and the underlying mechanisms require further study. These speculations are supported by the observation that newly formed cardiomyocytes after myocardial infarction in the infarct region were reported to be significantly smaller compared with normal adult cardiomyocytes.39

The mechanism by which pressure overload increases numbers of EPC and promotes angiogenesis remains to be further elucidated. Bone-marrow-derived cells may potentially transdifferentiate into endothelial cells.40,41 Chimerism studies in patients after gender-mismatched heart and bone marrow transplantation observed that small numbers of extracardiac cells may differentiate to cardiomyocytes and endothelial cells.29,42–45 In addition, EPCs have been shown to surround vessels as pericytes and augment neovascularization through paracrine effects.46 Both mechanisms could be responsible for the effects on neoangiogenesis in our model, since we found cells positive for CD31 (respectively von Willebrand factor) and GFP in cardiac capillaries simultaneously. The number of these double-positive cells coming from the bone marrow increased significantly after transaortic constriction, underlining the concept of transdifferentiation of bone-marrow-derived EPCs to endothelial cells. We rarely found endothelial cells positive for GFP in the large vessels, which can be explained by the lack of large vessel injury in the model of transaortic constriction. Despite the upregulation of myocardial CD31pos/GFPpos cells in transaortic constriction, the number of capillaries per cardiomyocyte remained unaltered. Factors that may impair EPC function within the diseased heart but do not hamper extra-cardiac angiogenesis remain to be identified, candidates include Hif-1 and p53.48

In summary, pressure-induced cardiac hypertrophy is a regulator of bone-marrow-derived EPCs. The intriguing perspective of these findings is that interventions enhancing EPC numbers and their function within the cardiac muscle may represent novel strategies for the treatment of pressure-induced hypertrophy to eventually delay the maladaptive progression into ventricular dysfunction and heart failure.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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