Nollet E, Hoymans VY, Rodrigus IR, De Bock D, Dom M, Vanassche B, Van Hoof VOM, Cools N, Van Ackeren K, Wouters K, Vermeulen K, Vrints CJ, Van Craenenbroeck EM. **Bone marrow-derived progenitor cells are functionally impaired in ischemic heart disease.** *Journal of Cardiovascular Translational Research.*

DETAILED METHODS

Study Population

Thirty-two patients undergoing elective cardiac surgery via median sternotomy (coronary artery bypass grafting, mitral valve surgery or implantation of ventricular assist device) were stratified into groups according to the presence of significant ischemic heart disease (IHD; coronary angiography), and thus requiring revascularization, and / or HF (symptoms, signs and systolic/diastolic dysfunction). Hence, there was a first group of patients with IHD without HF (i.e. CAD group), a second group with HF due to IHD (i.e. ischemic cardiomyopathy or ICM group) and a third group with HF due to other reasons than IHD (i.e. non-ischemic HF group). Eleven subjects without significant past history of cardiovascular disease, served as controls.

Exclusion criteria were anemia (Hb levels <12 g/dl for women and <13 g/dl for men)[1], chronic inflammatory or malignant disease, chronic kidney disease (eGFR<30 ml/min), diabetes mellitus, recent acute coronary syndrome (<2 weeks) and age <18 years and ≥75 years. The study was conducted in accordance with the Helsinki Declaration, approved by the local Ethics Committee and all patients gave written informed consent.

Sampling and isolation of bone marrow mononuclear cells

In patients, BM (2x10 ml) was aspirated by sternal puncture (15G Jamshidi T-handle BM aspiration needle with safety lock, Hospithera SA/NV, Belgium) under general anesthesia as described previously.[2] Arterial PB (12 ml) was sampled simultaneously via arterial catheter. In control subjects, BM was aspirated at the time of cortical and spongious bone prelevation of the crista iliaca prior to further jaw reconstruction using a 8G aspiration needle (Medax Medical Devices, Poggio Rusco, Italy); venous PB was sampled by venepuncture. Both BM and PB were collected sequentially in a small EDTA tube (3 ml; used for plasma isolation; Becton Dickinson (BD) Benelux, Belgium), a serum tube (3 ml) and 2 large EDTA tubes (6 ml each; used for BM- and PB-mononuclear cells (MNC) isolation). BM- and PB-MNC were isolated by density gradient centrifugation (Lymphocyte Separation Medium, Lonza Braine SA, Belgium), immediately after sampling.

Clinical and biochemical assessment

All patients underwent coronary angiography and standard transthoracic echocardiography pre-operatively in order to determine the complexity of IHD (Syntax score) and LVEF (Simpson's biplane method) respectively. The Syntax score, an angiographic tool to quantify the complexity of coronary artery lesions, was calculated using the online Syntax Score calculator by 1 independent observer.[3] Assessment of physical capacity was evaluated by the 6-minute walk test (6-MWT) in 15 patients.[4] Furthermore, the Framingham risk score representing the 10-year risk for cardiovascular disease, was calculated.[5] C-reactive protein (CRP), hemoglobin (Hb), creatinine, leukocyte, total cholesterol, as well as HDL and LDL circulating levels were measured pre-operatively by routine hematological and biochemical assessment. The glomerular filtration rate (eGFR) was estimated by the Chronic Kidney Disease Epidemiology Collaboration quotation, based on creatinine levels, age, gender and race.[6]

Assessment of BM purity

The percentage of bone matrix vesicle-bound bone alkaline phosphatase (ALP) of total ALP activity, as marker for the purity of BM aspirates, was quantified in BM serum as described in previous work.[7] BM aspirates in which the percentage of bone matrix vesicle-bound ALP was less than 15%, were excluded from the study for quality reasons.

Transwell migration assay of BM-MNC

Freshly isolated BM-MNC were resuspended in Endothelial Basal Medium-2 (EBM-2 Basal Medium, Lonza Benelux B.V., the Netherlands) with 0.5% bovine serum albumine and pipetted into the upper chamber of a transwell (Transwell, PC membrane, 8.0 μm pore size, Elscolab N.V., Belgium). In vitro migratory capacity of BM-MNC towards stromal cell-derived factor-1α (SDF-1α, 100 ng/ml, R&D Systems Europe Ltd., United Kingdom) and vascular endothelial growth factor (VEGF, 50 ng/ml, R&D Systems Europe Ltd.) was evaluated after 4 hours using a hemocytometer. Migrated BM-MNC were expressed as a percentage of the total cell number, corrected for spontaneous passive migration (negative control, no addition of chemo-attractants). All analyses were performed in duplicate.

Colony-forming unit assays

Freshly isolated BM-MNC were resuspended in IMDM (Invitrogen, Belgium) with 2% fetal bovine serum (FBS) and aliquoted in duplicate in Methocult H4535 Enriched without EPO medium (Stem Cell Technologies, France), containing recombinant human stem cell factor, granulocyte and granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-6 for the induction of differentiation into the myeloid lineage (granulocyte/macrophage- colony forming units, GM-CFU). In addition, BM-MNC

were also aliquoted in duplicate in Methocult H4434 Classic medium (Stem Cell Technologies), containing recombinant human stem cell factor, GM-CSF, IL-3 and EPO for the induction of differentiation into the erythroid lineage (erythroid-burst forming units, BFU-E). After incubation for 14 days, the number of GM-CFU and BFU-E were counted by 2 independent investigators and averaged.

Paracrine activity of BM-MNC

Freshly isolated BM-MNC were resuspended in IMDM (Invitrogen) supplemented with 10% FBS and seeded on a gelatin-coated 24-well plate ($5x10^6$ cells/well). After incubation for 24 hours, supernatant was collected and centrifuged. Levels of VEGF in the supernatant were measured using a human VEGF ELISA kit (R&D Systems Europe Ltd).

Flow cytometric assessment of the cellular composition of the BM-MNC pool

After cryopreservation in 10% DMSO freeze solution in liquid nitrogen, isolated BM-MNC were thawed, washed and directly incubated with FITC-labeled mouse anti-human CD71 (BD), FITC-labeled mouse anti-human CD3 (BD), ECD labeled mouse anti-human CD34 (Beckman Coulter (BC) Nederland B.V., the Netherlands), PE-Cy7-labeled mouse anti-human CD10 (BD), APC-labeled mouse anti-human CD117 (BD), APC-Alexa Fluor 700-labeled mouse anti-human CD56 (BC), APC-Alexa Fluor 700-labeled mouse anti-human CD16 (BC), APC-Alexa Fluor 750-labeled mouse anti-human CD19 (BC), Pacific Blue-labeled mouse anti-human CD4 (BC), Pacific Blue-labeled mouse anti-human CD20 (BC), and Krome Orange-labeled mouse anti-human CD45 (BC). Viability was assessed by 7-AAD (BC). For each sample, a total of 500 000 events was acquired and the different cell phenotypes as mentioned in the table below, were expressed among viable CD45+ BM-MNC. Samples were measured by Navios flow cytometer (BC) and analyzed using Kaluza software (BC).

Cell type	Cellular phenotype
Erythroblasts	Viable CD45- CD71+ BM-MNC
T cells	Viable CD45+ CD3+ BM-MNC
NK cells	Viable CD45+ CD3- CD56+ CD16+ BM-MNC
B cells	Viable CD45+ CD19+ BM-MNC
Mature B cells	Viabe CD45+ CD20+ CD10- CD34- B cells
Hematogones	Viable CD45dim CD10+ CD34- B cells
Lymfoblasts	Viable CD45dim CD10+ CD34+ B cells
Monocytes	Viable CD45+ CD33++ CD4dim BM-MNC
Granulocytes	Viable CD45+ CD33+ CD4- BM-MNC
CD34+ blasts	Viable CD45dim CD34+ BM-MNC
Lymfoblasts	Viable CD19+ CD10+ CD34+ blasts
CD33+ Myeloblasts	Viable CD19- CD33+ CD34+ blasts
CD117+ Myeloblasts	Viable CD19- CD117+ CD34+ blasts

Flow cytometry for hematopoietic and endothelial progenitor cell enumeration

Freshly isolated BM- and PB-derived MNC were directly pre-treated with Fc receptor blocking reagent (Miltenyi Biotec, Germany) and incubated with APC-H7-labeled mouse anti-human CD45 (BD), PE-Cy7-labeled mouse anti-human CD34 (BD) and APC-labeled mouse anti-human KDR (R&D Systems) for the enumeration of hematopoietic (HPC, $CD45^{dim}CD34^{+}SSC^{low}$, ISHAGE gating strategy [8]) and endothelial (EPC, $CD45^{dim}CD34^{+}KDR^{+}$, EPC-gating protocol standardized by our laboratory [9]) progenitor cells in both BM- and PB-MNC sample. For each sample, a total of 800 000 events was acquired and HPC and EPC numbers were expressed as relative numbers among viable CD45+ BM-MNC. Early and late apoptosis was assessed by 7-AAD (BD) and FITC-labeled Annexin V staining (BD). Samples were measured by FACSCanto II flow cytometer (BD) and analyzed using FACSDiva software (BD).

Inflammatory and angiogenic cytokine levels

Plasma from PB and BM was obtained by respectively 1 and 2 centrifugation step(s) at 1500g for 15 minutes and stored frozen at -80°C until further use. Levels of the inflammatory cytokines TNFα, IFNγ, and IL-6, and the angiogenic cytokines VEGF and bFGF were measured in both PB and BM plasma using Meso Scale Discovery multiplex platforms (Meso Scale Diagnostics, Maryland, USA) on the Meso Quickplex SQ 120 (Meso Scale Diagnostics).

Molecular expression of inflammatory and angiogenic receptors

RNA was extracted from BM-MNC stored in RNAprotect Cell reagent at -80°C using the Rneasy Mini Kit with additional Dnase I digestion (Qiagen Benelux B.V., Netherlands) and reverse transcribed into cDNA using the iScript Advanced cDNA Synthesis kit (Bio-Rad Laboratories N.V., Belgium).

Expression of TNFR1 and 2 (TNFα receptors), IFNGR1 (IFNγ receptor 1), IL6R (IL-6 receptor), CXCR4 (SDF-1α receptor) and VEGFR2 (VEGF receptor 2) was analyzed by real time qPCR using corresponding SYBR Green based Prime PCR assays (Bio-Rad, see **Supplementary Table II** for Genebank accession numbers). All reactions were run in duplicate on a CFX96 PCR System (Bio-Rad) according to the manufacturer's instructions. After testing the stability of the genes encoding for beta-2-microglobulin (B2M), TATA box binding protein (TBP), actin beta (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in BM-MNC from 4 patients of each patient group, GAPDH was selected as most stable reference gene and used for data normalization. PCR data were analyzed using the comparative ΔΔCT method.

Statistical analysis

Results are expressed as median (Interquartile range-IQR). IBM SPSS Statistics version 22.0 was used for statistical analysis. Normality of the variables was tested with Shapiro-Wilk and by visual inspection of the Q-Q plots. Logarithmic transformation of skewed data was done to obtain a normal distribution. Comparison between different groups was performed using ANOVA (normally distributed data), Kruskal-Wallis (skewed data) or chi-square test (nominal data), as appropriate. To evaluate the specific influence of either IHD or HF, a two-way ANOVA was performed for each parameter with IHD, HF and the interaction between IHD and HF as independent factors. All models were adjusted for age. When the interaction between IHD and HF was not significant, it was removed from the model. Post-hoc pairwise comparison was obtained by Mann-Whitney U and adjusted with the Bonferroni-Holm correction for multiple testing. Correlations were evaluated by the Pearson or Spearman test, as appropriate. A p-value of <0.05 was considered statistically significant.

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