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Accelerated cellular senescence as underlying mechanism for functionally impaired bone marrow-derived progenitor cells in ischemic heart disease

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ABSTRACT

Background and aims: Bone marrow (BM)-derived progenitor cells are functionally impaired in patients with ischemic heart disease (IHD), thereby hampering the outcome of autologous stem cell therapy. In search for underlying mechanisms for this BM dysfunction, accelerated cellular senescence was explored.
Methods: We analysed telomere length of BM-derived mononuclear cells (MNC) by MMqPCR in patients with coronary artery disease (n = 12), ischemic heart failure (HF; n = 9), non-ischemic HF (n = 7) and controls (n = 10), and related it to their myeloid differentiation capacity. Expressions of senescence-associated genes *p53*, $p21^{Cip1}$ and $p16^{Ink4A}$; and telomere maintenance genes *TERT, TRF1/2, Sirt1* in BM-MNC were evaluated using qPCR. Pro-inflammatory cytokine levels (TNFα, IFNy, IL-6) in BM were measured by MSD.
Results: BM-MNC telomere length was shortened in patients with IHD, irrespective of associated cardiomyopathy, and shortened further with increasing angiographic lesions. This telomere shortening was associated with reduced myeloid differentiation capacity of BM-MNC, suggesting accelerated senescence as underlying cause for progenitor cell dysfunction in IHD. Both $p16^{Ink4A}$ and $p21^{Cip1}$ were activated in IHD and inversely related to myeloid differentiation capacity of BM-MNC; hence, the BM-MNC functional impairment worsens with increasing senescence. While BM-MNC telomere attrition was not related with alterations in *TERT, TRF1/2* and *Sirt1* expression, IFNy levels were associated with $p21^{Cip1}/p16^{Ink4A}$ upregulation, suggesting a link between inflammation and cellular senescence. Still, the trigger for telomere shortening in IHD needs to be elucidated.
Conclusions: Accelerated replicative senescence is associated with a functional impairment of BM-derived progenitor cells in IHD and could be targeted to improve efficacy of stem cell therapy.

1. Introduction

In search for an explanation for the poor outcome of autologous stem cell therapy in ischemic heart disease (IHD) and heart failure (HF), we have recently documented a functional impairment of bone marrow (BM)-derived progenitor cells in patients with coronary artery disease (CAD) irrespective of left ventricular

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dysfunction. [1] The underlying mechanisms, however, are still unclear. In the present study, we hypothesize that a process of accelerated replicative senescence might contribute to BM cell deterioration in IHD, and thereby hampers the clinical efficiency of autologous stem cell therapy in this patient group. This hypothesis is based on previous research by Van der Harst et al. demonstrating increased telomere shortening and senescence in circulating leukocytes from patients with HF compared to healthy age and gender-matched controls. In this study, leukocyte telomere shortening was also more pronounced in patients with ischemic HF than with HF of non-ischemic etiology. [2] In addition, patients with CAD and acute myocardial infarction show shorter leukocyte telomere lengths than healthy controls. [3–5].

Telomeres are TTAGGG repeats located at the distal ends of chromosomes that are important in the maintenance of genomic stability during cell division. With each cell division, telomere length (TL) shortens. The enzyme telomerase is able to add new telomere repeats onto the chromosome ends and prevents loss of TL. The expression of telomerase, however, is rather low in the majority of mature human somatic cells, whereas it is upregulated in committed progenitor cells, activated immune cells and cancer cells [6,7]. In addition to specific intrinsic genetic factors associated with the process of biological aging, endogenous stimuli such as systemic chronic inflammation and mild oxidative stress may aggravate telomere shortening and promote cellular senescence. [8] When a critical TL is reached, cell cycle inhibitors *p53*/*p21*^{Cip1} and *p16*^{Ink4A} become activated, which halts cell division and induces a state of replicative cell senescence. This telomere shortening mechanism could, thereby, compromise the proliferative and differentiation potential of cells. [2,8,9].

The present study compares TL of BM-derived mononuclear cells (MNC) of patients with different types of heart disease, including coronary artery disease (CAD), ischemic HF, non-ischemic HF, and control subjects. Furthermore, the relationship between telomere shortening and BM-MNC dysfunction is investigated. We evaluated the activation of senescence-associated genes *p16*^{Ink4A}, *p21*^{Cip1} and *p51*, as well as the expression of telomerase reverse transcriptase *(hTERT)*, telomeric repeat binding-factor 1 *(TRF1)* and *2 (TRF2)*, [10] and Sirtuin1 *(SIRT1)*; [11] all involved in telomere maintenance. [8] Furthermore, chronic inflammation was explored as potential trigger for telomere shortening in this study cohort. [12]

2. Materials and methods

Detailed methods are available as Supplementary material.

2.1. Study population

Thirty-eight subjects were included in the study and divided into 4 groups according to the presence of significant CAD and/or HF. Group 1 consisted of patients with CAD without HF (i.e. CAD group; n = 12), group 2 consisted of patients with HF due to ischemic cardiomyopathy (i.e. ischemic HF group; n = 9), group 3 were patients with HF due to other reasons (i.e. non-ischemic HF group; n = 7) and group 4 were healthy subjects without significant past history of cardiovascular disease (i.e. healthy controls; n = 10). All cardiovascular patients underwent coronary angiography and cardiac ultrasound to determine the CAD complexity (Syntax score [13]) and left ventricular ejection fraction (LVEF), respectively. The study complied with the Helsinki Declaration and was approved by the local Ethics Committee. Written informed consent was obtained from all subjects.

2.2. Sampling and isolation of BM-MNC

In patients, BM (2 × 10 ml) was aspirated by sternal puncture under general anesthesia prior to cardiac surgery. In control subjects, BM was aspirated at the time of bone prelevation of the iliac crest prior to jaw reconstruction.

The purity of BM aspirates by means of peripheral blood contamination was quantified as described previously. [14] BM aspirates with a bone matrix vesicle-bound alkaline phosphatase <15% were excluded from the study for quality reasons. From the 50 samples, 38 pure BM samples were retained for further analyses. Next, BM-MNC were isolated by density gradient centrifugation immediately after sampling.

2.3. Monochrome multiplex qPCR for TL measurement

The average TL was measured in a gDNA sample extracted from BM-MNC using the monochrome multiplex qPCR method, as originally developed by Cawthon et al. [15] and optimized by Van der Harst et al. [16] Telomeric DNA was amplified using specific telomere primers (TelC and TelG, see Supplementary material); the reference gene albumin using albumin primers (AlbUgc and AlbDgc). The relative T/S ratio of the telomere (T) signal to the albumin (S) signal was calculated for each sample, with all samples being compared to the same reference DNA sample. The mean T/S ratio is considered to be proportional to the average BM-MNC TL.

2.4. Real time qPCR for telomerase, regulators of telomerase activity and senescence-associated genes

Expression of *hTERT*, the catalytic subunit of the enzyme telomerase and considered as a good correlate of telomerase expression; [6,17] *TRF1 and 2*, which are known regulators of the telomerase activity; senescence-associated genes encoding for *p53, p21*^{Cip1} and *p16*^{Ink4A}; as well as *hSIRT1* were all normalized to *GAPDH* and calculated by real time qPCR using the ΔΔCT method. A fold change of ≥1.5 or ≤ -1.5 was considered as relevant up- or downregulation.

2.5. Colony-forming unit (CFU) assays

Isolated BM-MNC were aliquoted in duplicate in Methocult H4535 Enriched without EPO medium (Stem Cell Technologies, France) for induction of differentiation into the myeloid lineage (granulocyte/macrophage-colony forming units, GM-CFU); as well as in Methocult H4434 Classic medium (Stem Cell Technologies) for induction of differentiation into the erythroid lineage (erythroid-burst forming units, BFU-E). After 14-day incubation, the GM-CFU and BFU-E numbers were counted.

2.6. Inflammatory cytokine levels in BM

Levels of the inflammatory cytokines TNFα, IFNy and IL-6 were measured in BM plasma using Meso Scale Discovery multiplex platforms (Meso Scale Diagnostics, USA).

2.7. 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 Q-Q-plot inspection. 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