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Telomere/telomerase system impairment in circulating angiogenic cells of geriatric patients with heart failure

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ABSTRACT

Background: The functional characteristics of circulating angiogenic cells (CACs) are impaired in congestive heart failure (CHF) patients, suggesting that CAC dysfunction could contribute to CHF pathogenesis. However, the underlying mechanisms are only partly unraveled. No data are currently available regarding telomere/telomerase system in CACs of CHF patients.
Methods: CACs were obtained from 80 subjects: 40 healthy control subjects (CTR) [median age (IQR), 80 (76–85 yrs)] and 40 patients affected by post-ischemic cardiomyopathy CHF [median age (IQR), 82 (77–89)]. CAC and leukocyte telomere length, assessed as T/S ratio, and telomerase (TERT) activity were determined in all the enrolled subjects. Specificity and sensitivity of CAC and leukocyte T/S in discriminating between CHF and CTR were evaluated using Receiver Operator Characteristic (ROC) curve analysis and reported as AUC values. CD34+/VEGFR2+ number and pro-inflammatory cytokines plasma levels, such as IL-6 and TNF-α, were also measured.
Results: CAC T/S and TERT activity were significantly reduced in CHF patients compared to CTR subjects. In leukocytes, only a significant T/S reduction was observed. AUC values were higher for CAC T/S with respect to leukocyte T/S (AUC = 0.89, and AUC = 0.73, $P<0.01$, respectively). In multivariate analysis, leukocyte T/S, CAC T/S, CAC TERT activity and NT-proBNP levels were confirmed as parameters significantly associated with CHF. CD34+/VEGFR2+ number, IL-6 and TNF-α plasma levels were significantly increased in CHF patients.
Conclusions: CACs from CHF patients are characterized by telomere/telomerase system impairment, providing new insight into the clinical relevance of CACs in CHF pathogenesis.

1. Introduction

Endothelial dysfunction has been widely reported in patients with heart failure [1,2]. The endothelium can be considered a highly dynamic tissue in equilibrium with a circulating compartment composed of different circulating endothelial progenitor cell (CEPC) subpopulations [3–6]. Circulating CD34+/VEGFR2+ cells represent the cellular phenotype that more closely approximates the stem cells/progenitors of an endothelial lineage in terms of detection accuracy, biological meaning and clinical usefulness [7]. Unfortunately, CD34+/VEGFR2+ cells are rare in peripheral blood, making it difficult to assess their functional alterations in human diseases. Alternatively, circulating angiogenic cells (CACs) deriving from the monocyte-macrophage lineage, can be easily obtained from peripheral blood and can be used for functional studies. Although the proliferative capacity of CACs is low and they fail to form blood vessels in vivo, they contribute to vascular homeostasis by secreting angiogenic growth factors, including vascular endothelial cell growth factor (VEGF), at sites of vascular injury [8,9].

The evaluation of CAC functional capacity and number of circulating CD34+/VEGFR2+ progenitor cells has been proposed as a strategy to assess vascular regeneration in cardiovascular diseases, including CHF [10]. Moreover the effect of exercise training on CAC migratory capacity in CHF patients has been largely investigated [11–13].

The number of circulating CD34+/VEGFR2+ progenitor cells was also related to CHF and its outcomes; a low number was reported to be

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an independent predictor of cardiovascular death and cardiovascular hospitalization in patients with chronic HF [14].

However, data regarding the prognostic value of these progenitor cells number in CHF are contrasting most likely because of the different etiology and the different degrees of CHF severity [15,16].

In vitro studies indicate that both cell turnover and oxidative/inflammatory stress may contribute to cell senescence by inducing telomere shortening [17]. Interestingly, telomere shortening in leukocytes and bone marrow-residing stem cells has been associated with cardiovascular diseases, including myocardial infarction and CHF [18–22]. To counter telomere shortening, cells can express the enzyme telomerase (TERT), a reverse transcriptase enzyme that uses an RNA molecule (TERC) as the template to elongate the 3′ ends of telomeres, in order to maintain telomere length. The telomerase-deficient (TERC−/−) mice provided new insight into the involvement of telomere biology in cardiovascular diseases, including CHF [23].

However, no data are currently available regarding CAC telomere/telomerase system function in CHF patients, although recently, the accumulation of senescent monocytes, having shortened telomeres and exhibiting features of activated cells, was related to the development of chronic inflammation and atherosclerosis in elderly subjects [24]. We focused our study on CACs, since these cells can contribute to vascular repair and can be easily obtained from peripheral blood of CHF patients. We aimed to investigate if CAC telomere length, assessed as T/S ratio by real-time PCR, and TERT activity were impaired in CHF patients. Considering that leukocyte telomere lengths were reported in association with CHF, we aimed also to compare data on telomere/telomerase system function between CACs and leukocytes.

2. Methods

2.1. Study population

A total of 80 subjects were enrolled: 40 healthy CTR subjects and 40 post-ischemic CHF patients in the New York Heart Association (NYHA) functional class II (n = 17) and III (n = 23). All patients had a well documented history of previous Non ST-Elevation Myocardial Infarction (NSTEMI) according to the definition in the European Society of Cardiology (ESC) guidelines. All patients gave their informed consent before participating in the study, which was approved by the INRCA Institutional Ethical Board. All CHF patients were recruited from the Cardiology Unit of the INRCA Hospital at the time of the admission for acute exacerbation of their CHF. Blood was collected during the first 6 h from hospital admission. Concurrently, healthy CTR subjects were enrolled by the same team in a cardiovascular disease prevention program.

The diagnosis of CHF was confirmed in all patients by clinical findings and assessment of cardiac function. In order to eliminate a possible bias due to the different causes of impaired cardiac function, only post-ischemic systolic heart failure CHF patients were enrolled, while those with isolated diastolic dysfunction were excluded.

Left ventricular ejection fraction (LVEF) was determined by 2D echocardio-color-doppler evaluation. Patients with severe renal, hepatic or chest disease with a poor prognosis or with a diagnosis of cancer with a predictable short follow-up, were excluded from the study. All enrolled subjects underwent a complete physical, as well as hematological, biochemical and instrumental examination. Arterial hypertension was defined as a systolic blood pressure >140 mm Hg and/or a diastolic blood pressure >90 mm Hg found on at least three different occasions measured in a seated position. Information regarding diabetes and the use of statins was also collected. All CHF patients received optimal standard therapy at hospital admission according to the 2008 ESC guidelines for the treatment of CHF.

Overnight fasting venous blood samples from all subjects were collected between 8:00 a.m. and 9:00 a.m. in plain, EDTA and citrate containing vials (Venoject, Terumo Europe NV). Samples were stored at −80 °C until testing.

2.2. Cell culture

CACs were isolated from approximately 14 mL of heparinized peripheral blood. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation with Ficoll (Ficoll-Paque™ PLUS, GE Healthcare Bio-Sciences Uppsala—Sweden), within 2 h after blood collection. 5×10^6 PBMC were plated on a 24-well fibronectin-coated plate (BD Biosciences, Mountain View, CA) and maintained in endothelial basal medium (EBM; Clonetics-Lonza, Walkersville, MD USA) supplemented with EGM SingleQuots and 20% FCS for 4 days. After 4 days in culture, non-adherent cells were removed by PBS wash, while adherent cells were lysed directly in the culture wells. DNA was purified according to the instruction manual of the All Prep DNA/RNA Mini kit (Qiagen GmbH, Hilden—Germany).

2.3. Characterization of cultured CACs

To confirm the CAC phenotype one well of cultured cells for each sample was tested for the ability to incorporate 1,1′-dioctadecyl-3,3,3′,3′ tetramethylindocarbocyanine-labeled acetylated LDL (DiLDL) and bind endothelial-specific lectins, such as *Ulex europaeus* agglutinin-1. Dual-stained cells positive for both UEA-1 and DiLDL were regarded as CACs. Briefly, to detect the DiLDL uptake, cells were incubated with DiLDL (2.4 μg/mL) (Molecular Probes, Eugene, OR) at 37 °C for 2 h. To detect the UEA-1 binding, the cells were fixed with 2% paraformaldehyde for 15 min and incubated with FITC labeled UEA-1 (10 μg/mL) (UEA-1-Sigma, St. Louis, MO) for 1 h.

2.4. Flow Cytometry Analysis

CD34+/VEGFR2+ cells, co-expressing CD34 and vascular endothelial growth factor receptor 2 (VEGFR2), were quantify by flow cytometry. 1×10^6 mononuclear cells were double labeled with FITC-conjugated anti-human CD34 (Becton Dickinson, San Jose, California) and PE-conjugated anti-human VEGFR2 (R&D System Inc, Minneapolis). Fluorescent isotype-matched antibodies (Becton Dickinson) were used as controls. Cell fluorescence was measured by flow cytometry using a fluorescence-activated cell sorter scan (EPICS XL Coulter, Miami, Florida) equipped with Cell Quest software (System II) setting a lymphomonocyte gate in the forward and side scatter dot plot, excluding debris. At least 300,000 events were measured, and analysis was consecutively performed three times for each subject. The absolute number of CD34+ and VEGFR2+ double-positive cells was calculated as the mean value of three replicate measurements and then compared to 1×10^6 PBMC.

2.5. Measurement of telomere length

For each sample, telomere length was measured for both leukocytes and CACs. The high molecular weight DNA from CACs was isolated as described above, while the high molecular weight DNA from leukocytes was isolated starting from 200 μl of whole blood using the QIAamp DNA Mini kit (Qiagen GmbH, D-40724 Hilden).

Telomere length was measured as the abundance of telomeric template (T) vs a single gene copy (S) by quantitative real-time PCR, according to a modified technique described by Cawthon et al. [25]. For the T/S analysis, a 5 μl aliquot containing 20 ng of DNA and 10 μl of master mix were added to each sample well. For each standard curve, one reference DNA sample was serially diluted in H_2O by 1.68 fold per dilution to produce five concentrations of DNA ranging from 30 ng to 2 ng in 5 μl. The composition of T and S PCRs were identical with the exception of oligonucleotide primers. Telomere template and single copy gene (36B4) were analyzed in the same plate in order to reduce inter-assay variability.

Measurements were performed in triplicate and reported as T/S ratio with respect to a calibrator sample (human genomic DNA, Roche). Both calibrator sample and no-template control samples were processed in duplicate per run. The same calibrator sample was used in all runs to allow comparison across runs. Every PCR was performed in the iCicler real-time (Biorad). The PCR profile for both amplicons began with a 95 °C incubation for 10 min followed by 30 cycles of 95 °C for 5 sec, 57 °C for 15 sec and 72 °C for 20 sec. The melting curve values for telomere length and single copy gene corresponded to the expected values. The coefficients of variation (CV) within duplicates of the telomere and single-gene assay were 2% and 1.8%, respectively. Approximately 30% of samples were repeated on different plates to assess T/S reproducibility. The inter-assay CV was <10%.

All analyses were performed blinded with regard to case–control status of the subjects. Correlation coefficient between T/S and Southern blot measurement of telomere length, calculated on a subgroup of samples, was $R^2 = 0.88$.

2.6. Telomeric repeat amplification protocol (TRAP) assay

For quantitative analysis of telomerase activity, a real time Telomeric Repeat Amplification Protocol was performed using Quantitative Telomerase Detection Kit (QTD, Allied Biotech Inc, Ijamsville, MD, USA), according to the manufacturer's protocol. This involved amplification of the telomerase reaction product by real-time PCR (iCicler real-time, Biorad).

Briefly, after 4 days in culture non-adherent cells were removed by washing two times with PBS, then CACs were lysed directly in the culture wells, using the lysis buffer provided in the kit. Protein lysate was kept on ice for 30 min and then centrifuged at 12,000 g for 30 min at 4 °C. Protein concentration was immediately measured using a Bradford assay. The supernatant was frozen and stored at −80 °C until use in the telomerase activity assay, performed with 1 μg of protein extract for each sample. Telomerase activity in the samples was calculated based on the threshold cycle, using a standard curve generated from serial dilutions of positive control template (TSR Allied Biotech, inc.). All experiments were performed in duplicate and heat-treated samples were used as negative controls.

2.7. Laboratory assays

High sensitive C-reactive protein (hsCRP) was determined by the particle-enhanced immunoturbidimetric assay (CRP High Sensitive, Roche-Hitachi), characterized by a lower limit of 0.1 mg/L and an upper limit of 160 mg/L. The inter and