Experimental Gerontology 47 (2012) 394–400

Contents lists available at SciVerse ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Circulating progenitor cells and the elderly: A seven-year observational study

Giuseppe Mandraffino [a], Maria A. Sardo [a], Stefania Riggio [a], Angela D'Ascola [b], Angela Alibrandi [c], Carlo Saitta [a], Antonio Versace [a], Maria Castaldo [a], Enricomaria Mormina [a], Egidio Imbalzano [a], Maurizio Cinquegrani [a], Michele Bonaiuto [a], Antonio David [d], Antonino Saitta [a,*]

[a] Department of Internal Medicine and Medical Therapy, University of Messina, Messina, Italy
[b] Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, Messina, Italy
[c] Department of Statistical Science, University of Messina, Messina, Italy
[d] Department of Neurosciences and Anaesthesiology, University of Messina, Messina, Italy

ARTICLE INFO

Article history:
Received 17 September 2011
Received in revised form 25 February 2012
Accepted 12 March 2012
Available online 17 March 2012

Section Editor: R. Westendorp

Keywords:
Circulating progenitor cells
Elderly
Life expectancy
Flow cytometry
Antioxidant enzymes

ABSTRACT

Cardiovascular (CV) diseases and related complications are the main causes of morbidity and mortality in the elderly. CV progenitor cells, including CD34+ cells, play a role in delaying the progression of atherosclerosis. In the present study we observed 100 octogenarians for seven years, in order to address the question of whether CD34+ cell number is a predictor of longevity in selected survivors. We also checked for associations of cell expression of manganese superoxide dismutase (Mn-SOD), catalase (CAT), and glutathione peroxidase type-1 (GPx-1) antioxidative enzymes, with number of CD34+ progenitor cells and mortality. We found that in very old subjects the number of CD34+ cells at baseline were higher in subjects who reached older age at death or were still living at the end of observation period, with respect to subjects who died from all causes, including CV deaths. On the other hand, HDL-C plasma levels and, with the exception of diabetes, the classic CV risk factors (hypertension, smoking, hypercholesterolemia) showed a loss of their predictive power. A significant association between the redox system of CD34+ cells and mortality was also observed. These data suggest that, even in the elderly, CD34+ cells maintain their role in predicting mortality. CD34+ cells could thus be considered as a biomarker of longevity.

1. Introduction

Persons who live until they are 80–90 years and over without serious disorders, including cardiovascular disease (CVD), are a testament to longevity (Franceschi et al., 2000; Marques et al., 2010; Rowe and Kahn, 1987). Typically considered as selected survivors, these subjects provide a model to investigate the protective factors which increase their resistance to develop diseases (Sprott, 2010; Walker and Herndon, 2010). Studies in this regard are, however, limited because of the complexity of the aging process and the difficulty in finding an adequate representation of adults at advanced ages. In previous studies, we enrolled a population of Sicilian octogenarians with no CVD and showed the contribution of paraoxonase (PON1) and platelet-activating factor acetylhydrolase (PAF-AH) enzymes and plasma HDL-C in delaying the development of CVD (Campo et al., 2004, 2008).

CVD and related complications are the main causes of morbidity and mortality in the elderly (Lloyd-Jones et al., 2010). Recent evidence suggests that the cardiovascular (CV) progenitor cells, including CD34+ cells and the more mature elements, endothelial progenitor cells (EPCs), play a role in delaying the progression of atherosclerosis. These populations of bone marrow-derived cells exhibit self-renewing capabilities and the capacity to differentiate into endothelial cell lineages, thus participating in the turnover of healthy and damaged endothelium, as well as in angiogenetic processes (Asahara et al., 1997; Fadini et al., 2007; Hristov and Weber, 2008; Urbich and Dimmeler, 2004). To contribute to tissue repair and survive in necrotic and ischemic tissue, CV progenitor cells express intrinsically high levels of antioxidative enzymes, including glutathione peroxidase (GPx-1), manganese superoxide dismutase (MnSOD) and catalase (CAT) (Dernbach et al., 2004; Gao and Mann, 2009; Haendeler and Dimmeler, 2006; He et al., 2004). In stress conditions, high concentrations of reactive oxygen species (ROS) induce activation of sensitive transcriptional pathways as well as molecule modifications leading to cell senescence and apoptosis (Pesce et al., 2011). The antioxidative enzymes have a specific scavenging activity, providing an important defense against oxidative stress and accumulation of intracellular ROS.

A decline in the number and function of progenitor cells has been found to be associated with the progression of several disorders, including CVD (Fadini et al., 2009, 2010; Hill et al., 2003; Maruyama et al., 2008; Schmidt-Lucke et al., 2005; Taguchi et al., 2004; Vasa et al., 2001; Werner et al., 2005). Similarly, the reduction of circulating progenitor cells has been associated with the Framingham risk score

* Corresponding author at: Department of Internal Medicine, Via Camiciotti, 82, 98123-Messina, Italy. Tel.: +39 090 2212376; fax: +39 090 2213900.
E-mail addresses: asaitta@unime.it, gmandraffinomd@libero.it (A. Saitta).

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doi:10.1016/j.exger.2012.03.007

(FRS) (Hill et al., 2003). Age is an important risk factor for cardiovascular disease, because with age several changes occur in the structure of organs and systems. In the elderly, for example, there is a reduction in the number of circulating EPCs as compared with younger adults (Jie et al., 2009). It has been suggested that the progressive depletion and/or functional impairment of bone marrow-derived EPCs might, at least in part, account for the effect of aging on the progression of atherosclerosis (Goldschmidt-Clermont et al., 2004).

In the present study we address the question of whether CD34+ cell number may be considered a predictor or at least a biomarker of longevity in selected survivors. We also checked for associations between cell expression of antioxidative enzymes, number of progenitor cells and mortality.

2. Subjects and methods

2.1. Subjects

The study population was composed of 100 octogenarians (49 men and 51 women, mean age at enrolment 84.18 ± 3.6 years). The patients were already enrolled in our previous studies investigating the role of PON1 and PAF-AH gene polymorphisms and activity in healthy aging (Campo et al., 2004, 2008). The follow-up was conducted for 7 years until May 2010. The subjects were enrolled from the surrounding areas of Messina, in Sicily. The baseline examination consisted of a home interview and a clinical examination. Specific details of medical history were confirmed by contacting the participant's physician. Informed consent was obtained from all subjects and the study was approved by the Ethics Committee of the University of Messina.

Inclusion criteria for the study population were normal ECG pattern and no history or clinical signs of arterial disease including CAD, stroke or peripheral arterial disease. Subjects with cognitive (Mini-Mental State Examination) and functional (Barthel Index and Instrumental Activities of Daily Living) impairment, thyroid, hepatic or kidney disease, infectious or autoimmune disease were excluded from the study. Blood pressure, height and weight were measured in all subjects by routine methods, and a venous blood sample for clinical chemistry and progenitor cells isolation was collected after overnight fasting. Hypertension was defined as systolic blood pressure (SBP) ≥140 and/or diastolic blood pressure (DBP) ≥90 mmHg or current use of anti-hypertensive medications. Diabetes mellitus was defined as fasting glucose ≥7.0 mmol/l or the need for oral anti-diabetic drug therapy or insulin use. Hypercholesterolemia was defined on the basis of total cholesterol (TC) levels ≥6 mmol/l. Plasma lipids and blood glucose levels were measured by routine enzymatic methods. HDL-cholesterol was determined after precipitation of the Apo B-containing lipoproteins with magnesium phosphotungstate. LDL-cholesterol was calculated by the Friedewald formula.

The 10-year CVD risk based on the Framingham CVD Risk Prediction Models was calculated in all subjects (D'Agostino et al., 2008).

The follow-up information was available for all patients. During follow-up, the patients were visited regularly at home by the family practitioner, and any clinical information, hospitalization or death was notified. Patients who had not been seen for more than 6 months were contacted and interviewed by one of the investigators. Causes of death were determined by examination of hospital records, and medical files of the patients' general practitioners. Deaths due to sudden death and death from acute myocardial infarction, coronary artery disease, congestive heart failure or stroke were considered as due to CV causes; other causes of deaths were considered as non-CV causes.

2.2. Methods

Flow cytometric evaluation, expression of MnSOD, GPx-1 and CAT (mRNA and protein) in circulating CD34+ cells, and ROS levels in CD34+ cells has already been explained elsewhere in detail (Mandraffino et al., 2011). Briefly, fresh blood flow cytometry was used (FACSCalibur; Becton Dickinson and Co., Franklin Lakes, NJ, USA) to identify the cells. Circulating cells that expressed the stem cells antigen CD 34 were defined as progenitor cells, estimated and counted.

After the isolation of mononuclear cells from peripheral venous blood by density centrifugation and enrichment of the samples in CD34+ cells by immunomagnetic sorting with the MiniMACS system (Miltenyi Biotec Inc, Auburn, CA, USA), the expression of MnSOD, GPx-1 and CAT- mRNA in circulating CD34+ cells was evaluated. CD34+ cell enrichment was validated by flow cytometry. After separation, total RNA was extracted and spectrophotometrically quantified (Biomate 3-Thermo Electron Corporation, Waltham, MA, USA). The retro-transcribed product was used to measure the gene expression of Mn-SOD, CAT, and GPx-1 by Real Time Polymerase Chain Reaction method (RT-PCR) using β-actin as the endogenous control for the final normalization, and the relative expression was performed by the $2^{-\Delta\Delta Ct}$ method. For this method, the average values of MnSOD, CAT and GPx-1 in samples from all subjects were considered as the calibrator (1× sample). The results were expressed as an n-fold difference relative to mean value (relative expression levels).

The enzyme protein levels were measured by Western blot. After image acquisition, results were expressed as relative amounts against the β-actin as endogenous control. After normalization, the average of MnSOD, GPx-1 or CAT concentration in samples from control subjects was considered as the calibrator (1× sample) and the results were expressed as an n-fold difference relative to normal controls.

ROS generation in CD34+ cells enriched samples was assessed using 2,7-dichlorofluorescin diacetate (DCFH-DA). Data were expressed as fluorescence intensity relative units (FU).

3. Statistical methods

The Kolmogorov Smirnov test verified that some variables (age, SBP, DBP, TC, CD34, MnSOD, CAT, GPx-1) had a non-normal distribution; consequently, given also the relatively small size of our sample, we chose a permutation test-based analysis, a subset of non parametric statistics; these tests are widely used in biomedical research, and are considered preferable to the classic non parametric approach used in larger sample sizes (Ludbrook and Dudley, 1998), since they are based on more realistic foundations, are intrinsically robust and resulting inferences are credible (Pesarin and Salmaso, 2010). This approach in fact makes it possible o estimate the whole data distribution, and to exploit all the information contained in the sample. The analyses were carried out by the Non Parametric Combination test (NPC test), that is based on a simulation or resampling procedure, conditional on the data, which provides a simulated estimate of the permutation distribution of any statistic, (Pesarin, 2001).

According to the statistical approach, data were expressed as mean ± standard deviation (SD).

Deaths were classified as due to CV or non-CV causes; comparisons between the CV and non-CV groups, and between deceased subjects and those still living at the end of the observation period, were performed by the Non Parametric Combination test. The correlations among the variables were assessed by Spearman's test. A stepwise multiple regression analysis (based on permutation tests) was used to assess the contribution of each selected study variable in determining variations of CD 34+ cell number, and to assess the influence of risk factors on duration of life. Furthermore, since the subjects had a different age at time of enrolment, and the age at death or at the end of the observation period could be different, we designed a statistical model to limit the influence of older age on the “death” event and on the age reached after seven years.

Accordingly, we performed the partial correlation test (controlling for age), then a logistic regression model, and finally a Cox regression model (Allison, 1995); moreover, to further control the influence of age, we performed the analysis in subjects aged less or more than 84 years (84 years was the mean age of whole study population), guaranteeing a similar age distribution in each stratum.