THE JOURNAL OF
SEXUAL MEDICINE

Serum From Patients With Erectile Dysfunction and Vascular Risk Factors Triggered an Oxidative Stress-Dependent Mitochondrial Apoptotic Pathway in Ex Vivo Expanded Circulating Angiogenic Cells of Healthy Men

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ABSTRACT

Introduction: Serum from men with erectile dysfunction (ED) and vascular risk factors inhibits circulating mononuclear cells (MNCs) from expanding ex vivo and differentiating circulating angiogenic cells (CACs), which are putatively involved in the repair of endothelial damage.

Aim: To explore the involvement of apoptosis in the inhibition of CAC differentiation from MNCs of healthy men exerted by serum from men with ED and vascular risk factors.

Methods: MNCs from healthy men were cultured in serum from 10 healthy men (median age = 45 years, 25th–75th quartiles = 38.5–48.5) and from 14 patients (median age = 58.0 years, 25th–75th quartiles = 52.5–62.0). CACs were identified by the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (DiLDL) and concomitant *Ulex europaeus* agglutinin I binding assessed by fluorescence microscopy.

Main Outcome Measures: Flow cytometric evaluation of mitochondrial membrane potential, assessed with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benimidazolyl carbocyanine iodide dye, and of activated caspase-8, -9, and -3 in DiLDL-positive cells.

Results: The number of CACs was significantly decreased by serum from patients compared with controls. This was associated with suppression of the mitochondrial membrane potential and activation of caspase-9 and -3 but not of caspase-8. This suggests an activation of the intrinsic (mitochondrial) pathway of apoptosis, whereas the death receptor activation of apoptosis was not involved. Activation of caspase-9 and -3 induced by serum from patients with ED was prevented by the exposure of MNCs to Trolox, a hydrophilic cell-permeable vitamin E analog with high antioxidant capacity.

Conclusion: An oxidative stress-dependent mitochondrial dysfunction was triggered in ex vivo expanded CACs of healthy men by serum from men with vascular risk factors and ED, the only clinical correlate for diffuse vascular disease. The activation of apoptosis and inhibition of CAC differentiation might generate a defective mechanism of vascular repair.

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Key Words: Erectile Dysfunction; Vascular Risk Factors; Circulating Angiogenic Cells; Oxidative Stress; Apoptosis

INTRODUCTION

Erectile dysfunction (ED) in men with vascular risk factors (VRFs) is an early clinical manifestation of systemic vascular disease[1] and is predictive for cardiovascular events.[2,3] Endothelial damage or dysfunction[4–6] and an impaired capacity for repairing endothelial damage[7–9] have been proposed as possible physiopathologic mechanisms that contribute to systemic vascular disease in men with ED and VRFs. Circulating mononuclear cells (MNCs) from monocytes and macrophages give rise to

Received February 24, 2016. Accepted April 13, 2016.

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http://dx.doi.org/10.1016/j.jsxm.2016.04.067

ex vivo expanded circulating angiogenic cells (CACs), which have been suggested to participate in vessel repair by releasing proangiogenic growth factors.[10] A smaller number of CACs has been associated with vascular diseases[11] and with ED and VRFs,[6] suggesting that dysfunction of the MNCs involved in vascular homeostasis might contribute to adverse cardiovascular outcomes. Of relevance is the observation that CAC expansion is improved by different interventions aimed at decreasing VRFs.[12,13]

The molecular mechanism(s) underlying the decreased ex vivo expansion of CACs in men with ED is undefined. We previously found that serum from men with ED and VRFs negatively modulated the number of ex vivo expanded CACs derived from MNCs of healthy men[13] and that this effect was related to the vascular risk score. In contrast, the relevance of circulating markers of endothelial damage and function, such as endothelin-1 or E-selectin, of the endothelial-dependent prothrombotic state, such as tissue type plasminogen activator, and of angiogenic modulators, such as vascular endothelial growth factors and its receptor-1, was limited.[14] Therefore, serum from men with VRFs associated with ED, the only clinical correlate for diffuse vascular disease, induced dysfunction of the cells putatively involved in vascular homeostasis[14] with an undefined mechanism.

Serum from men with advanced clinical arteriosclerosis had a proapoptotic effect on human umbilical vein endothelial cells (HUVECs),[15,16] and apoptosis in endothelial progenitor cells was elicited by proinflammatory and pro-oxidative serum factors involved in the pathogenesis of atherosclerosis.[17–19] In the present study, we explored the molecular pathways of apoptosis in ex vivo expanded CACs of healthy men and their involvement in the adverse effect exerted by serum from patients with ED and VRFs. Because oxidative stress mediates activation of apoptosis in different cell types,[20] we checked whether the adverse effects on CACs exerted by serum from patients with ED could be prevented by a hydrophilic cell-permeable vitamin E analog (Trolox) that exhibits a high antioxidant capacity.[21]

METHODS

Study Population

Patients seeking medical care at the Andrology Clinic of the University Hospital of L'Aquila (L'Aquila, Italy) because of ED lasting longer than 6 months were invited to participate in the study. The Sexual Health Inventory for Men was used to evaluate their erectile function.[22] Exclusion criteria were a history of endocrine diseases (other than type 2 or 1 diabetes), androgen deficiency (total testosterone level $<$ 300 ng/dL), pelvic surgery or trauma, penile curvature, neurologic or major psychiatric disorders, stroke and other overt cardiovascular diseases (CVDs), alcohol or substance abuse, or major hematologic, renal, or hepatic abnormalities. The 10-year risk assessment for developing heart disease was carried out using the Framingham risk score of Adult Treatment Panel III (ATP III).[23] Fourteen consecutive patients with ED and a 10-year risk for CVD of 20% (median; 25th–75th quartiles = 16–25) were enrolled in the study and 10 healthy men with no recognizable VRFs (ATP III score = 1%) and without ED were included as controls. Two patients were orally treated for type 2 diabetes, and nine patients were treated for hypertension. Participants were requested to sign an informed consent, and the local ethic committee of the University of L'Aquila approved the study.

Blood was drawn after overnight fasting and no smoking for at least 12 hours. All participants were occasionally physically active. Patients were asked to discontinue medications for ED, non-steroidal anti-inflammatory drugs, and antioxidants for 2 weeks before blood sampling. No patients were treated with statins. Common carotid artery intima-media thickness was evaluated in all participants as a reproducible marker of generalized early atherosclerosis[24] using a duplex scanner equipped with color flow imaging (ATL HDI 3000; Philips, Amsterdam, Netherlands) and a 10-MHz linear array transducer.[1]

Ex Vivo Expansion of MNCs and Identification of CACs

MNCs isolated by Ficoll density-gradient centrifugation from 20 mL of peripheral blood were washed three times in phosphate buffer saline (PBS) and suspended in endothelial basal medium (EBM-2; Euroclone SpA, Milan, Italy) supplemented with 20% fetal bovine serum, and 10^6 mononuclear cells/cm^2 were seeded on fibronectin-coated culture dishes (Becton-Dickinson, Milan, Italy). After 4 days of culture at 37°C in an atmosphere of 5% CO_2 and 95% air, the non-adherent cells were discarded by washing with PBS, and the adherent cells were maintained in culture for an additional 3 days, under different treatments, according to the study design described below, before undergoing cytochemical and cytofluorimetric analysis. After treatment, adherent cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (DiLDL; Invitrogen, Milan, Italy) at a concentration of 2.4 μg/mL for 1 hour at 37°C. Then, cells were fixed with 1% paraformaldehyde for 10 minutes and incubated for 1 hour with fluorescein isothiocyanate (FITC)-labeled *Ulex europaeus* agglutinin I (UEA-1; Sigma, Milan, Italy) at a concentration of 10 μg/mL. Dual-staining cells positive for DiLDL and FITC-labeled UEA-1 were judged as functional CACs[10] and counted manually in 10 randomly selected microscopic fields by two independent investigators using an inverted fluorescence microscope (20×; Zeiss, Oberkoken, Germany).

Study Design

Preliminary experiments (not shown) demonstrated that tumor necrosis factor-α, the activator of the extrinsic (death receptor) pathway of apoptosis,[25] decreased the number of CACs, induced the intracellular pathway of apoptosis, including a decreased mitochondrial membrane potential ($\Delta\Psi$m), and

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