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Detection of early endothelial damage in patients with Raynaud's phenomenon

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ABSTRACT

Objectives: Raynaud's phenomenon (RP) can be the first manifestation of systemic sclerosis (SSc) or other connective tissue diseases (CTDs), often preceding an overt disease by years. It is not known if markers of endothelial damage are detectable in those RP patients who subsequently develop a CTD.
Methods: We studied 82 RP patients at their first evaluation to correlate the levels of endothelial markers with the subsequent development of an overt disease 36 months later. We measured plasma levels of tissue-type plasminogen activator (t-PA) and von Willebrand factor (vWF), two markers of endothelial damage, and interleukin-6 (IL-6), a pro-inflammatory cytokine. Thirty sex- and age-matched healthy subjects (HS) served as controls.
Results: At baseline, 67 patients showed capillaroscopic normal pattern (CNP) and 15 patients, of which 11 were very early SSc, had capillaroscopic scleroderma pattern (CSP). Plasma levels of t-PA, vWF and IL-6 were higher in patients with CNP ($p = 0.0001$) than in HS and even much higher in patients with CSP ($p = 0.0001$). In patients with CNP and RP of recent onset (<18 months), vWF plasma levels were higher when autoantibodies were present ($p = 0.020$). After 36 months, among 48 RP patients with CNP who remained in follow-up, 24 were diagnosed as primary and 24 as secondary RP. In secondary RP, basal levels of t-PA, IL-6 and particularly vWF were higher than in primary RP ($p = 0.005$, $p = 0.004$, $p = 0.0001$ respectively) and HS ($p = 0.0001$ for all).
Conclusions: Our findings indicate that markers of endothelial damage are elevated in RP patients who subsequently develop SSc or other CTDs, even in the absence of capillaroscopic abnormalities.

1. Introduction

Raynaud's phenomenon (RP) is characterised by episodes of digital ischemia induced by exposure to cold temperatures or emotional stress; it is due to the vasospasm of the digital arteries, arterioles and arteriovenous anastomoses (Wigley and Flavahan, 2016). RP is usually characterised by three phases: the first is a well-demarcated ischemia leading to the blanching of the fingers; the second is the consequent deoxygenation leading to cyanosis; the third is the post-ischemic red flushing upon reperfusion of the digits (Wigley and Flavahan, 2016). RP events may be accompanied by different degrees of paraesthesia, numbness, and pain (Block and Sequeira, 2001). RP may be primary (uncomplicated) when it occurs without an underlying disease, or secondary to an underlying connective tissue disease, representing the first manifestation in 90% of patients with systemic sclerosis (SSc), a chronic systemic disease associated with progressive disability and reduction of life expectancy (Harper et al., 1982; Kallenberg, 1990; Suter et al., 2005). In particular, RP may precede by years the occurrence of an overt disease (Wigley, 2002), but to date the causes and the time of development of a full-blown connective tissue disease (CTD) have not been completely clarified. Anti-nuclear antibodies (ANA) and anti-extractable nuclear antibodies (anti-ENA) such as anti-centromere and anti-topoisomerase-Scl-70, together with typical capillaroscopic abnormalities, are very important tools to identify those patients who could potentially develop a CTD (Koenig et al., 2008; Ingegnoli et al., 2010; Castelino and Varga, 2013; Emrani et al., 2016). These patients may already present endothelial damage in the absence of capillaroscopic abnormalities and detectable serum autoantibodies (Ingegnoli et al., 2010).

With this as background, we studied 82 patients with RP at their first evaluation, measuring at baseline two reliable markers of endothelial

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activation and damage, which are known to be elevated in patients with secondary RP (Mercie et al., 1995; Marasini et al., 1991; Marasini et al., 1992; Ames et al., 1997; Muangchant and Pope, 2013), i.e. von Willebrand factor (vWF), a glycoprotein responsible for platelet adhesion and tissue plasminogen activator (t-PA), the most important activator of fibrinolysis. Although a number of biomarkers of endothelial damage and dysfunction have been employed in the last three decades, in our experience vWF and t-PA are the plasmatic antigens that better correlate with a functional endothelial test, i.e. brachial artery flow-mediated vasodilation (Cugno et al., 2010). We also tested interleukin-6 (IL-6), a pro-inflammatory cytokine whose role is considered to be important in SSc pathogenesis since the very early phases of the disease (O'Reilly et al., 2013). After a follow-up of 36 months, we re-evaluated patients clinically to verify whether those patients with higher levels of endothelial markers at baseline had developed a CTD.

2. Materials and methods

2.1. Patients

This is an observational prospective study of 82 patients with RP (76 women and 6 men; median age 46 years, age range: 21–71 years) recruited from an Italian Rheumatology Clinic (Division of Rheumatology, Gaetano Pini Hospital in Milano, University of Milan), studied at their first rheumatologic evaluation and followed-up for 36 months. The inclusion criteria were age > 18 years and presence of RP. Patients with an established diagnosis of CTD or with an intercurrent acute illness were excluded. Thirty healthy subjects (27 women and 3 men, median age 43.5 years, age range: 22–68 years) served as normal controls. Active smokers were 3 out of 15 RP patients with capillaroscopic scleroderma pattern at baseline (20%) and 16 out of 67 RP patients with capillaroscopic normal pattern (24%). In the latter group re-evaluated after 36 months, smokers were 5 out of 24 primary RP patients (21%) and 4 out of 24 secondary RP patients (17%). Smokers were 6 out of 30 healthy controls (20%).

The study was approved by the ethics committee of the Hospital and informed consent was obtained from all patients. Patients were followed according to the guidelines of good clinical practice.

RP was diagnosed with screening questions (Wigley, 2002) and defined as an episodic, reversible vasospastic ischemia of the digits upon exposure to cold and/or in association with emotional stress, and characterised by blanching, possibly followed by cyanosis and post-ischemic red flushing upon rewarming (Ingegnoli et al., 2015). The episodes could be associated with varying degrees of paraesthesia, numbness or pain. Full medical history (e.g. symptoms typically associated with an underlying CTD, current drug treatment, exposure to toxic agents, occupational history, and family history) was collected. At baseline, all subjects underwent complete physical examination with particular focus on digital ulcerations, pitting scars, puffy hands and sclerodactyly. Patients were classified as having primary RP based on the International consensus criteria by Maverakis et al. (Maverakis et al., 2014), which require: all criteria for the diagnosis of RP as described above; capillaroscopic normal pattern such as “normal” and “perfect normal” patterns as previously described (Ingegnoli et al., 2013); absence of findings suggestive of secondary causes such as ulcerations, tissue necrosis or gangrene, sclerodactyly, calcinosis, or skin fibrosis at physical examination; no history of existing CTD and ANA titre ≤1:40 by indirect immunofluorescence. Very early SSc was diagnosed based on accepted classification criteria, in particular, the presence of RP, puffy fingers and ANA, in addition to specific antibodies (anti-centromere antibodies, anti-topoisomerase I antibodies), and/or capillaroscopic scleroderma pattern (Avouac et al., 2011; Minier et al., 2014). Patients were classified as having SSc in the presence of a 9-point score of the 2013 EULAR/ACR classification criteria (van den Hoogen et al., 2013). These are based on the only presence of skin thickening of the fingers extending proximal to the metacarpophalangeal joints (which is sufficient to formulate the diagnosis of SSc), or a combination of skin thickening of the fingers, fingertip lesions, telangiectasia, abnormal nailfold capillaries, interstitial lung disease or pulmonary arterial hypertension, RP, and SSc-related autoantibodies (van den Hoogen et al., 2013). Patients were classified as having undifferentiated connective tissue disease (UCTD) based on the classification criteria which consist of signs and symptoms suggestive of a CTD, but not fulfilling criteria for defined CTDs, positive ANA and a disease duration of at least 3 years (Mosca et al., 1999). We classified patients as having other CTDs, such as Sjoegren syndrome (SS) or mixed connective tissue disease (MCTD) based on the correspondent classification criteria (Shiboski et al., 2012; Sharp et al., 1972). Patients with RP were re-evaluated clinically and instrumentally after a 36 month-follow-up using the above-mentioned criteria.

2.2. Methods

2.2.1. Nailfold capillaroscopy

Nailfold capillaroscopy was performed using an equipment with a 200× optical probe. Images were captured, coded, and stored using Videocap software (DS-Medica, Milano, Italy). All of the recordings were made with the subjects sitting in a room at a temperature of 22° to 25 °C and with their hands at heart level. The procedure was explained and a drop of immersion oil was applied to the nailfold to maximize the translucency of the keratin layer. In each subject all the images available (at least two) in each finger of both hands were examined. An experienced observer evaluated the capillaroscopic images. Scleroderma pattern was considered in the presence of more than one giant capillary, microhemorrhages or loss of capillaries as described in previous studies (Cutolo et al., 2000; Ingegnoli et al., 2008). Then, a second observer reviewed all the images blindly. The inter-observer agreement is good as assessed by a previous study by our group reporting high values of weighted kappa statistic ($k_w > 0.70$) (Ingegnoli et al., 2009).

2.2.2. Blood sampling

Blood samples were obtained at the first evaluation both in patients and in controls by clean puncture of an antecubital vein with minimal stasis. Serum and plasma samples were stored at a temperature of −80 °C until assayed.

2.2.3. Autoantibodies

Anti-nuclear antibodies (ANA) were tested by indirect immunofluorescence on HEp2 cells, considering positive those samples with a dilution ≥1:160 (Agmon-Levin et al., 2014); anti-extractable nuclear antibodies (anti-ENA) were detected by DotBlot EUROLINE Systemic Sclerosis and Myositis profile IgG (EUROIMMUN AG, Lubeck, Germany), anti-U1 ribonuclear protein (RNP) and anti-ribosomal P protein were evaluated with Phadia Elia kit (Thermo Fisher Scientific Inc., Chicago, IL, USA); anti-double strand (ds) DNA were detected by in house ELISA; rheumatoid factors (RF) were assessed by immunoturbidimetric method (Cobas 8000, Roche Diagnostics, Basel, Switzerland); anti-cardiolipin (aCL) and anti-beta2-glycoprotein I (anti-beta2GPI) antibodies were tested by home-made ELISA, as previously reported (Di et al., 2010).

2.2.4. Tissue plasminogen activator antigen

Tissue plasminogen activator (t-PA) antigen was measured by a commercial enzyme-linked immunoassay method (Imunolyse tPA; Biopool, Umea, Sweden) according to the manufacturer's instructions. Intra- and inter-assay CVs were 6.5% and 8.0%, respectively.

2.2.5. von Willebrand factor antigen

von Willebrand factor (vWF) antigen was measured in citrated plasma by a sandwich “in-house” ELISA that used two monoclonal antibodies directed against different vWF epitopes (11B6.18 and 7G10.8). Intra- and inter-assay CVs were both lower than 8.0%.