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The differential expression of VEGF, VEGFR-2, and GLUT-1 proteins in disease subtypes of systemic sclerosis

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Summary Our aim was to evaluate (*a*) whether there is differential expression of the endothelial regulator vascular endothelial growth factor (VEGF), its receptor (VEGFR-2), and the hypoxia-associated glucose transporter molecule, GLUT-1, in skin biopsies from different disease subtypes of systemic sclerosis (SSc) and (*b*) whether they associate with dermal calcinosis, a significant complication of SSc. Skin punch biopsies were taken from the forearms of 66 SSc patients including 18 with limited cutaneous disease without calcinosis (lcSSc), 23 with calcinosis (lcSSc/cal), and 25 with diffuse cutaneous disease (dcSSc) and from 12 healthy control subjects. The histological appearance of the skin was graded as G0 (normal), G1 (dermal edema), or G2 or G3 (increasing fibrotic changes). Immunohistochemistry was performed with antibodies to VEGF, VEGFR-2, and GLUT-1. Staining was assessed in the epidermis, microvessels, and fibroblasts. The Kruskal-Wallis 1-way analysis of variance was used to compare the data between disease groups. VEGF protein was located in the epidermis and in dermal endothelial cells, pericytes, fibroblasts, and inflammatory cells. In dcSSc only, there was a significant increase in VEGF staining intensity in the keratinocytes and pericytes and the lowest percentage of microvessels with VEGF-positive endothelial cells. GLUT-1 protein was located in the epidermis, erythrocytes, and perineurium. In both lcSSc/cal and dcSSC, but not lcSSc, there were significant increases in GLUT-1 staining intensity of keratinocytes. We propose that in patients with dcSSc, there is a net increase in unbound VEGF in skin that may account for the raised levels of VEGF in serum reported by others. Increased GLUT-1 expression in lcSSc/cal and dcSSc indicates that hypoxia is an associated factor.

1. Introduction

In systemic sclerosis (SSc, scleroderma), skin is one of the target organs affected by microvascular compromise. Endothelial cell (EC) dysfunction is thought to be an early, if not the primary, event in the disease process before obvious signs of fibrosis appear [1-7]. Damaging effects to the endothelium are associated with perivascular inflammation and edema followed by fibrosis and, eventually, endothelial cell death with the resultant loss of microvessels [2,3,8]. In a number of pathological states, damage to the

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endothelium and loss of microvessels lead to chronic hypoxia, which up-regulates the expression of several genes, mediated by the transcription factor hypoxia-inducible factor-1α (HIF-1α) [9]. These include vascular endothelial growth factor (VEGF), its receptors 1 and 2 (VEGFR-1, Flt-1; VEGFR-2, Flk), and the human erythrocyte glucose transporter-1 (GLUT-1) [10,11]. VEGF exists as 5 amino acid variants and it is regulated directly by hypoxia. Its up-regulation induces EC hyperplasia, increased permeability of vessels, and angiogenesis. Those responses are mediated mainly by VEGFR-2; the role of VEGFR-1 is less clear but it appears to be involved in hematopoietic cell development [12-15].

The differential expression of VEGF protein in the two main subtypes of SSc—limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc) disease [16]—has been reported [17-19], suggesting that VEGF may be a useful indicator of endothelial dysfunction. Several studies have documented increases in VEGF levels in the serum from SSc patients compared with healthy controls, with the highest values found in the patients with dcSSc [17-19], but others claim that VEGF levels in plasma are not related to disease activity [20].

Microvascular involvement, as measured by laser Doppler and nailfold video capillaroscopy, is more marked in lcSSc patients [8,21] and it is recognized that patients with lcSSc are more prone to develop subcutaneous calcinosis than those with dcSSc [22]. Anticentromere antibody, which is associated with severity of digital ischemia [23,24], is also associated with calcinosis [25]. Furthermore, a group of lcSSc and dcSSc patients with calcinosis have been shown to have fewer nailfold capillaries than a similar group without calcinosis [26]. Supporting evidence for the association of microvascular disease with the pathogenesis of calcinosis comes from another rheumatological disorder, juvenile dermatomyositis, in which similar nailfold abnormalities to those in SSc occur and subcutaneous calcinosis can be a prominent feature later in the course of the disease [27]. In addition, hypoxia has been implicated in the development of calcification in cerebral hypoxia and calcific shoulder lesions [28,29]. There has been very little research addressing the mechanisms of calcinosis, but a contributory factor could be related to hypoxia.

VEGF and GLUT-1 proteins have been verified as indicators of hypoxia in correlative studies with pimonidazole binding and direct Po_2 measurements [30-32]. GLUT-1 expression has not been investigated in SSc. Therefore, given that there is very little in vivo evidence for tissue hypoxia in SSc, the aim of this study was to look for evidence of hypoxia in skin biopsies from groups of patients with SSc, differentiated according to disease subtype (lcSSc without calcinosis, lcSSc with calcinosis [lcSSc/cal], or dcSSc) and degree of skin involvement. We used immunohistochemistry to evaluate the expression of VEGF, VEGFR-2, and GLUT-1 proteins in different dermal cell populations.

2. Patients and methods

2.1. Patients

Full clinical details including disease subtype and duration and autoantibody status were available for all patients. Ethical approval was obtained from Salford and Trafford Local Research Ethics Committee and informed written consent was given by the patients to have skin biopsies taken. The patients with SSc were subdivided into those with lcSSc and dcSSc, those with and without calcinosis, and by histological skin grade. All patients experienced Raynaud phenomenon. There were 74 patients in the original cohort but 8 were excluded because they constituted 3 histological subgroups that were too small for meaningful statistical comparison. The biopsies were divided into 4 experimental groups (Table 1): (1) healthy control subjects; (2) lcSSc patients without calcinosis, that is, skin involvement restricted to distal to the elbows, knees, and neck; (3) lcSSc patients with calcinosis (lcSSc/cal), that is, evident upon examination or by a convincing history of previous extrusion of calcinotic nodules; and (4) patients with dcSSc, that is, skin involvement extending proximal to the elbows and knees, none of whom had calcinosis.

2.2. Tissue preparation

Skin punch biopsies (4 mm) were taken from the flexor aspect of the forearm. The fresh tissue was immediately placed into 10% neutral buffered formalin, routinely processed and embedded in paraffin wax. Serial sections were cut at 5 μm from each block and mounted on APES (3-aminopropyl-triethoxysilane, A-3648, Sigma-Aldrich, Poole, UK) coated slides.

2.3. Histological classification

The histological appearance of the skin was graded on hematoxylin and eosin–stained sections by an experienced pathologist (AJF) blinded to the clinical grade/diagnosis. The grades comprised the following: grade 0 (G0) = normal skin ± minimal perivascular edema and/or pigmentary incontinence; grade 1 (G1) = normal appearance of the dermal matrix but with marked perivascular edema ±

Table 1 Clinical characteristics of patients and controls

	Control	lcSSc	lcSSc/cal	dcSSc
No. of patients	12	18	23	25
Age (y), mean ± SD	40 ± 11	52 ± 14	50 ± 13	50 ± 13
Female, n (%)	9 (75)	14 (78)	20 (87)	15 (60)
Duration of Raynaud phenomenon (y), median (Q1, Q3)		10 (2, 16)	19 (15, 27)	3.5 (1, 6)