

## Testing for Hypoxia in Forearm Skin of Patients with Systemic Sclerosis, Assessed by Pimonidazole

To the Editor:

It is often assumed that sclerodermatous (thickened) skin in patients with systemic sclerosis (SSc) is hypoxic. This is because many of the clinical features of the disease, e.g., digital ulceration and/or pitting, are attributed to ischemic atrophy, and hypoxia activates a number of genes implicated in the SSc disease process, e.g., transforming growth factor- $\beta$  and endothelin-1<sup>1</sup>. Studies have shown that hypoxia induces several extracellular matrix proteins in both SSc and healthy dermal fibroblasts<sup>2</sup>. However, there is very little direct evidence that sclerodermatous skin is hypoxic. Silverstein, *et al* showed a skin thickness-related reduction in transcutaneous oxygen pressures<sup>3</sup>. Valentini, *et al* subsequently reported reduced transcutaneous oxygen pressure in both sclerotic and nonsclerotic skin of patients with SSc at 44°C but not at 37°C<sup>4</sup>. We tested the hypothesis that skin of patients with SSc is hypoxic, especially where the skin is abnormal. The hypoxic cell marker pimonidazole can be detected and quantified using immunohistochemistry on formalin-fixed, paraffin-embedded biop-

sies. Although there are many examples of pimonidazole being used to quantify hypoxia in tumors<sup>5</sup>, pimonidazole has been very little used in the study of nonmalignant disease. Recently, pimonidazole staining was demonstrated in a sample of irradiated breast tissue, the pathological features of which included sclerosis<sup>6</sup>.

Nine patients with SSc (7 women; median age 51 years, range 41–68 years) were recruited for study. Four had limited cutaneous and 5 diffuse cutaneous disease subtype. All had well established disease, with median duration of Raynaud's phenomenon 9 years (range 14 months to 41 years).

Pimonidazole (Hydroxyprobe-1, Natural Pharmacia International Inc., Belmont, MA, USA) was infused intravenously over 20 minutes, at a dose of 500 mg/m<sup>2</sup> in 100 ml of 0.9% saline. Six hours after infusion, 2 skin punch biopsies (4 mm) were taken, one from forearm and one from buttock (always clinically uninvolved). Samples were immediately placed into 10% neutral buffered formalin, processed, and embedded in paraffin wax. The study was approved by the Salford and Trafford Research Ethics Committee, and all patients provided signed informed consent. It was not believed to be justifiable to inject pimonidazole into controls but another hypoxia marker, glucose transporter-1 (GLUT-1)<sup>7</sup>, was examined in fore-

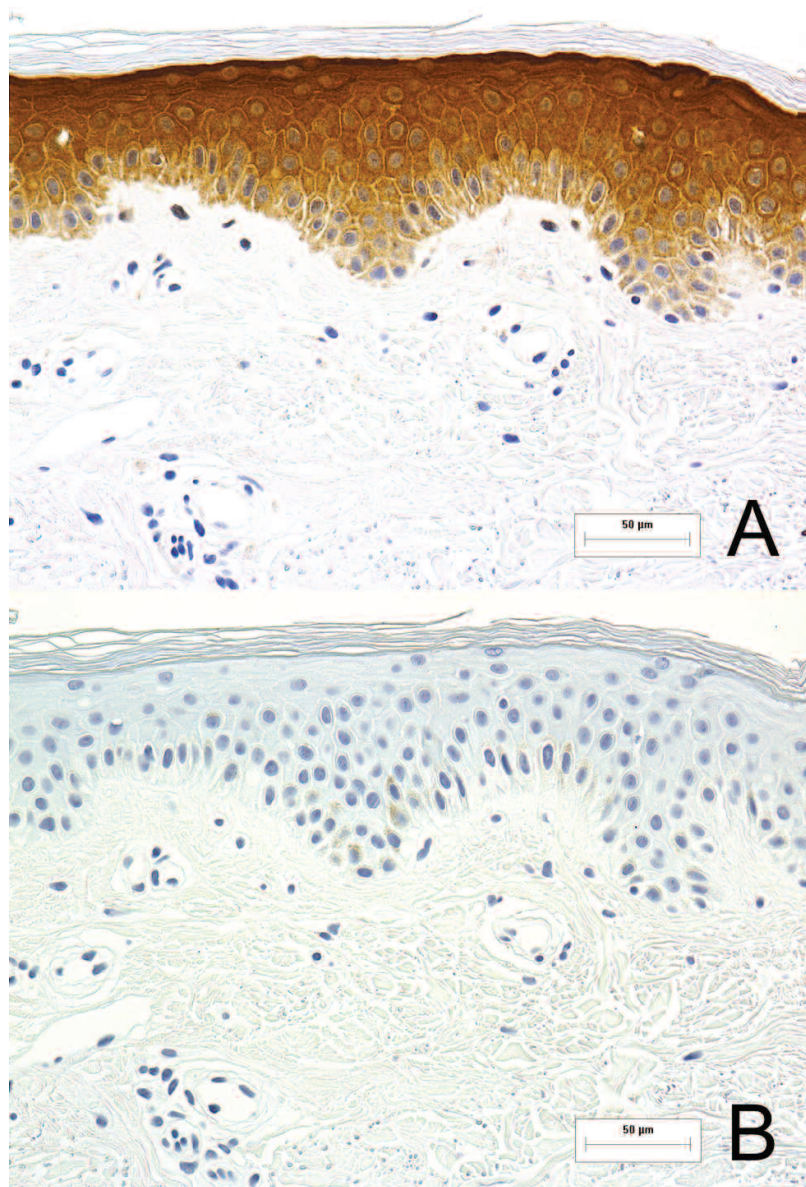


Figure 1. Pimonidazole staining in skin of a patient with SSc. A. Intense staining for pimonidazole (score 3) was localized to epidermis. B. Consecutive section incubated with buffer only shows no positive staining. Bars = 50  $\mu$ m.

arm skin from 12 healthy controls (9 women; median age 39 years, range 26–59 years).

Pimonidazole adducts and GLUT-1 proteins were determined as described<sup>5</sup>. Negative controls consisted of replacing the primary antibodies with phosphate buffered saline or TRIS-buffered saline/bovine serum albumin, or with mouse or rabbit immunoglobulins (Dako Chemical).

The intensity of staining for pimonidazole (Figure 1) and GLUT-1 was assessed in the granular, prickle cell, and basal cell layers of the epidermis on coded slides, so that the observer was blind to the site/origin of the biopsy (forearm vs buttock, or SSc vs healthy control). Intensity was scored from 0 (no staining) through 1, 2, or 3 for low, medium, and high staining, respectively.

Pimonidazole infusions were well tolerated by all patients. In the SSc biopsies, there were no differences in either pimonidazole or GLUT-1 staining between forearm and buttock in any of the epidermal layers, nor was there any difference in GLUT-1 forearm biopsy staining between SSc patients and healthy controls (Table 1). Six patients had Grade 1 sclerodermatous change on skin histology<sup>8</sup>, 3 of whom had mild forearm skin thickening clinically (another patient had mild skin thickening without histological change).

By demonstrating pimonidazole staining in the skin of patients with SSc we have shown that this skin is, to some extent, hypoxic. Without a healthy control group, it is not possible to state whether the pimonidazole staining we did observe was clinically relevant: only that in patients with SSc there were no differences between forearm and buttock skin. A limitation of our study was that no patient had more than mildly thickened forearm skin, and any further studies should include patients with greater degrees of skin thickening. Our results are consistent with those of Evans, *et al*<sup>9</sup>, who assessed oxygenation in human skin using the hypoxia marker EF5: the epidermis was “modestly” hypoxic with more severe hypoxia in portions of sebaceous glands and hair follicles.

GLUT-1 staining in the prickle and basal layers is consistent with our previous findings examining GLUT-1 in forearm skin from patients with SSc<sup>8</sup>, although in our previous study we found that GLUT-1 staining was increased in patients with SSc. Again, the fact that the 9 patients included in the present study had only very minimal skin involvement may have been a contributory factor. The reason for the difference between pimonidazole and GLUT-1 staining in the granular layer is not known.

This was a small pilot study showing the feasibility of using pimonidazole as a marker of hypoxia in non-neoplastic diseases. Future studies in patients with greater degrees of skin thickening, with biopsies at different sites, and also studies in animal models of SSc could provide new insights into the relation between the extent of clinical and histological skin involvement and hypoxia/pimonidazole staining.

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Table 1. Pimonidazole and GLUT-1 staining in epidermis. Results are median (range). 0 = no staining; 1 = low staining, 2 = medium staining, 3 = high staining.

	Granular Layer	Prickle Layer	Basal Layer
Pimonidazole			
SSc forearm (n = 9)	3.0 (2.0–3.0)	1.0 (0.5–2.0)	1.0 (0.0–2.5)
SSc buttock (n = 9)	3.0 (2.0–3.0)	1.5 (1.0–2.5)	1.0 (0.0–1.0)
GLUT-1			
SSc forearm (n = 9)	0.0 (0.0–0.5)	2.0 (1.8–3.0)	2.0 (1.0–3.0)
SSc buttock (n = 9)	0.0 (0.0–0.0)	2.5 (1.8–3.0)	2.5 (1.5–3.0)
Healthy control forearm (n = 12)	0.0 (0.0–0.0)	1.6 (0.5–2.5)	1.6 (0.5–2.5)