HLA-G Expression in the Skin of Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** To determine HLA-G expression in skin biopsies from patients with systemic sclerosis (SSc), and its association with epidemiological, clinical, and laboratory variables and survival.

Methods. Paraffin-embedded skin biopsies obtained from 21 SSc patients (14 limited SSc, 7 diffuse SSc) and from 28 healthy controls were studied. HLA-G expression was evaluated by immunohistochemistry.

Results. HLA-G molecules were detected in 57% of skin biopsies from patients with SSc (9 from limited SSc, 3 from diffuse SSc), whereas no control sample expressed HLA-G ($p = 0.000004$). In patients, HLA-G molecules were consistently observed within epidermal and some dermal cells. HLA-G expression was associated with a lower frequency of vascular cutaneous ulcers ($p = 0.0004$), telangiectasias ($p = 0.008$), and inflammatory polyarthralgia ($p = 0.02$). After a 15-year followup, SSc patients who exhibited HLA-G survived longer than patients who did not.

Conclusion. HLA-G is expressed in skin biopsies from patients with SSc, and this is associated with a better disease prognosis. This suggests a modulatory role of HLA-G in SSc, as observed in other skin disorders. (First Release April 15 2009; J Rheumatol 2009;36:1230–4; doi:10.3899/jrheum.080552)

Key Indexing Terms:
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Systemic sclerosis (SSc) is a multisystem connective tissue disorder of unknown etiology that is highly heterogeneous in its clinical manifestations, following a variable and unpredictable course. The hallmarks of SSc are autoimmunity and inflammation, widespread vasculopathy affecting multiple vascular beds, and progressive interstitial and perivascular fibrosis[1]. Patients with SSc display a relative shift in the Th1-Th2 cytokine balance toward Th2 predominance. This constellation of seemingly disparate yet interlinked features differentiates SSc from other connective tissue diseases and organ-specific fibrosing disorders[2].

Patients with SSc are commonly classified into 2 distinct clinical variants based on the extent of skin involvement. Diffuse cutaneous SSc (dcSSc) is dominated by rapidly progressive fibrosis of the skin and internal organs. In limited cutaneous SSc (lcSSc), skin and organ fibrosis exhibit slow progression and the clinical picture is dominated by vascular manifestations only in the late stages of disease[3]. Survival is influenced by the clinical variant, with limited SSc presenting a better prognosis than diffuse SSc. Other factors associated with a poor prognosis are male sex, visceral involvement (predominantly interstitial lung disease, pulmonary arterial hypertension, and scleroderma renal crisis), autoantibodies (anti-Scl-70), anemia, and elevated erythrocyte sedimentation rate (ESR)[4].

Several pathogenic mechanisms have been proposed for

SSc, including excessive perivascular collagen deposits, hypoxia, cytokine imbalance[5], and the presence of fetal microchimerism[6], which may be related to modulation of the maternal immune system by persistence of alloreactive fetal progenitor cells. Moreover, HLA-G has been associated with the modulation of the immune system[7], and HLA-G expression has been shown in cutaneous inflammatory diseases.

HLA-G is a nonclassical MHC class I molecule characterized by restricted tissue expression (cytotrophoblasts, amnion and thymic epithelial cells, pancreas, proximal nail matrix[8] and erythroblasts[9], low polymorphism, and 7 isoforms (HLA-G1 to G7).

HLA-G1 has a structure similar to that of classical HLA class I molecules, i.e., a heavy-chain noncovalently associated with ß$_2$-microglobulin displaying a nonameric peptide bond in its groove. HLA-G expression is induced by interleukin 10 (IL-10)[10] or interferon-γ[11] and exerts an overall negative immune regulatory function inhibiting allogeneic proliferation of T cells, natural killer cell cytotoxicity, and antigen-specific T cell cytotoxicity. HLA-G inhibits the immune response by at least 3 inhibitory receptors, ILT2 (CD85j), ILT4 (CD85d), and KIR2DL4 (CD158d). Because of these effects on immune system cells, the expression of HLA-G may have beneficial or harmful effects. Under physiological conditions, expression of HLA-G is limited to the placenta, protecting the fetus against maternal immune system attack against the foreign paternal antigens. Similarly, in solid-organ transplantation the expression of HLA-G on allograft cells has been associated with a decreased number of rejection episodes, yielding a better prognosis. On the other hand, HLA-G expression on tumor or virus-infected cells permits evasion of attack by the immune system, facilitating tumor progression and virus protection[7]. Little attention has been devoted to the role of HLA-G in autoimmune and chronic inflammatory disorders; however, accumulating evidence demonstrates that HLA-G expression in chronic inflammatory cutaneous diseases including psoriasis and atopic dermatitis has been associated with a better clinical course[12,13].

We evaluated HLA-G expression and its possible correlation with the epidemiological, clinical, and laboratory variables of each clinical SSc variant. Since HLA-G expression has been associated with a better clinical course in other cutaneous inflammatory diseases like psoriasis and atopic dermatitis, and considering its immunological modulatory properties, we investigated whether HLA-G expression was correlated with a better prognosis of the disease, independently of the form of SSc.

MATERIALS AND METHODS

Patients. All patients were evaluated according to the standard investigation protocol for SSc at the Unit of Rheumatology of the State University of Campinas. According to the skin color, patients were stratified into Caucasians and African Brazilians (Black patients of unmixed ancestry and Mulattos, i.e., originating from the mixture of White and Black individuals). Extent and severity of skin thickening were evaluated by the modified Rodnan skin score (mRSS) that analyzes 17 anatomical sites, graded from 0 (normal skin) to 3 (intense skin thickening). The following definitions were used to characterize specific organ involvement: (1) articular: inflammatory polyarthralgia or arthritis; (2) vascular: ischemic ulcers of fingertips or extensive cutaneous necrosis, amputation, or both; (3) esophageal: dysphagia with radiological evidence of distal esophageal hypomotility; (4) intestines: altered intestinal habit, associated with radiological evidence of motility disturbances in the small or large bowel; (5) interstitial lung disease: dyspnea associated with radiological evidence of interstitial lung involvement, restrictive defect of lung function tests (forced vital capacity < 70%), or both; (6) pulmonary vascular disease: pulmonary artery systolic pressure > 40 mm Hg on Doppler echocardiogram, alone or associated with interstitial lung disease; (7) heart: congestive heart failure or symptomatic pericarditis or symptomatic arrhythmia; and (8) kidney: scleroderma renal crisis, characterized by rapidly progressive renal insufficiency associated with malignant arterial hypertension. Raynaud's phenomenon, calcinosis, and telangiectasis were also determined. Laboratory investigation included complete blood cell count, ESR, and antinuclear, anticentromere and anti-Scl-70 autoantibodies.

The study protocol was approved by the local Ethics Committee (protocol 282/2008).

Skin specimens. Paraffin-embedded skin biopsies from SSc patients were retrospectively selected from the archives of the Department of Pathology, State University of Campinas, from 1985 to 2005. Skin biopsies were obtained from 21 untreated patients (15 women) with a longterm followup (median 15 years). All skin specimens were obtained from the dorsal region of the third proximal phalanx. All patients fulfilled the American College of Rheumatology classification criteria for SSc[14] and were divided into diffuse and limited SSc groups according to LeRoy, *et al*[15]. Fourteen patients presented limited SSc (10 women) and 7 diffuse SSc (5 women; Table 1).

Table 1. Epidemiological, clinical, and laboratory data of patients with systemic sclerosis (SSc).

	No. of Patients		
	Limited SSc	Diffuse SSc	Total
Male/female	4/10	2/5	6/15
Age, mean yrs	45	47	
Race (Caucasian/African Brazilian)	11/3	5/2	16/5
Mean disease duration prior to the skin biopsy, yrs	5	4	—
Followup, mo	148	107	127
Raynaud's phenomenon	14	7	21
Vascular cutaneous ulcers	6 (5)*	3 (3)*	9 (8)*
Telangiectasis	7 (5)**	4 (3)**	10 (8)**
Calcinosis	8	1	9
Esophageal disease	13	5	18
Polyarthralgia	9 (5)†	3 (3)†	13 (8)†
Intestinal	2	0	2
Pulmonary			
Interstitial lung disease	10	4	14
Vascular disease	1	2	3
Heart	3	5	8
Scleroderma renal crisis	0	1	1
Antinuclear antibody	11	6	17
Anticentromere antibody	4	0	4
Anti-Scl-70	3	3	6

* HLA-G-negative patients with vascular cutaneous ulcers (p = 0.0004); ** HLA-G-negative patients with telangiectasias (p = 0.008); † HLA-G-negative patients with inflammatory polyarthralgia (p = 0.02).

A total of 28 normal skin specimens were obtained from healthy women who underwent breast reduction plastic surgery (21 samples) or who had traumatic skin lesions on the third proximal phalanx (7 samples). (Normal skin specimens were kindly provided by Dr. Renata Nahas Cardili, Department of Dermatology, Faculty of Medicine of Ribeirão Preto, University of São Paulo.)

Immunohistochemistry. Four-micrometer sections were cut from paraffin-embedded specimens. The streptavidin-biotin universal detection system (Immunotech, Westbrook, ME, USA) was used. Briefly, after rinsing the sections in phosphate buffered saline with 0.1% saponin, endogenous peroxidases were inhibited using H_2O_2. Samples were initially incubated with specific or irrelevant antibodies for 1 hour at room temperature and subsequently with a solution containing a peroxidase-labeled polymer conjugated with a goat anti-mouse immunoglobulin for 30 minutes. Diaminobenzidine plus chromogen-substrate was used to develop antibody fixation. The specific monoclonal antibody (4H84) used reacts with the 61–83 residues of the α-1 domain of denatured HLA-G of all HLA-G isotypes (kindly provided by Dr. E. Carosella, Hôpital Saint-Louis, Paris, France). To support our data, we also used the MEM-G/2 antibody that recognizes the free heavy-chain of all HLA-G isoforms (Exbio, Praha, Czech Republic). An identical IgG1 isotype antidesmin antibody that was run simultaneously with each sample served as a negative control. Invasive (intermediate) cytotrophoblasts from third-trimester human placenta served as a positive protein control.

Statistical analysis. Data are reported as arithmetic mean and standard deviations or median (range) values. Variables were compared using the nonparametric Mann-Whitney and Spearman rank correlation tests. The 2-sided Fisher exact test was also used when 2×2 contingency tables were analyzed. Statistical analyses were performed using the GraphPad Instat 3.05 software.

RESULTS

Histology. The histopathological findings in SSc skin biopsies included fibrosis in the deep dermis and replacement of subcutaneous fat by newly formed collagen, which was hypocellular, thick, and with hyalinized bundles. A mild inflammatory infiltrate was present around small blood vessels and eccrine coils. Vascular changes consisted of vascular cell wall hyalinization and thickening, and narrowing of the vascular lumen, particularly in the subcutis.

HLA-G protein expression on skin specimens. Considering the patients as a group, HLA-G molecules were detected in 12 cases out of 21 (57%), whereas HLA-G expression was consistently negative in the 28 healthy human skin samples ($p = 0.000004$). These results are illustrated in Figure 1. Among specimens that presented HLA-G, 9 exhibited limited SSc, while the remaining 3 exhibited diffuse SSc.

HLA-G expression in SSc skin biopsies was observed in basal and suprabasal cell layers of the epidermis (keratinocytes) (Figure 1C). The number of HLA-G-positive cells in the epidermis was variable, being very dense in 4 cases, 2 of them with limited and 2 with diffuse skin involvement. HLA-G expression was also observed in epithelial eccrine sweat glands (Figure 1E).

HLA-G protein expression and disease severity. HLA-G expression was correlated with a lower frequency of vascular cutaneous ulcers ($p = 0.0004$), telangiectasias ($p = 0.008$), inflammatory polyarthralgia ($p = 0.02$), and heart involvement (trend; $p = 0.08$), when the patients were analyzed as a group. Similar results were observed when we analyzed only the group with limited SSc. Patients of this group that expressed HLA-G presented lower frequency of vascular cutaneous ulcers ($p = 0.02$), telangiectasias ($p = 0.02$), and polyarthralgia (trend; $p = 0.08$).

In addition, we observed statistically significant differences when we compared the mRSS of patients that expressed and did not express HLA-G ($p = 0.04$). However, when we evaluated the same patients considering the limited or diffuse clinical variants, there were no significant differences in the mRSS of the patients with and those without HLA-G. The average modified Rodnan scores were as follows: HLA-G-positive patients with limited SSc, 7.5; HLA-G-negative patients with limited SSc, 11; HLA-G-positive patients with diffuse SSc, 15; HLA-G-negative patients with diffuse SSc, 27 (Figure 2).

HLA-G protein expression and survival. The mean followup time of the patient group was 127 months (148 months for limited SSc, 107 months for diffuse SSc). Eleven patients (52%) died, with 9 cases (5 diffuse and 4 limited disease) considered to be related to SSc. In this group, only one patient presenting diffuse SSc expressed HLA-G. When we compared the survival of each clinical variant separately depending on the presence or absence of HLA-G, we observed a significant improvement ($p = 0.005$) in the survival of patients with limited SSc that expressed HLA-G.

DISCUSSION

The immune system utilizes several strategies to neutralize self-reactive T cells, a critical step for the development of self-tolerance. Central and peripheral clonal deletions or anergy are part of these immunoregulatory pathways[16]. HLA-G may prevent various primary and secondary phases of immune reactions[17]. A broader immune-inhibitory action of HLA-G molecules was described recently in association with autoimmune diseases such as multiple sclerosis, psoriasis, and atopic dermatitis[12,13].

We have shown for the first time the expression of HLA-G at lesional sites of skin biopsies from patients with SSc. HLA-G expression was observed in epidermal cells and in the dermis. In the epidermis, HLA-G was observed in keratinocytes of basal and suprabasal cell layers, which are cells involved in many immunological and homeostatic skin mechanisms. Expression of HLA-G in the dermis was observed in sweat eccrine glands, which are completely entrapped by broad collagen fibers during the progression of SSc.

As demonstrated by Caumartin, *et al*[17], only a few cells expressing HLA-G are necessary to exert significant inhibitory effects on target destruction through the trogocytosis mechanism, i.e., a few HLA-G-positive cells can protect a larger number of negative cells. This can be observed in kidney allografts, where HLA-G expression is

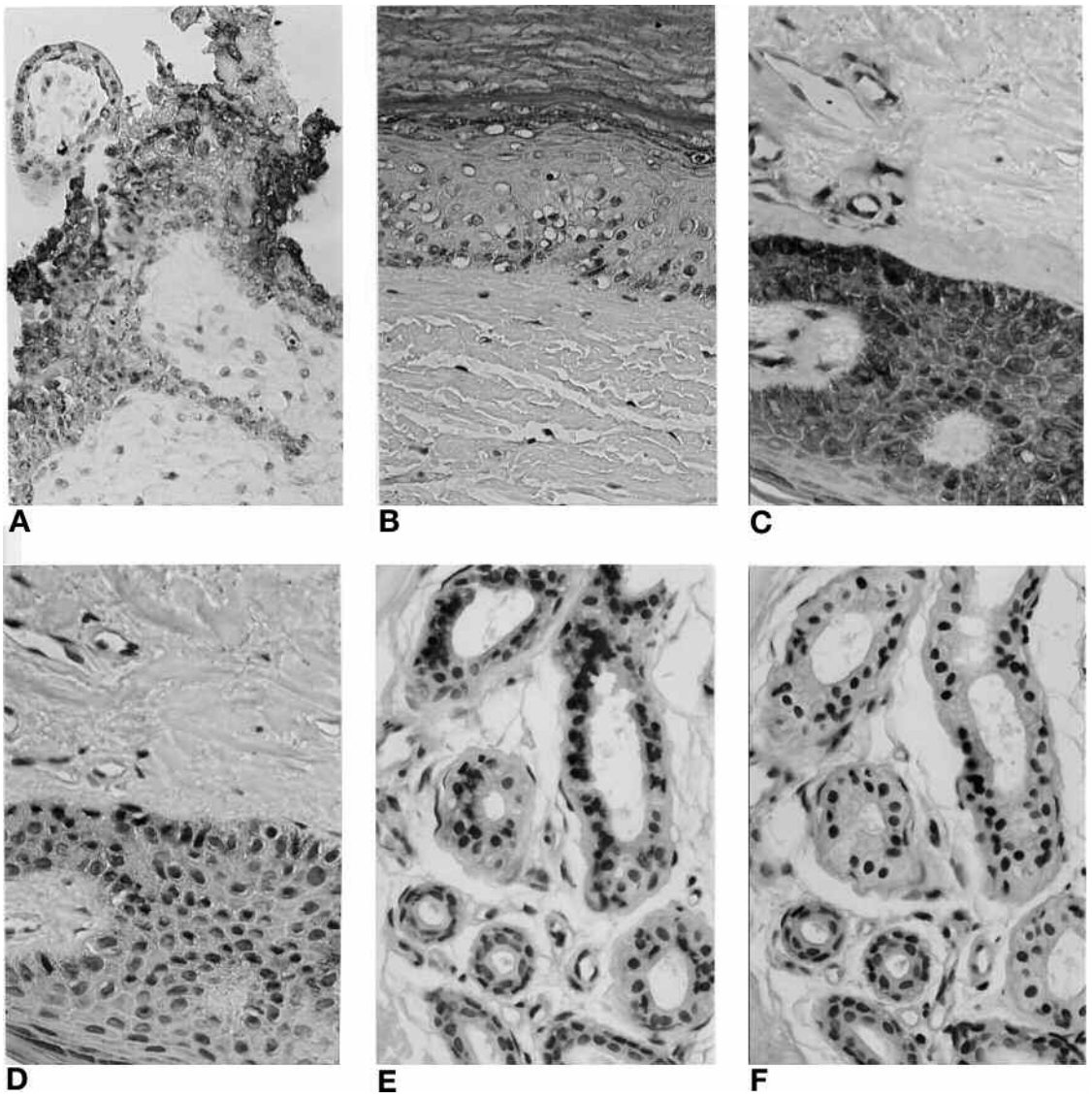

Figure 1. HLA-G expression in the skin of patients with SSc. Skin biopsies were analyzed by immunohistochemistry. (A) Invasive (intermediate) cytotrophoblast from third-trimester human placenta served as a positive protein control. (B) Specimen obtained from a healthy subject. (C and E) Specimens from a patient with SSc: labeling of cells within the epidermis and in-dermis eccrine gland cells. (D and F) SSc patient isotype control (irrelevant antibody). Original magnifications ×40.

detected in tubular epithelial and endothelial cells and not in the whole kidney. However, this expression seems to be sufficient to reduce kidney graft rejection[18]. Considering these data and the fact that the cutaneous involvement in SSc usually has been associated with widespread involvement of the viscera, we could suggest that, although localized, HLA-G expression may exert inhibitory effects on the SSc skin environment that could result in a better prognosis. Indeed, when we correlated clinical and laboratory data with HLA-G expression, we observed that HLA-G expression was associated with a lower frequency of cutaneous (calcinosis and telangiectasis), vascular (cutaneous ischemic ulcers), and visceral involvement (heart; trend to significance).

Finally, we observed that patients expressing HLA-G in their skin biopsies had a higher probability of being alive after a longterm followup. Together, these findings agree with other studies[12,13] that showed the protective role of HLA-G in chronic inflammatory skin disorders.

Although the mechanisms that drive HLA-G production have not been elucidated, many factors related to immune function may be implicated, including cytokines, stress conditions, and drugs.

HLA-G may be upregulated by the antiinflammatory cytokine IL-10[10]. Interestingly, patients with SSc presenting with the limited variant, with the better prognosis, exhibited high serum levels of IL-10[2].

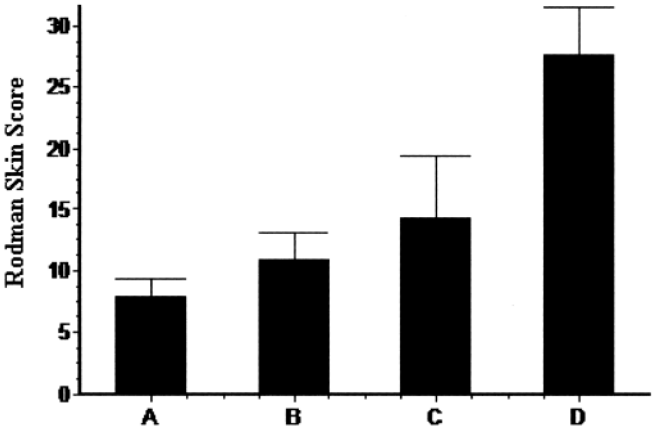

Figure 2. Modified Rodnan skin scores of SSc patients with and without HLA-G: (A) HLA-G-positive patients with limited SSc, 7.5. (B) HLA-G-negative patients with limited SSc, 11. (C) HLA-G-positive patients with diffuse SSc, 15. (D) HLA-G-negative patients with diffuse SSc, 27.

HLA-G expression may also be upregulated by stress conditions, including hypoxia[19] and heat shock[20], among others. The cellular response to hypoxia is driven mainly by a key transcription factor, hypoxia-inducible factor 1 (HIF-1α), involved in angiogenesis and cell survival. Mouillot, *et al*[19] demonstrated the influence of hypoxia on HLA-G gene induction (at the transcriptional level only) of an HLA-G-negative cell line, M8 melanoma cells. Modulation of HLA-G gene expression in hypoxic tumors seems to be dependent on the stabilization of HIF-1α[20]. Distler, *et al*[21] have shown that chronic hypoxia in SSc induces expression of connective tissue growth factor through the activation of HIF-1α in dermal fibroblasts, contributing to the progression of skin fibrosis in SSc. Since HIF-1α expression is stimulated by chronic hypoxia in the skin of patients with SSc, these data suggest that HLA-G expression might be induced by HIF-1α-dependent mechanisms.

Several drugs may influence HLA-G expression, especially the immunosuppressive drugs usually included in therapy for SSc, mainly the glucocorticoids[22]. In our study, all skin samples were collected before the beginning of treatment, supporting the idea that the HLA-G expression observed in patients with SSc was not a consequence of treatment.

HLA-G has been proposed to serve as a fundamental mechanism of immune surveillance. It has been hypothesized that the upregulation of immune-inhibitory HLA-G at sites of inflammation contributes to the limitation of organ damage and plays a role in the maintenance of tissue integrity[7]. Our results suggest that HLA-G expression in SSc could be associated with a more favorable disease course. Although the beneficial role of HLA-G has been demonstrated in some autoimmune diseases, no previous study had evaluated HLA-G expression in SSc. Further studies are needed to fully understand the mechanisms associated with the function and regulation of HLA-G in SSc.

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