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ARTÍCULO ORIGINAL

Expression of transforming growth factor-beta and platelet-derived growth factor in linear scleroderma

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Linear scleroderma (LS) is a localized form of scleroderma characterized by mononuclear cell infiltration and fibroblast proliferation. In the later stages of the disease, excessive collagen is deposited with concomitant skin and appendage atrophy. These symptoms suggest a breakdown of fibroblast cell function, and consequently, growth factors have been thought to play a role in the pathogenesis of LS. The present study examined the expression of TGF-ß and PDGF in skin biopsies obtained from patients with LS and from normal subjects. Samples were prepared for immunohistochemistry. To identify TGF-ß, two polyclonal antibodies were used: TGF-ß1 (RaB4) and TGF-ß2 (CL-B1/29) and, to identify PDGF, two monoclonal antibodies were used: PDGF-AA (3E-205) and PDGF-BB (1F-133). Staining for TGF-ß1 and TGF-ß2 was observed around blood vessels (endothelial cells), and sweat glands in both LS and normal skin. Staining for PDGF-AA and PDGF-BB was intense in endothelial cells and sweat glands in LS and normal skin. Mononuclear cell infiltrates and abnormal collagen bundles did not stain for TGF-ß or PDGF. The strength and extent of staining was evaluated in tissues using a scale from zero (no staining) to four (strong staining). The amount of TGF-ß1, TGF-ß2, PDGF-AA and PDGF-BB was found similar in LS and normal skin. These results do not support the hypothesis that the excessive fibroblast cell activity and abnormal collagen deposition observed in LS are associated with downregulation of TGF-ß or PDGF.

Key words: linear scleroderma, growth factors.

Expresión del factor β transformador del crecimiento (TGF-β) y del factor de crecimiento derivado de las plaquetas (PDFG) en la esclerodermia lineal

La esclerodermia lineal (EL) es una forma localizada de la enfermedad, caracterizada por la proliferación fibroblástica y los infiltrados linfocitarios perivasculares en la dermis. En los estadios terminales de la enfermedad, hay depósito de colágeno en exceso con atrofia cutánea y de los anexos, todo lo cual sugiere una alteración de la regulación de la función fibroblástica, con participación de los factores de crecimiento en la patogenia de la enfermedad. Este trabajo se planeó para examinar la expresión de los factores TGF-ß y PDFG en biopsias de piel de pacientes con EL y de controles sanos, con estudio inmunohistoquímico. Para idenficar el TGF-ß se emplearon dos anticuerpos policlonales TGF-ß1 (RaB4) y TGF-ß2 (CL-B1/29) y dos anticuerpos monoclonales para identificar el PDFG: PDFG-AA (3E-205) y PDFG-BB (1F-133). La tinción para TGF-ß1 y TGF-ß2 se observó alrededor de los vasos sanguíneos pequeños y de las glándulas sudoríparas tanto en la EL como en la piel normal. La tinción para PDGF-AA y PDGF-BB fue intensa en las células endoteliales y en las glándulas sudoríparas en la EL y en la piel normal. El infiltrado linfocitario y los haces colágenos anormales no se tiñeron para TGF-β ni para PDGF. La intensidad y la extensión de la reactividad evaluada en los tejidos con una escala de 0 (ausencia de tinción) a 4 (tinción notoria) indican que la expresión de TGF-ß1, TGF-ß2, PDGF-AA y PDGF-BB son similares en la EL y en la piel normal. Nuestros hallazgos no apoyan la hipótesis según la cual la actividad fibroblástica excesiva y el depósito de colágeno

anormal observados en la esclerodermia lineal estén asociados con alteraciones de la regulación del TGF-ß o del PDGF.

Palabras clave: esclerodermia lineal, factores de crecimiento.

Linear scleroderma (LS) is a distinct form of dermal induration confined to an asymmetrical area of the skin. Althought some clinico-pathological findings are similar, LS bear minimal relationship to systemic sclerosis. The lesion may appear spontaneously but, in most cases, it is preceded by injury. Early manifestations are difficult to diagnose. An ill defined plaque of edema with occasional erythema and pain precedes the typical skin lesions (1). Later, the lesions may appear with distinct features. The plaque ranges from 1 to 15 cm in its largest dimension, are sharply defined and asymmetrical (2) . The center is smooth and whitish with various shades of pigmentation. The surface appears shiny and atrophic.

The lesions are characterized by its cord-like distribution involving superficial as well as deeper layers of the skin. The advanced form of the disease may result in fixation of the skin to underlying structures. Histological studies have identified that the initial inflammatory cell infiltrate is replaced by fibroblast proliferation and excessive collagen deposition, progressing to fibrosis and kin atrophy (3,4). Fleischmajer *et al.* (5,6) have characterized the cellular infiltrate of this disease. The histological sequence of LS is initiated by an inflammatory cell infiltrate (macrophages and lymphocytes) that progresses later towards fibroblast proliferation and collagen synthesis. Thus, it may be that the initial wave of cellular infiltration by inflammatory cells sets the stage for the second wave of infiltration by fibroblasts, which may result in collagen synthesis and fibrous tissue accumulation in the local fashion characteristic of LS. The local and progressive proliferation of fibroblasts and collagen deposition that correlates with LS progression suggest an alteration of the control of fibroblast growth-promoting activities, a process regulated by growth factors. Transforming growth factor beta (TGF-ß) and platelet-derived growth factor (PDGF) are polypeptides widely distributed and critically involved in cell proliferation and differentiation of fibroblasts and control of collagen synthesis necessary for growth, development and repair (7,8). The present study was designed to examine the expression of TGF-ß and PDGF in skin biopsies obtained from patients with LS and from normal skin obtained from affected patients and from normal subjects.

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Materials and methods

We selected 10 patients from a cohort of 25 patients with clinical and pathologic diagnosis of LS, according to the classification of Tufffanely (9) and Leroy (3).

After informed consent, skin incisional biopsies (1 x 0.5 cm of diameter that included subcutaneous tissue) were obtained from 10 patients with LS (affected and normal skin) and from 2 normal subjects. The normal skin specimens were obtained from the forearm. The 10 patients have the subset form of *coup du sabre* and facial hemiatrophy. The lesions were well established and with different time of evolutions: 3 patients with less than a year; 2 between 1 and 5 years, and 5 patient with 5 or more years of evolution. Specimens were fixed in formaldehyde, embedded in paraffin and in 5 µ cross sections.

Antibodies

Two polyclonal antibodies raised in rabbit (Celtrix Laboratories, Palo Alto, CA) were used recognize TGF-ß: RaB4 was used to detect intracellular TGF-ß1, and CL-B1/29, directed against a peptide with a sequence identical to the amino terminus residues 1-29 of TGF-ß2 and detecting intra and extracellular TGF-ß2. No cross-reaction was detected between these two antibodies. Two monoclonal antibodies raised in mouse (Amgen Inc, Thousand Oaks, CA) were used to recognize PDGF: clone 3E-205 detecting the AA isoform of PDGF, and clone 1F-133 detecting the BB isoform

and with reactivity towards the AB isoform of PDGF.

Reagents

Phosphate buffered saline (PBS) hydrogen peroxide, diaminobenzidine tetrahydrochloride (DAB), Tween-20 and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Avidin biotin immunoperoxidase labeling kit (ABC kit), normal mouse IgG and normal rabbit IgG were purchased from Vector Laboratories (Burlingame, CA).

Immunohistochemical analysis for TGF-ß1 and TGF-ß2. These studies were performed as previously described (5,10). Briefly, sections were fixed in cold acetone for 30 min and air dried. Endogenous peroxidase was inactivated by 30 minute incubation in hydrogen peroxide in PBS. Sections were incubated with 10% normal goat serum (ABC kit) in PBS with 0.5% BSA and 0.05% Tween-20 for 45 min. After draining, samples were incubated with primary antibodies RaB4 and CL-B1/29 and subsequently diluted 1:40 in PBS at room temperature for 1 h. Samples were rinsed three times in PBS and incubated with secondary antibody biotinylated goat anti-rabbit (ABC kit) diluted 1:50 for 30 min. Slides were washed and incubated with streptavidin peroxidase (ABC kit) diluted in PBS with 0.5% BSA and 0.05% Tween 20 during 45 min. Samples were exposed to DAB for 2 min, washed in running water for 5 min, counterstained with hematoxylin and mounted under coverslips. Controls were carried out by replacing the primary antibodies with normal rabbit IgG.

Immunohistochemical analysis for PDGF-AA and PDGF-BB

After fixation in cold acetone and inactivation of endogenous peroxidase, samples were incubated with 10% normal horse serum (ABC kit) in PBS for 30 min. After draining, samples incubated with primary antibodies PDGF-AA, 3E-205 and PDGF.BB 1F-133 diluted 1:200 in PBS for 15 hours at room temperature. Samples were rinsed three times in PBS and incubated with secondary antibody biotinylated horse anti-mouse IgG (ABC KIN) for 30 min. After washing, slides were incubated with streptavidin peroxidase (ABC kit), exposed to DAB and counterstained with hematoxylin. Controls were carried out by replacing the primary antibodies with normal mouse IgG.

Histological analysis

To provide a semi-quantitative comparison of the expression of these growth factors between LS lesions and controls, an arbitrary grading scale from zero to four was used to rate the intensity and extent of staining in each sample, as previously described (5). Zero was scored when staining was absent or very weak; one when the staining was weak; two when moderate staining was observed; three, strong staining; and, four for the strongest staining observed. Evaluation was performed in a blinded manner by two independent observers using the same criteria.

Statistics

Results were averaged and expressed as mean ± standard error of the mean (SEM). Comparisons were performed by one way analysis of variance (ANOVA) with Bonferroni's correction. Differences were considered significant when $p<0.05$.

Results

The clinical characteristics of our patients were skin involvement consisting of purple, asymmetric and linear indurated plaques. Over half of the patients had more than one lesion. At the time of the skin biopsy, the disease had been present a year or less in three patients, one to five years in two patients, and more than five years in the remaining five patients. Despite the localized nature of the disease, three patients had severe fibrosis and atrophy near a major joint that was associated with significant decrease in the range of motion of body segment (table 1). Most of the skin biopsies were obtained from the lower extremities. By definition, these patients did not have any manifestations of systemic scleroderma.

Light microscopy confirmed the findings of this localized form of scleroderma (5,6) (figure 1) with the inflammatory cellular infiltrate (mononuclear cells) more obvious around blood vessels and sweat glands.

The skin obtained from LS lesions displayed intense staining for PDGF-AA and PDGF-BB predominantly in endothelial cells and sweat glands.

Table 1. Clinical characteristics of patients with linear scleroderma.

Age (years)	Gender	Appearance	Duration	Localization	Comments
10	F	plaque	5 years	leg	M, U
20	F	plaque	8 years	leg	M, U
9	F	plaque	6 months	gluteus	I
17	F	plaque	1 years	thigh	I
24	F	plaque	9 years	leg	M, U
35	F	plaque	3 years	thigh	I
28	F	atrophy	10 years	thigh	I
46	F	plaque	4 years	leg	M, U

F: female; M: multiple; I: isolated; u, unique.

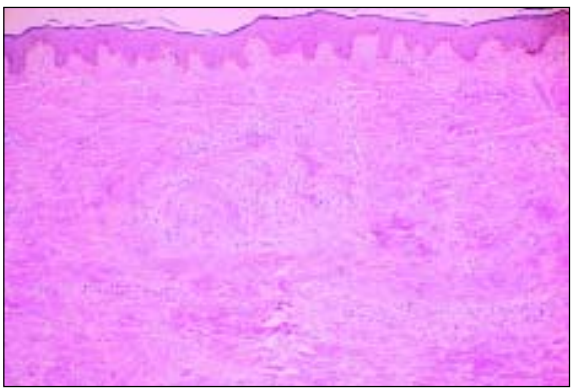

Figure 1. Pathological changes of linear scleroderma are observed: accumulation of inflammatory cells among collagen bundles and abnormal collagen deposition. HE, 40X.

Mononuclear cell infiltrates and abnormal collagen bundles were negative for TGF-ß or PDGF. The strength and extent of staining of TGF-ß1, TGF-ß2, PDGF-AA and PDGF-BB were similar between LS lesions and the skin obtained from normal-looking skin from these patients or the two control subjects (table 2).

TGF-ß1: Staining was intense in LS lesions and normal skin. The expression of TGF-ß1 and TGF-ß2 was cytoplasmic, primarily in perivascular endothelial cells and around sweat glands (figure 2).

PGDF-AA: Staining was also intense in LS lesions and normal skin. Staining was observed in perivascular endothelial cells, fibroblasts, keratinocytes and sweat glands (figure 3).

PDGF-BB: staining was weaker but the distribution was similar to that observed for PDGF-AA (not shown).

Table 2. Overall tissue staining and intensity for PDGF-AA, PDGF-BB, TGF-ß1 and TGF-ß2 in linear scleroderma and control samples**.**

	Linear (n=10) Scleroderma	Control (n=10)	Normal (n=2) Skin	t-test
PDGF-AA	2.4±0.2	2.3±0.2	2.4±0.2	NS
PDGF-BB	1.8±0.1	1.6±0.1	1.5±0.1	NS
TGF-ß1	2.5±0.3	2.6±0.2	2.4±0.2	NS
TGF-ß2	1.5±0.1	1.6±0.1	1.4±0.1	NS

NS: not significant

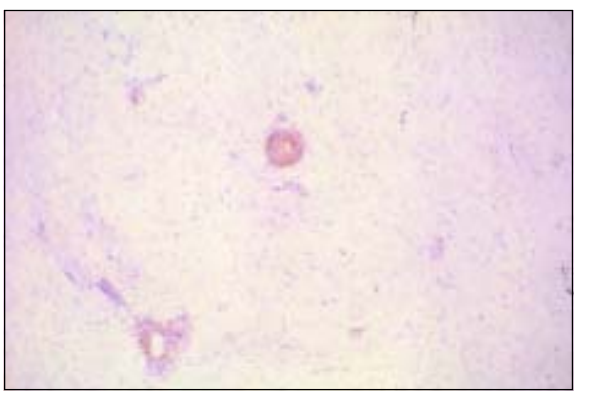

Figure 2. Staining of TGF-ß1 with RAB antibody in linear scleroderma. Hematoxylin counterstained, 40X.

As it was noted above, neither PDGF or TGF-ß staining was associated with mononuclear cell infiltrates or abnormal collagen bundles in LS lesions (figure 4). The specificity of the reactions was confirmed by the lack of staining in negative controls incubated with IgG instead of primary antibody (not shown).

Discussion

The pathogenesis of LS remains undetermined. Since the disease is characterized by local fibrosis

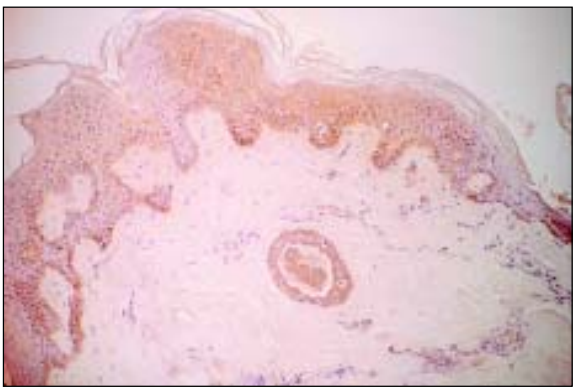

Figure 3. Staining of PDGF-AA with 3E 205 antibody in linear scleroderma. Hematoxylin counterstained, 40X.

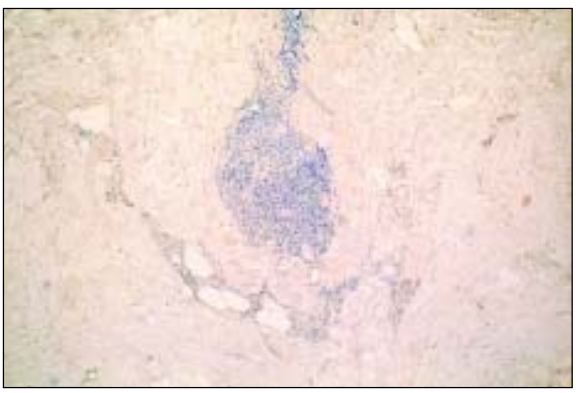

Figure 4. Staining of PDGF-AA with 3E 205 antibody. Patchy inflammation stains negative for PDGF-AA. Hematoxylin counterstained, 160X.

due to collagen deposition, it is assumed that fibroblasts may play a key pathogenetic role (3). Published evidence indicates that among the cytokines produced by inflammatory cells, and in particular by macrophages, growth factors play an important role in regulation of fibroblast function (11-14). *In vitro* studies have demonstrated that fibroblasts incubated with PDGF and TGF-ß obtained from macrophages in culture, exhibit significantly more migration (chemotaxis), more proliferation and greater ability to synthesize collagen than fibroblasts incubated with media alone (11). Also in *in vitro* studies it has been shown that stimulation of the NIH3T3 fibroblast with TGF-ß resulted in increased expression of the gen promoters for á1 and á2 collagen (12). In addition, Bolstein *et al.* (15) demonstrated that fibroblasts isolated from skin lesions with scleroderma may be programmed to have increased synthesis of collagen. Our results are in agreement with those reported by Gay *et al.* (16) who reported expression of PDGF and TGF-ß in LS lesions, with expression of PDGF found predominantly in endothelial cells lining small capillaries. Taken together, those studies suggest that the dysfunction of the fibroblasts seen in LS resulting in excessive rate of collagen synthesis may be mediated, at least in part, by increased expression of growth factors.

We designed the present study to examine localization of TGF-ß and PDGF in human skin obtained from ten patients with linear scleroderma. These observations were compared with those made on normal-looking skin samples obtained from the same patients and from two normal volunteers. The expression of TGF-ß and PDGF of the skin samples obtained from the lesions of LS was not different than the once seen in the samples obtained from normal-looking skin obtained from the same patients or those obtained from the normal volunteers. In all samples examined, PDGF and TGF-ß were expressed primarily in perivascular endothelial cells, fibroblasts, keratinocytes and around sweat glands with the same distribution and intensity of observed in the different samples.

Different interpretations may be given to the lack of difference in the expression of growth factors between normal-looking skin and skin affected with LS. First, it may be suggested that PDGF and TGF-ß do not play a role in the pathogenesis of this disease. This argument may be sustained by the lack of difference in the expression of these factors in the skin samples with LS, compared with normal looking skin or skin obtained from normal subjects. In agreement with this view are the reports by Needlemann *et al.* (17), Takehara *et al.* (18), Feldman *et al.* (19), and Gruschwitz *et al.* (20) . Needlemann (17) isolated fibroblasts from skin obtained from patients with systemic scleroderma and reported that secretion of TGF-ß by fibroblasts and the number or receptors TGF-ß in these cells were not significantly different from fibroblasts obtained from normal-looking skin obtained from volunteers. *In vitro* studies by Takehara (18) showed fibroblasts obtained from lesions of patients affected with LS had less proliferation af-

ter incubation with PDGF or TGF-ß than those obtained from normal controls. Feldman *et al.* (19), and Gruschwitz *et al.* (20), working independently, demonstrated that fibroblasts obtained from skin lesions of patients affected by LS had a similar mitogenic response after stimulation with PDGF or TGF- ß. Taken together, these studies may be interpreted as to suggets that PDGF or TGF-ß do not play a key role in the pathogenesis of LS.

Second, it also may be argued that the time of the skin biopsies may have played a decisive role in the expression of these growth factors. In essence, the expression of PDGF and TGF-ß may have been associated with the initial wave of the infiltrate by immune cells, in particular macrophages that disappeared by the time of the infiltration by fibroblasts and the active phase of collagen synthesis. Since these events may precede the clinical manifestations, the predominant infiltration by immune cells associated with greater synthesis of cytokines that induce collagen synthesis by fibroblasts may have been missed at the time of the skin biopsy. Although that may have been the case, the different time of evolution of the clinical manifestations in our patients argue against that contention: three of our patients had clinical manifestations of less that a year duration, two patients had clinical manifestations one to five years duration, and five patients had clinical manifestations for five years or longer. Thus, we may argue that we had access to skin samples with different stages of the disease and that in none of those expression of PDGF and TGF-ß was significantly different that in the normal controls.

The role, if any, of these growth factors, an in particular that of TGF-ß, may not be dismissed in the context of the present evidence. *In vitro* studies have demonstrated that TGF-ß stimulate chemotaxis, proliferation and synthesis of collagen by fibroblasts. Also, its role in scar formation is increased by its toxicity to endothelial cells (11) and it inhibits collagenase synthesis. The latter characteristics may increase the chances of replacement of normal-looking tissue by scar tissue. *In vitro*, it has been demonstrated that PDGF stimulate chemotaxis and proliferation of fibroblasts (11,14,16,21,22). In addition, *in vitro* studies have shown that TGF-ß activates the mouse a2 (3) and a1 (13) collagen promoters by fibroblasts in culture due to its effects on nuclear binding sites (13). In another study by Kubo *et al.* TGF-ß receptor I and II (RI and RII) were up regulated in dermal fibroblasts in the affected skin of patients with localizes scleroderma (23). Zheng *et al.* performed both *in vivo* studies on the expression of PDGF ß-receptor protein in scleroderma tissue and *in vitro* studies on the expression of PDGF ß-chain and PDGF ß-receptor mRNA in cultured fibroblast derived from both lesions of scleroderma and normal skin. Immunohistochemistry staining showed that PDGF ß-receptor expression was greatly elevated in the dermis of scleroderma lesion whereas PDGF ß-receptor were expressed at low levels in normal skin. Northern blot analysis showed that cultured fibroblast from scleroderma had higher expression of PDGF ß-chain and PDGF ß-receptor mRNA than those from normal control (24). Taken together, these studies suggest that PDGF and TGF-ß may play a role in the pathogenesis of LS. However, noted by Smith and LeRoy (10,13), the *in vitro* effects of these growth factors has not been accompanied by the same observations *in vivo* in scleroderma, suggesting that other factors may also play a role in the pathogenesis of LS.

Kawakami *et al.* also found that immunoreactivity with TGF-ß3 tends to increase in the dermal and subcutaneous fibrosis, fibroblasts and inflammatory cells of patients with solitary morphoea profunda, however we did not look for that specified molecule (25).

Finally, it is also possible that PDGF and TGF-ß play a role in the pathogenesis of LS, but that this role may not be as important as initially postulated. Observations by Claman (26) and Mican (27) suggest that cells other than immune cells, and possibly other mediators, may play a more important role in the pathogenesis of LS. In this respect, mast cells have been described in this disease in association with endothelial structures and sweat glands (23). Since one common pathway of the pathogenesis of LS is the damage of the endothelial cell and capillary obliteration, it is possible that these events my have been mediated by mast cells. To the best of our knowledge this postulated has not been either proved or disproved.

The end-result of this pathway is local ischemia and tissue death with subsequent replacement by fibroblasts and scar all of which are characteristic of LS.

The results of this study suggest that increased expression of these growth factors do not play a significant role in the pathogenesis of this disease. However, since we obtained only one sample from each lesion per patient, our results do not permit us to rule out definitively a role of these factors in early stages of the disease, especially in view that the number of our sample is small.

In summary, our results demonstrate that the expression and distribution of TGF-ß and PDGF is similar in LS lesions and normal skin. TGF-ß and PDGF may play a role in the pathogenesis of linear scleroderma at early stages, probably mediating initial fibroblast activation and collagen deposition, followed by subsequent down-regulation of their expression when the disease is established. Alternatively, is it possible that these growth factors do not play any role at all in the pathogenesis of the disease.

These findings do not support the hypothesis that the excessive fibroblast activity and abnormal collagen deposition observed in linear scleroderma are dependent on abnormal regulation of TGF-ß and/or PDGF.

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