Distinct Expression of Adhesion Molecules on Skin Fibroblasts from Patients with Diffuse and Limited Systemic Sclerosis. A Pilot Study

FLORENZO IANNONE, MARCO MATUCCI-CERINIC, PAOLA CHIARA FRANCESCA FALAPPONE, SERENA GUIDUCCI, MARINA CINELLI, OLIVER DISTLER, and GIOVANNI LAPADULA

ABSTRACT. ***Objective***. Systemic sclerosis (SSc) is characterized by excessive production of collagen and other components of the extracellular matrix (ECM) by fibroblasts. The ECM receptors, integrins and CD44 (hyaluronan receptor), play a key role in the homeostasis of connective tissue and may also have a role in the pathogenesis of fibrosis. We investigated the expression of integrins and CD44 on skin fibroblasts from patients with limited and diffuse SSc.

Methods. We studied 13 patients with SSc, 8 with limited SSc, and 5 with diffuse SSc, and 8 control subjects. Fibroblasts were isolated and cultured from biopsies taken from the lesional skin of the second finger of the left hand. Cell-surface expression of ß1, ß3, α1–α6, αv integrins, and CD44 was evaluated by immunofluorescence and flow cytometry analysis.

Results. Fibroblasts from limited SSc showed significantly decreased expression of α2, α3, α4 integrins, while diffuse SSc fibroblasts had significantly reduced expression of α5, αv integrins, and CD44. Diffuse SSc also had significantly increased expression of α6 integrin on fibroblasts. In controls, the expression of α4 and α5 correlated positively, while in limited and diffuse SSc it did not.

Conclusion. This is the first study evaluating separately the expression of adhesion molecules on skin fibroblasts from limited and diffuse subsets of SSc. We detected a distinct pattern of expression with decrease of collagen and fibronectin receptors in limited SSc, and downregulation of fibronectin and hyaluronan receptors in diffuse SSc. These results suggest that changes of fibroblasts/ECM interactions and mechanisms underlying the pathogenesis of fibrosis in SSc may differ in the single subset of the disease. (J Rheumatol 2005;32:1893–8)

Key Indexing Terms:
SYSTEMIC SCLEROSIS CD44 ß1 INTEGRIN ß3 INTEGRIN
α INTEGRINS EXTRACELLULAR MATRIX

Systemic sclerosis (SSc) is a connective tissue disease characterized by excessive fibrosis in the skin and internal organs[1]. According to the extent of skin involvement, SSc has been clinically divided into limited SSc and diffuse SSc subsets with different clinical outcome and prognosis. Although SSc pathogenesis is still poorly understood, one hypothesis suggests that growth factors and cytokines trigger an abnormal connective tissue metabolism, eventually inducing skin fibrosis[2]. An overproduction of type I collagen and fibronectin at transcriptional and posttranscriptional levels has been shown in activated SSc fibroblasts[3].

Fibroblasts play a key role in maintaining the homeostasis of connective tissue by regulating the synthesis, assembly, and breakdown of type I collagen and extracellular matrix (ECM). This balance is maintained by the inside-out/outside-in signaling interplay between cell and cell and cell and ECM and is modulated by cell-surface receptors, named integrins[4]. Integrins are heterodimers constituted by a common (ß) and a variable (α) chain, with high affinity for matrix proteins such as collagen, fibronectin, vitronectin, and laminin[5]. Among integrins, α2 is the receptor that recognizes type I collagen: it has been shown that the α2 integrin promotes the internalization and subsequent degradation of collagen fibrils by human fibroblasts. α2 integrin is then rapidly recycled or synthesized following the internalization of α2/collagen complex[6]. In addition to integrins, CD44 is another cell membrane receptor involved in modu-

From the Rheumatology Unit — DIMIMP, University of Bari, Bari; SOD Internal Medicine 1 and Rheumatology, AOUC Careggi, University of Florence, Florence, Italy; and Center of Experimental Rheumatology, Department of Rheumatology, University Hospital Zurich, Zurich, Switzerland.

Supported by grants from the University of Bari and University of Florence.

F. Iannone, MD, PhD, Consultant in Rheumatology; P.C.F. Falappone, MD, Research Assistant in Rheumatology; G. Lapadula, MD, Full Professor of Rheumatology, Rheumatology Unit — DIMIMP, University of Bari; M. Matucci-Cerinic, MD, PhD, Full Professor of Rheumatology; S. Guiducci, MD, Research Assistant in Rheumatology; M. Cinelli, MD, Research Assistant in Rheumatology, SOD Internal Medicine 1 and Rheumatology, AOUC Careggi, University of Florence; O. Distler, MD, Consultant in Rheumatology, Center of Experimental Rheumatology, Department of Rheumatology, University Hospital Zurich.

Address reprint requests to Prof. G. Lapadula, Rheumatology Unit–DIMIMP, Piazza G. Cesare 11, 70124 Policlinico, Bari, Italy. E-mail: g.lapadula@reumbari.uniba.it

Accepted for publication May 9, 2005.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2005. All rights reserved.

Downloaded on April 23, 2024 from www.jrheum.org

lating ECM remodeling. CD44 binds hyaluronic acid and anchors cells to proteoglycans, and activates intracellular metabolic processes, cell growth, and development[7].

It is conceivable that in SSc skin the abnormal connective tissue remodeling may be related to changes in integrin expression on fibroblasts, with subsequent aberrant ECM-fibroblast crosstalk. The expression of integrins has been studied in SSc skin fibroblasts, with conflicting results. α2 integrin concentrations were found to be decreased[8-11] or normal[12]. In these studies, however, SSc patients were not stratified according to disease subset, and we believe that fibroblast metabolism and phenotype can differ as a function of the extent of skin involvement. We have recently shown that skin fibroblasts from subjects with diffuse SSc had a higher proliferation rate and lower CD10/neutral endopeptidase surface expression than those from limited SSc *in vitro*[13]. In the present study we evaluated the surface expression of ß1, ß3, α1–α6, αv integrins, and CD44 on skin fibroblasts from SSc patients in limited and diffuse disease subsets.

MATERIALS AND METHODS

Patients. Thirteen patients with SSc were consecutively selected and classified into the limited (8 patients) or diffuse (5 patients) cutaneous subset[14]. Patients with limited SSc had a mean disease duration of 11 years (range 7–19) and were treated with prostaglandins (8 patients), cyclophosphamide (2 patients), and azathioprine (one patient). Patients with diffuse SSc had a mean disease duration of 9 years (range 3.1–12) and were treated with prostaglandins (5 patients) and cyclophosphamide (one patient). Punch skin biopsies were taken from the second finger of the left hand with informed consent. Skin biopsies were obtained from the same site from 8 control subjects matched for sex and age, who had undergone posttraumatic hand surgery.

Fibroblast cultures. Tissue from the skin biopsies was placed in 60 mm tissue culture plates and cultured in complete minimal essential medium (MEM; Gibco, Grand Island, NY, USA) containing 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37°C with 5% CO_2. The medium was changed weekly. When fibroblasts were near confluence they were trypsinized with 0.05% trypsin and 0.01% EDTA in phosphate buffered saline (PBS), resuspended in culture medium, and plated in 25 cm^2 plastic tissue culture flasks. After the third passage, confluent fibroblasts were trypsinized, washed twice in PBS, and counted. More than 95% of the fibroblasts were viable (Trypan blue exclusion test).

Fibroblast phenotype analysis. The following monoclonal antibodies (Mab) were used in the study: anti-ß1 integrin, anti-α1 integrin, anti-α2 integrin, anti-α3 integrin, anti-α4 integrin, anti-α5 integrin, anti-α6 integrin, anti-αv integrin (from Serotec, Kidlington, UK); and anti-ß3 integrin, anti-CD44 (Becton-Dickinson, Mountain View, CA, USA). Freshly isolated fibroblasts were resuspended in PBS containing 0.1% sodium azide and 0.2% bovine serum albumin, and blocked by incubating with 2% normal human serum (Advanced Protein Products, Brierly Hill, UK). After centrifugation (5 min/1300 rpm) cells were incubated with 5 μl of Mab for 20 min at 4°C. When unconjugated Mab were used, a second incubation with fluorescein-conjugated affinity goat anti-mouse immunoglobulin $F(ab')_2$ fragments (GAM-FITC; Becton-Dickinson) was performed. Control samples were incubated with mouse IgG1-FITC/IgG2-PE (Dako, Glostrup, Denmark) or GAM-FITC alone. Stained cells were analyzed on a FACScan (Lysis 2; Becton-Dickinson). Dead cells and debris were excluded by gating live fibroblasts on forward and 90° light scatter. The FACS setting was identical throughout the study.

Statistical analysis. Results are shown as mean ± 1 standard deviation. The statistical difference in antigen expression was assessed by Mann-Whitney U test. Correlation analysis was carried out using Spearman's correlation test. The significance level was set at $p < 0.05$.

RESULTS

The percentages of fibroblasts expressing the surface receptors assessed in limited SSc, diffuse SSc, and control subjects are shown in Figure 1.

ß1 integrin common chain was largely expressed on fibroblasts, although the proportion of positive cells was similar in the 3 groups: limited SSc (80.0% ± 8%), diffuse SSc (74.5% ± 11%), and controls (79.1% ± 7%). Similarly, α1 chain was expressed in limited SSc (68.9% ± 13%), diffuse SSc (64.9% ± 8%), and controls (70.6% ± 12%) and the difference was nonsignificant. The expression of α2 integrin subunit was significantly lower in limited SSc (29.6% ± 16%) than diffuse SSc (51.5% ± 9%; $p < 0.01$) and controls (46.6% ± 9%; $p < 0.01$). The percentage of fibroblasts bearing α3 chain was significantly reduced in limited SSc (31.7% ± 12%) in comparison with diffuse SSc (54.4% ± 14%; $p < 0.01$) and controls (50.3% ± 11%; $p < 0.05$). As well, α4 integrin subunit expression was significantly lower in limited SSc (32.1% ± 13%) than in control samples (54.5% ± 8%; $p < 0.01$), while it did not differ significantly from diffuse SSc (47.5% ± 15%).

Diffuse SSc samples had a significant reduction of α5 integrin chain (54.5% ± 5%) in comparison with controls (70.1% ± 11%; $p < 0.05$), but did not differ from limited SSc (65.8% ± 7%). αv integrin subunit expression was significantly lower in diffuse (55.2% ± 6%) than limited SSc (74.4% ± 13%; $p < 0.01$) or controls (82.6% ± 5%; $p < 0.01$). The proportion of fibroblasts expressing α6 chain was significantly higher in diffuse SSc (35.7% ± 15%) than limited SSc (9.4% ± 4%; $p < 0.01$) or controls (14.6% ± 3%; $p < 0.01$). Yet CD44 expression was significantly downregulated in diffuse SSc (51.4% ± 10%) in comparison with limited SSc (66.6% ± 13%; $p < 0.01$) or controls (67.8% ± 5%; $p < 0.01$). Finally, ß3 integrin common chain was weakly expressed on skin fibroblasts from diffuse SSc (3.1% ± 0.1%), limited SSc (1.6% ± 0.7%), and control samples (3.0% ± 2.4%).

We correlated expression of the integrin receptors on fibroblasts of each group. As shown in Figure 2, a significant positive correlation between α4 and α5 was found only in controls, whereas this correlation was absent in fibroblasts from patients with limited and diffuse SSc.

DISCUSSION

Our data provide clear evidence that skin fibroblasts of patients with limited and diffuse SSc bear a different pattern of ECM receptors. Although it has been reported that serially cultured fibroblasts may change their phenotype *in vitro*[15], the identical procedure was used for the different fibroblast explants and thereby the differences we detected

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2005. All rights reserved.

Downloaded on April 23, 2024 from www.jrheum.org

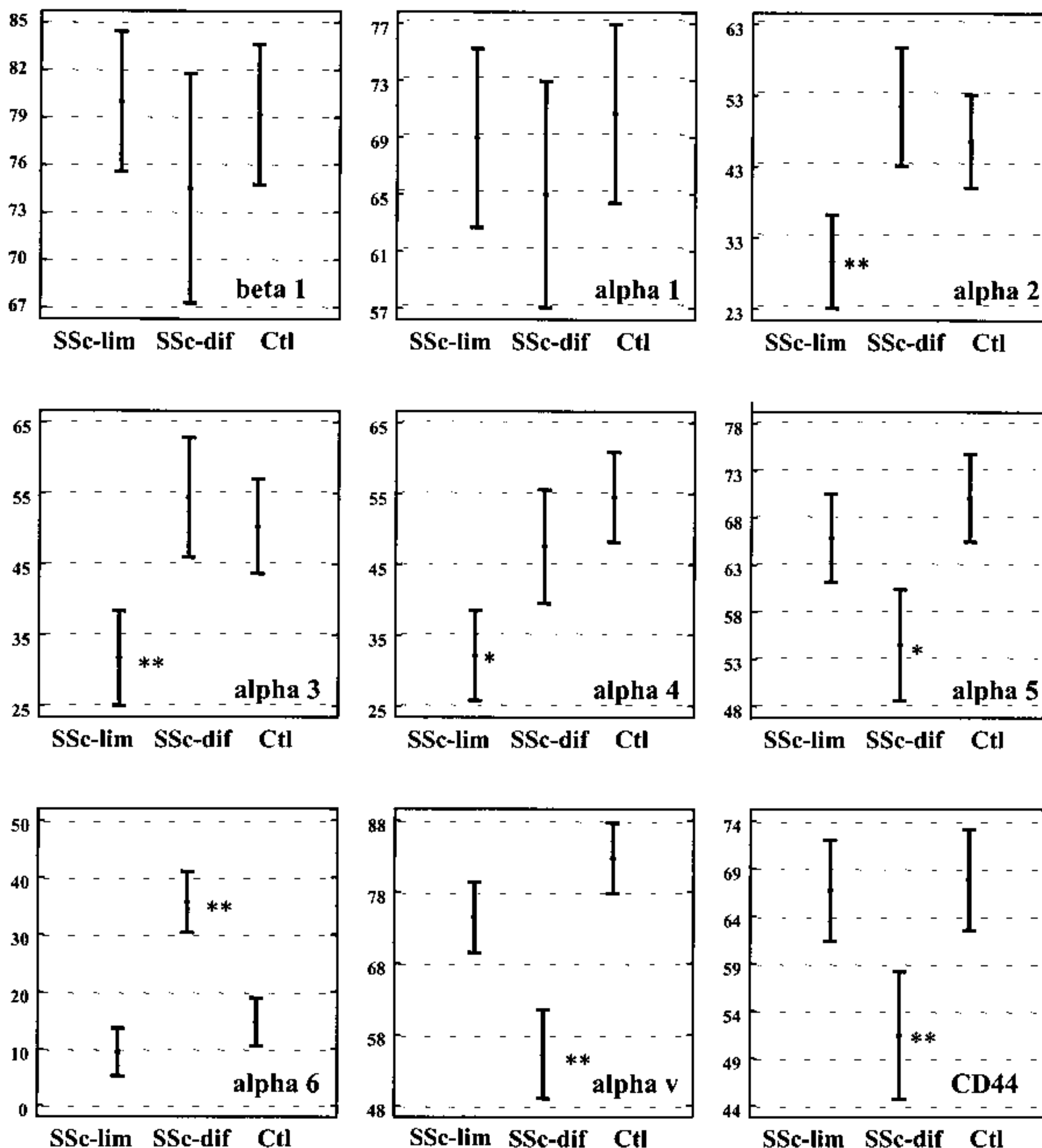

Figure 1. Percentage (mean ± 1 SD) of skin fibroblasts expressing extracellular matrix receptors from 8 controls, 8 limited SSc patients (SSc-lim), and 5 diffuse SSc patients (SSc-dif). **p < 0.01, *p < 0.05. See text for statistical comparisons between groups.

can still be considered noteworthy. Limited SSc samples had decreased expression of α2, α3, and α4 integrins, while diffuse SSc showed a reduced expression of α5 and αv integrins and CD44, but in addition had an enhanced expression of α6 integrin. These results are in agreement with data reporting decreased expression of α2 integrin mRNA in SSc fibroblasts[8,9] or low levels of ß1α1 integrin[10]. In another study, changes of ß1α1 and ß1α2 integrins on SSc fibroblasts were not detected[12]. This discrepancy may be due to cell culture conditions, investigation techniques, disease duration, and mainly the activation state of fibroblasts. Kozlowska, *et al*[8] showed that SSc fibroblasts producing excessive type I collagen expressed lower levels of α2 integrin mRNA than low collagen-producing SSc fibroblasts. This suggests that α2 integrin surface expression will depend on the functional state of fibroblasts and accordingly on the subset of the disease. A relevant variable may also be the stage of the evolution of skin involvement. Indeed, kinetic experiments on human skin wounds at different stages of healing showed in the early phases of scar formation an upregulation of α2 and αv integrin expression in dermal fibroblasts, which decreased when healing was completed[16].

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2005. All rights reserved.

Downloaded on April 23, 2024 from www.jrheum.org

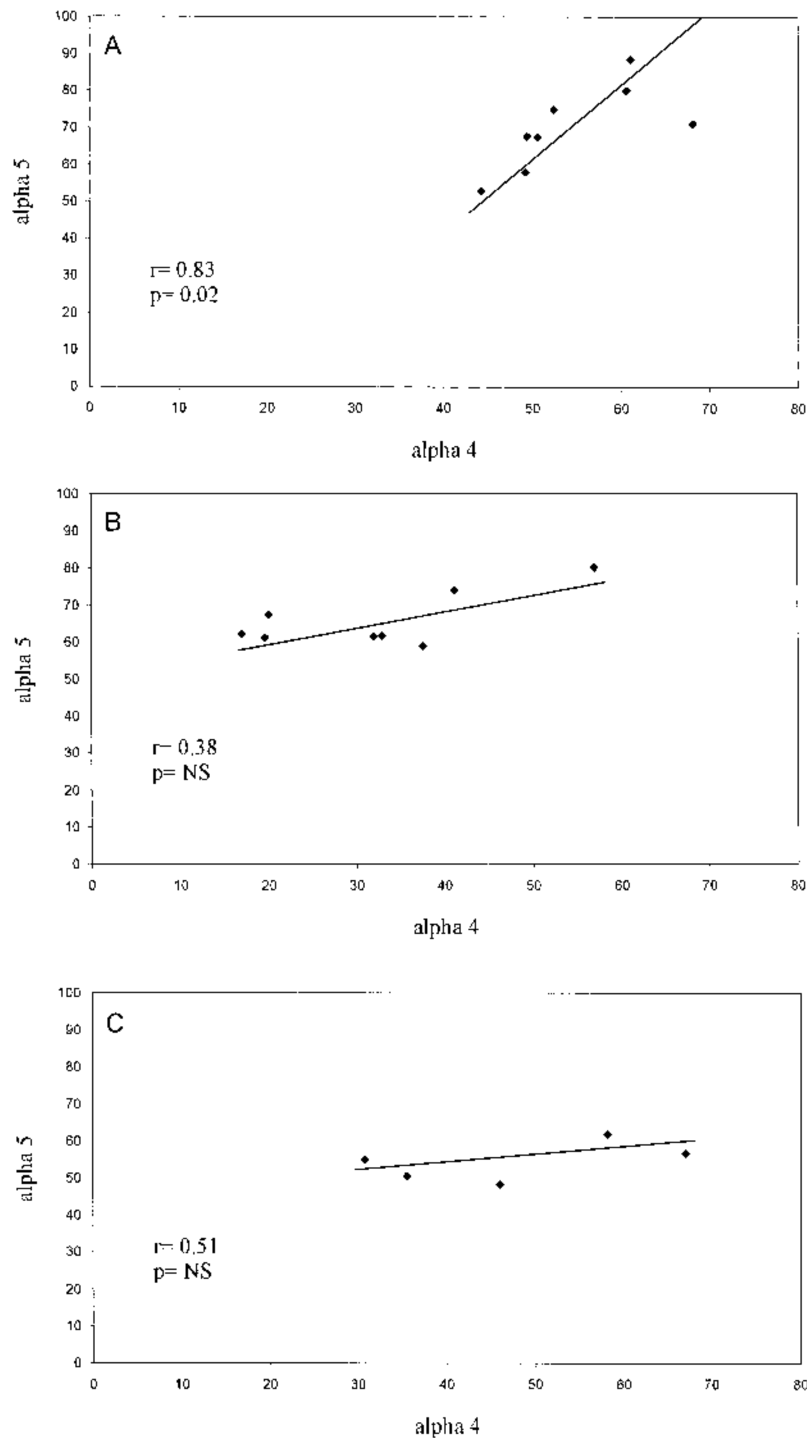

Figure 2. Correlation analysis between the expression of α4 and α5 integrin subunits on skin fibroblasts from 8 controls (A), 8 limited SSc patients (B), and 5 diffuse SSc patients (C). Spearman correlation test. r: correlation coefficient, NS: not significant.

The ß1 common chain of integrins was normally expressed on dermal fibroblasts with no differences between limited and diffuse SSc, while the collagen receptor α2 and α3 integrins are significantly downregulated only in limited SSc. α2 integrin is the main collagen receptor, involved in binding, phagocytosis, and degradation of collagen fibrils[6]. Further, α2 integrin plays an important role in matrix turnover by regulating secretion of 72 kDa gelatinase[17].

The α2 integrin downregulation, resulting in abnormal fibroblast-ECM interactions, may be relevant in promoting

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2005. All rights reserved.

Downloaded on April 23, 2024 from www.jrheum.org

the aberrant matrix remodeling and collagen accumulation occurring in SSc. α4 integrin plays an important role in cell-cell signaling by binding its counter–receptor vascular cell adhesion molecule-1, and also in cell-ECM interactions by binding fibronectin[5]. Decreased expression of α4 integrin on skin fibroblasts from limited SSc samples would also suggest that cell-cell interactions are altered in SSc skin. However, while α4 integrin is well described in hematopoiesis and immune responses[5], its role in skin fibroblasts is less well known.

In diffuse SSc, α5 and αv integrins and CD44 were decreased on skin fibroblasts. α5 integrin is the major fibronectin-binding receptor, recognizing the Arg-Gly-Asp (RGD) peptide sequence of fibronectin, and under physiologic conditions its expression decreases when fibronectin synthesis is reduced[18]. This is in contrast with the finding of an increased amount of fibronectin in SSc skin[3]. However, a mutant fibronectin gene has been found in SSc skin, suggesting that α5 integrin/fibronectin interactions are altered in SSc fibroblasts. As well, αv integrin recognizes the RGD sequence and binds vitronectin, osteopontin, and tenascin when coupled to ß3 or ß5 integrin chain, while it interacts with fibronectin when coupled to ß1 integrin chain[19]. The lack of ß3 integrin on SSc and normal fibroblasts indicates that the downregulation of αv integrin may be due to changes of fibroblast/fibronectin interactions. However, our fibroblasts may have lost ß3 integrin through *in vitro* passaging. Indeed, fibroblasts grown to confluence can lose surface expression of ß3[19]. Further, αv-ß5 integrin, a receptor for vitronectin that inhibits the plasmin-mediated pericellular proteolytic cascade, is reported to be upregulated on SSc skin fibroblasts[20], and this makes interpretation of our data more difficult. Further studies using immunohistochemical techniques should be performed on normal and SSc skin to clarify this issue. The hyaluronan receptor CD44 is expressed on a wide range of cells, and plays a pivotal role in modulating cell metabolism and in the turnover of hyaluronic acid[7]. The striking downregulation of CD44 on fibroblasts from diffuse SSc may be correlated to the abnormal metabolism of hyaluronic acid found in serum[21] and skin of patients with SSc[22]. Finally, the expression of laminin receptor α6 integrin was significantly increased on diffuse SSc but not on limited SSc fibroblasts. α6 integrin is thus the only ECM receptor found to be overexpressed on SSc fibroblasts: this finding is corroborated by the evidence that the percentage of α6-positive fibroblasts was almost 3-fold higher in diffuse SSc samples than in limited SSc or controls. These data deserve further investigation, as very little is known about laminin, a protein located almost exclusively in basement membranes. It has been shown that SSc dermal fibroblasts have an increased adhesion capacity to collagens I, IV, VI, fibronectin, and laminin[23], and that the levels of laminin fragment P1 are elevated in serum of patients with SSc[24]. The meaning of these results remains obscure.

Evidence of the dysregulation of ECM receptors in SSc fibroblasts was strengthened by studying the correlation of single α integrin chains expressed on fibroblasts. We found that the fibronectin receptors α4 and α5 correlated positively in control fibroblasts, while they did not in SSc samples. This clearly indicates that α4 and α5 act synergistically in normal conditions and that SSc fibroblasts have disturbed expression of integrins, with changes of their capacity for interacting with fibronectin and other ECM proteins.

The reason for this aberrant distribution of integrin receptors on SSc skin fibroblasts is unknown. Phenotype disturbance may be an outcome of the inflammatory/fibrotic process occurring in the affected skin, but we cannot exclude that it may be an essential feature of SSc fibroblasts. This hypothesis could be explored by evaluating integrin pattern expression on SSc fibroblasts from uninvolved skin as well as from patients with SSc "sine scleroderma."

Fibroblasts from skin of patients with SSc have abnormal integrin-mediated interactions with ECM molecules. Interestingly, diffuse and limited SSc have a distinctive pattern of expression of the adhesion molecules, confirming the hypothesis that fibroblasts from limited and diffuse SSc skin have a different phenotype[13]. This suggests that the mechanisms underlying the pathogenesis of fibrosis in SSc may differ in the diverse subsets of the disease.

REFERENCES

1. Takehara K. Hypothesis: pathogenesis of systemic sclerosis. J Rheumatol 2003;30:755-9.
2. Morino N, Mimura T, Hamasaki K, et al. Impaired collagen gel contraction with cultured skin fibroblasts from patients with systemic sclerosis. Scand J Rheumatol 2000;29:124-6.
3. Eckes B, Mauch C, Huppe G, Krieg T. Differential regulation of transcription and transcript stability of pro-alpha 1(I) collagen and fibronectin in activated fibroblasts derived from patients with systemic scleroderma. Biochem J 1996;315:549-54.
4. Damsky C, Tremble P, Werb Z. Signal transduction via the fibronectin receptor: do integrins regulate matrix remodeling? Matrix Suppl 1992;1:184-91.
5. Springer TA. Adhesion receptors of the immune system. Nature 1990;346:425-34.
6. Lee W, Sodek J, McCulloch CA. Role of integrins in regulation of collagen phagocytosis by human fibroblasts. J Cell Physiol 1996;168:695-704.
7. Laurent TC, Laurent UBG, Fraser JRE. Function of hyaluronan. Ann Rheum Dis 1995;54:429-32.
8. Kozlowska E, Sollberg S, Mauch C, Eckes B, Klein CE, Krieg T. Decreased expression of alpha 2 beta 1 integrin in scleroderma fibroblasts. Exp Dermatol 1996;5:57-63.
9. Osada K, Seishima M, Kitajima Y, Yaoita H, Mori S. Decreased integrin alpha 2, but normal response to TGF-beta in scleroderma fibroblasts. J Dermatol Sci 1995;9:169-75.
10. Ivarsson M, McWhirter A, Black CM, Rubin K. Impaired regulation of collagen pro-alpha 1(I) mRNA and change in pattern of collagen-binding integrins on scleroderma fibroblasts. J Invest Dermatol 1993;101:216-21.
11. Gruschwitz M, von den Driesch P, Kellner I, Hornstein OP, Sterry W. Expression of adhesion proteins involved in cell-cell and cell-matrix interactions in the skin of patients with progressive systemic sclerosis. J Am Acad Dermatol 1992;27:169-77.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2005. All rights reserved.

Downloaded on April 23, 2024 from www.jrheum.org

12. Herzhoff K, Sollberg S, Huerkamp C, Krieg T, Eckes B. Fibroblast expression of collagen integrin receptors alpha 1 beta 1 and alpha 2 beta 1 is not changed in systemic scleroderma. Br J Dermatol 1999;141:218-23.
13. Matucci-Cerinic M, Iannone F, Carossino A, et al. Discrepant expression of neprilysin on fibroblasts in diffuse systemic sclerosis. J Rheumatol 1999;26:347-51.
14. Le Roy EC, Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;15:202-5.
15. Szulgit G, Rudolph R, Wandel A, Tenenhaus M, Panos R, Gardner H. Alterations in fibroblast alpha 1 beta 1 integrin collagen receptor expression in keloids and hypertrophic scars. J Invest Dermatol 2002;118:409-15.
16. Noszczyk BH, Klein E, Holtkoetter O, Krieg T, Majewski S. Integrin expression in the dermis during scar formation in humans. Exp Dermatol 2002;11:311-8.
17. Ishibashi Y, Ito H, Seyama Y, Kubota S. Anti-alpha 2 integrin antibody induces secretion and activation of 72-kDa progelatinase by human fibroblasts. IUBMB Life 2001;51:25-31.
18. Dalton SL, Marcantonio EE, Assoian RK. Cell attachment controls fibronectin and alpha 5 beta 1 integrin levels in fibroblasts. Implications for anchorage-dependent and -independent growth. J Biol Chem 1992;267:8186-91.
19. Bates RC, Rankin LM, Lucas CM, Scott JL, Krissansen GW, Burns GF. Individual embryonic fibroblasts express multiple beta chains in association with the alpha v integrin subunit. Loss of beta 3 expression with cell confluence. J Biol Chem 1991;266:18593-9.
20. Asano Y, Ihn H, Yamane K, Kubo M, Tamaki K. Increased expression levels of integrin alpha v beta 5 on scleroderma fibroblasts. Am J Pathol 2004;164:1275-92.
21. Neudecker BA, Stern R, Connolly MK. Aberrant serum hyaluronan and hyaluronidase levels in scleroderma. Br J Dermatol 2004;150:469-76.
22. Sondergaard K, Heickendorff L, Risteli L, et al. Increased levels of type I and III collagen and hyaluronan in scleroderma skin. Br J Dermatol 1997;136:47-53.
23. Majewski S, Hunzelmann N, Schirren CG, Mauch C, Aumailley M, Krieg T. Increased adhesion of fibroblasts from patients with scleroderma to extracellular matrix components: in vitro modulation by IFN-gamma but not by TGF-beta. J Invest Dermatol 1992;98:86-91.
24. Guseva NG, Anikina NV, Myllyla R, et al. Markers of collagen and basement membrane metabolism in sera of patients with progressive systemic sclerosis. Ann Rheum Dis 1991;50:481-6.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2005. All rights reserved.

Downloaded on April 23, 2024 from www.jrheum.org