Dermal fibroblasts derived from types I and IV Ehlers-Danlos syndrome (EDS) patients, carrying mutations in COL5A1 and COL3A1 genes, respectively, synthesize aberrant types V and III collagen (COLL) and show defective organization of these proteins into the extracellular matrix (ECM) and high reduction of their functional receptor, the α2β1 integrin, compared with control fibroblasts. EDS cells also show reduced levels of fibronectin (FN) in the culture medium and lack an FN fibrillar network. Finally, EDS cells prevalently organize FN in the culture medium and lack an FN fibrillar network. Treatment of EDS cells with purified FN and pFN, plasma fibronectin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; COL, collagen; EDS, Ehlers-Danlos syndrome; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FTIC, fluorescein isothiocyanate; FN, fibronectin; FNR, fibronectin receptor; IF, indirect immunofluorescence; IO, integrated optical density; mAb, monoclonal antibody; pFN, plasma fibronectin; PBS, phosphate-buffered saline; VN, vitronectin.

The extracellular matrix (ECM) is a complex structure formed by distinct molecular networks that interact with specific cell receptors, triggering numerous responses that play essential roles in cell behavior regulation (1). The ECM provides a substrate for cell migration and wound healing, regulating tissue architecture and morphogenesis (2), and is also signaling in the cell microenvironment affecting cell proliferation, differentiation, and death (3–7).

Collagens (COLLs) and fibronectin (FN) are major ECM protein components (1, 8–10). COLLs, the most abundant proteins of connective tissues, are formed by three polypeptide chains, synthesized as propeptide (pro-α chains), coiled into triple helices, and encoded by the same or different genes (11). Types I, III, V, and XI COLLs are organized in fibrillar structures and are therefore referred to as fibrillar COLLs. In particular, COLLIII is an α(III)1 homotrimer encoded by the COL3A1 gene and is mainly distributed in skin, tendon, aorta, and cornea (12), whereas COLLV is a quantitatively minor fibrillar COLL with a broad tissue distribution that regulates COLLI fibrillogenesis (13). COLLV molecules may contain α1(V), α2(V) and α3(V) or α1(V)α2(V) chains (13).

Mutations in COLLs are related to a variety of hereditary connective tissue disorders one of which is Ehlers-Danlos syndrome (EDS), a group of heterogeneous diseases (at least 11 types) caused by alterations in different COLL genes, COL1A1, COL1A2, COL3A1, COL5A1, and COL5A2 (14–17), and to mutations in lysyl oxidase (18) and N-proteinase genes (19), altering the post-translational modification of COLLs. In particular, mutations in COL5A1 and COL5A2 genes have been reported in EDSS patients showing classical signs of the syndrome, i.e., widespread scarring and bruising, skin hyperextensibility, and joint laxity (15–16). Mutations in COL3A1 genes have been disclosed in EDSS patients showing as common features vascular rupture (vascular type), colonic perforation, thin, translucent skin, and severe bruising (15–20).

FN is a dimeric glycoprotein that triggers cell adhesion, migration, cell cycle progression, and differentiation (4, 7, 21). FN deposition in vitro represents the initial event during fibrogenesis of connective tissue matrices occurring during embryogenesis and wound healing (3, 22, 23). Human skin fibroblasts adhere in vitro through the organization of an ECM mainly composed of FN and types III, V, and VI COLLs (3, 24). FN can bind to COLLs through several COLL binding sites (25), and FN binding sites in COLL molecules have been reported (26). Many data indicate the interdependence of FN and COLL network assembly; in particular, in several cell systems, the assembly of COLLs has been shown to depend on FN organization (3, 27), whereas, in others, COLLs have been shown to influence FN fibrillogenesis (26, 28). FN and COLLs fibrils interact with the cells through specific plasma membrane receptors belonging to the integrin family.
**α2β1 Integrin Regulates FN Receptors in COL5A1 and COL3A1 Mutants**

(29, 30). Integrins are αβ heterodimeric transmembrane receptors with specific ligand binding potential involved in structural and regulatory functions such as linking ECM to actin cytoskeleton at focal adhesion sites and providing bidirectional transmission of signals across the plasma membrane (31, 32). Cultured dermal fibroblasts express αβ integrin (mediating cell adhesion to types I-VI COLLS), αβ integrin (a minor COLL receptor that preferably binds to COLLI and COL-LXIII) (33, 34), αβ integrin (as primary FN receptor (FNR), organized either in focal adhesions or in fibrillar adhesions) (35), and αβ integrin (as minor FNR) (36). The αβ integrin, highly expressed by dermal fibroblasts in cutaneous wound repair, supports their migration in the provisional matrix (36, 37) and mediates adhesion to FN, vitronectin (VN), and fibrinogen (38). Another receptor binding either to FN or VN in cultured fibroblasts is the αβ integrin (39, 40).

We have previously demonstrated that all types of EDS fibroblasts (EDSI to EDSVII) do not organize the FN-ECM in *vitro* and express low levels of FN and αβ integrin, compared with control fibroblasts (41–43). We report that EDSI and EDSIV skin fibroblasts, which carry mutations in *COL5A1* and *COL3A1* genes, respectively, do not organize the altered COLL molecules they encode in the ECM and show a type in control fibroblasts. This evidence shows that grin receptor with specific antibodies induces an EDS phenotype in both EDS fibroblasts, whereas perturbation of collagen, VN, or polylysine coating (Sigma) by mouse anti-human IgG, or anti-FN, is as minor FNR integrin, (mediating adhesion to FN, VN, or polylysine (Sigma)) and EDS fibroblasts allowed to adhere in the absence and in the presence of serum. To study EDS cell proliferation, DNA synthesis was evaluated by immunofluorescence detection of BrdUrd (Roche Applied Science), according to De Petro et al. (45). 4 × 105 cells were seeded on coverslips for 3 h and grown to semi-confluence in complete medium. After washing three times with PBS, the cells were maintained in serum-free medium for 48 h to induce the non-proliferating G0 phase. The cells were stimulated with 10% FBS-supplemented medium for 20 and 40 h at 37°C and fixed with 10 μl BrdUrd Ab (1:10 v/v in 66 mM Tris buffer, 0.66 mM MgCl2, 1 mM 2-mercaptoethanol) at 37°C for 30 min. After washing three times in PBS, a sheep FITC-conjugated anti-mouse secondary Ab (1:10 v/v in PBS) was added for 30 min at 37°C, and the coverslips were analyzed at ×20 magnification. The number of fluorescent nuclei over all the cells was determined, and the data were expressed as percentage of labeled nuclei.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents—**Human control skin fibroblasts were established in our laboratory from an arm biopsy from an 18-year-old healthy donor. Types I and IV EDS fibroblasts strains were established and characterized for missense dominant mutations in the gene coding for the α1 chain of COLLI (44) and in the gene for the α1 chain of COLLI (22), respectively. Two other EDS fibroblast strains were also analyzed: type IV EDS (ATCC CRL 1215 JS) (43), type IV EDS (ATCC CRL 1243 CP). All cell strains, used at similar in *vitro* passages (4–6), were grown in *vitro* at 37°C in modified Eagle’s medium (In-vitrogen) supplemented with 10% FBS (Invitrogen), 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Polyclonal anti-FN Ab was provided by Sigma Aldrich; anti-αβ (clones JS55 and H5A), anti-αβ (clone LM609), anti-αβ (clone BHA.2) integrin mAbs, polyclonal anti-COLLI, anti-COLLI, and anti-COLLI Abs, purified human COLLI and COLLI were from Chemicon Int. Inc. (Temecula, CA). Anti-αβ integrin subunit serum was kindly donated by F. S. Retta (Turin). FITC-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse secondary Abs were from Calbiochem-Novabiochem INTL. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG, BSA, and purified mouse IgG were from Sigma Chemical Co.

**Cell Adhesion, Migration, and Proliferation—**To study the adhesion properties of control and EDS fibroblasts, the cells were seeded on tissue culture wells not coated or coated overnight at 4°C with 10 μg/ml human FN (New York Blood Center Inc., NY, or polylysine (Sigma Chemical Co.) and blocked for 60 min at 37°C by the addition of 1% BSA in PBS. 2 × 10⁵ cells were detached using 500 μg/ml trypsin and 200 μg/ml EDTA. Trypsin activity was inhibited by washing the cells with 1 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.). Cells in the presence of serum-free medium were allowed to adhere in the absence of FN for 30, 60, or 120 min at 37°C. Adhered cells were stained with crystal violet in 20% methanol and counted with a light microscope. The reported values are means ± S.D. of three independent measurements.

The *in vitro* migration potential of control and EDS cells was analyzed in Transwell 8-μm filters (Corning Costar Corp. Cambridge, MA) by using 5 × 10⁵ fibroblasts, resuspended in serum-free medium, were plated onto the upper chambers and allowed to migrate for 6 h through the polycarbonate filter into the bottom wells filled with complete medium. The cells that migrated into the lower chambers were collected and counted. Each assay was performed in triplicate.

**IF and FACS Analysis—**The study of COLLS, FN, and their receptor integrins was performed by IF on control and EDS fibroblasts grown in monolayer. The cells were maintained with 10% FBS-supplemented medium for 20 and 40 h at 37°C and fixed with 10 μl BrdUrd. The cells were fixed in methanol for 20 min at −20°C, blocked with 0.3% (v/v) BSA in PBS for 5 min at room temperature and incubated with mouse monoclonal anti-BrdUrd Ab (1:10 v/v in 66 mM Tris buffer, 0.66 mM MgCl2, 1 mM 2-mercaptoethanol) at 37°C for 30 min. After washing three times in PBS, a sheep FITC-conjugated anti-mouse secondary Ab (1:10 v/v in PBS) was added for 30 min at 37°C, and the coverslips were analyzed at ×20 magnification. The number of fluorescent nuclei over all the cells was determined, and the data were expressed as percentage of labeled nuclei.

Quantitative evaluation of fluorescence associated with the FN- and COLL-ECM organized by control and EDS cells, was performed as previously reported (25). The fluorescence images, with the same spatial resolution and comparable light intensity, were captured by a CCD black and white video camera (SensiCam-PCO Computer Optics Gmbh, Germany) mounted on a Zeiss fluorescence microscope and acquired by Image Pro Plus program (Media Cybernetics, Silver Spring, MD).

To study the role of FN integrin receptors in cell adhesion, control and EDS fibroblasts allowed to adhere in the absence and in the presence of 10 μg/ml FN, VN, or polylysine coating, for 30, 60, and 120 min, were rinsed twice with PBS, fixed in 3% paraformaldehyde and 60 μM succrose for 7 min, permeabilized in 0.5% (v/v) Triton X-100 at 90°C, and the coverslips were stained with 1:50 dilution of labeled nuclei. The IF signals were acquired by a CCD black and white video camera (SensiCam-PCO Computer Optics Gmbh, Germany) mounted on a Zeiss fluorescence microscope and acquired by Image Pro Plus program (Media Cybernetics, Silver Spring, MD).
washed twice with 0.15 M glycine/PBS, and immunoreacted with mouse anti-αβ1, and anti-αβ3 integrin mAbs as reported above.

In Vitro Treatment of Skin Fibroblasts with COLLS, Antibody Perturbation of COLLS, and αβ Integrin Function—To test the role of COLLS on ECM assembly and on FN and COL integrin receptor clustering, control and EDS fibroblasts were cultured for 48 h in serum-free medium in the presence of 5 μg/ml human purified COLL or COLIII. After treatment with COLLS and immunoreaction with anti-COLL, anti-COLLIII, and anti-FN serum, anti-αβ1, αβ3, and αβ2, integrin mAbs, the cells were analyzed by IF for the organization of COLLS and FN into the ECM and for αβ1, αβ3, and αβ2 integrin distribution. The levels of integrin receptors organized after COLL treatment were evaluated by FACS analysis, as reported above. The effect of human purified pFN on COL- and FN-ECM and on COLLS and FN integrin receptor organization was tested by culturing control and EDS cells in the presence of 10 μg/ml pFN for 48 h.

To study the effect of COLL inhibition on ECM assembly, human control fibroblasts were cultured on glass coverslips in complete medium supplemented with 20 μg/ml goat anti-COLL and anti-COLLIII polyclonal antibodies for 4 days. Confluent cell cultures were fixed in 3% paraformaldehyde and 60% sucrose for 7 min, permeabilized in 0.5% (v/v) Triton X-100 for 90 s, immunoreacted with rabbit anti-FN, goat anti-COLL, goat anti-COLLIII polyclonal antibodies, mouse anti-αβ1, anti-αβ3 and anti-αβ2 integrin mAbs, and analyzed by epifluorescence as reported above.

In parallel, human control fibroblasts were treated for 4 h with 10 μg/ml anti-human αβ1 integrin mAb recognizing the collagen binding site of the receptor and competing with the specific integrin ligand (46) and with a polyclonal anti-human-α2 cytoplasmic tail subunit Ab.

Western Blot Analysis—The evaluation of FN in control and EDS cells was performed either in the culture medium or in deoxycholate-soluble and deoxycholate-insoluble fraction of each cell strain as follows: 3 × 10^6 control, EDSI, and EDIV cells were seeded for 2, 4, 8, 16, 24, and 48 h in complete medium. At each time complete medium was collected in the presence of protease inhibitors (25 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM amonobenzamidine). The cell layers were rinsed three times in cold PBS and treated for 10 min at 4 °C in the sodium deoxycholate lysis buffer (0.5% deoxycholate in 10 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA) to collect the FN cytoplasmic soluble fraction. The pericellular matrices attached to the culture dishes (insoluble FN fraction) were scraped into a mixture of 4% SDS, 20% glycerol, 25 mM Tris-HCl, pH 8.0 and 0.002% bromphenol blue (47). Alternatively, the FN-ECM was washed in PBS, fixed in methanol, and immunoreacted with the polyclonal anti-FN Ab, as reported above.

The analysis of FN synthesized and secreted by control and EDS fibroblasts cultured with and without 5 μg/ml COLL and COLLIII was performed as follows: control, EDSI and EDIV cells were washed with PBS twice and collected 24 h after seeding, sonicated for 30 s, and centrifuged at 12,000 rpm for 30 min. Cell extracts and complete medium were collected in the presence of protease inhibitors (25 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM amonobenzamidine); 20 μg of total proteins were diluted in Laemmli’s buffer and separated by electrophoresis in 8% SDS-PAGE under non-reducing conditions. After nitrocellulose membrane (Schleicher & Schuell) transfer, the membranes were blocked overnight at 37 °C with 3% nonfat milk (w/v) in 0.1 M Tris-HCl, pH 8.1), immunoreacted for 2 h at 37 °C with anti-FN f33 (25) mAb (final concentration 1 μg/ml in 0.5% BSA, 0.01% NaN3 in PBS), washed 3 × 10 min in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS), 0.1% Tween 20 (TBS-T) at room temperature, and reacted for 2 h at 37 °C with horseradish peroxidase-conjugated anti-mouse IgG.

The analysis of integrins synthesized by control and EDS fibroblasts was performed on cell cultures grown for 24 h in complete medium. The cells were kept at 4 °C overnight in 0.5% (v/v) Triton X-100, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml pepstatin, and 0.1 KIU/ml aprotinin. After centrifugation at 14,000 rpm, 10 min at 4 °C, the supernatants were recovered, 3 mg of proteins were immunoprecipitated with anti-αβ1, and anti-αβ3 mAbs (10 μg of Ab/1 mg of protein) at +4 °C for 3 h in the presence of protein G-Sepharose beads (Pierce) diluted 1:1 in TBS and the immunocomplexes were recovered by centrifugation. After washing, the bound proteins were recovered by boiling in 1% SDS. Equal amounts of extracts were separated by 7% SDS-PAGE under non-reducing conditions, transferred to nitrocellulose sheets and reacted for 2 h at room temperature with anti-αβ1 and anti-αβ3 mAbs (1 μg/ml) diluted in TBS-0.1% Tween 20 (TBS-T). The detection was performed incubating 2 h at room temperature with horseradish peroxidase-conjugated anti-mouse secondary Ab diluted in TBS-T.

Detection of FN and integrin subunits was carried out using the enhanced chemiluminescence (ECL) method (Pierce). The bands obtained were evaluated as IOD by the Gel-Pro 3.1 Analyzer software (Media Cybernetics, Silver Spring, MD) or by Analytical Imaging Station (AIS) software (Image Research INC., St. Catherine, Ontario, Canada).

RESULTS

Mutations in COL5A1 and COL3A1 Genes Affect the Organization of COLLS into the ECM and Clustering of αβ Integrin Receptor in Cultured Fibroblasts—We analyzed the assembly of COLL and COLLIII in the ECM in EDSI and EDIV fibroblasts, which carry mutations in COL5A1 and COL3A1, respectively, as compared with control fibroblasts. IF analysis on control fibroblasts shows that these cells organize COLV and COLIII in large fibrils (Fig. 1A, c and d) distributed in the intercellular spaces. On the contrary, EDSI-COL5A1 mutated cells do not organize COLV in the ECM and retain this protein in the cytoplasm (Fig. 1A, b). These cells also show a large amount of COLIII in the cytoplasm (Fig. 1A, e). EDIV-COL3A1-mutated cells lack a COLLIII-ECM (Fig. 1A, f) but organize COLV fibrils, as well as control fibroblasts (Fig. 1A, c).

Fig. 1. Organization of COLLV, COLLIII, COLI, and αβ Integrin receptor in control and EDS skin fibroblasts. Control (C), EDSI, and EDIV cells, grown to confluence, were immunoreacted with polyclonal anti-COLLV (a–c), anti-COLLIII (d–f), anti-COLLI Ab (g–i), and anti-αβ1, αβ3 integrin mAbs (A). EDSI and EDIV fibroblasts do not organize COLLIV and III in the ECM (b and f), do not secrete COLLv (h and i), and show reduced amount of αβ1 integrin patches (m and n) compared with control fibroblasts (a, d, g, and i, respectively). Scale bar, 5 μm. B, quantitative evaluation, performed by image analysis, of COLL and COLLIII detected by IF. The IOD expressed in pixels ×10^3 are average numbers obtained when evaluating the fluorescent signals in 5–7 fields of 1.3 × 10^6 pixels for each sample. The average number of cells counted in each field was 6 ± 2 control fibroblasts and 3 ± 1 EDSI and EDSIV cells. In and out refer to the intracellular and extracellular fluorescence evaluated by image analysis.
lar storage of the abnormal proteins. The IF analysis of COLLI, the main collagen synthesized by skin fibroblasts, shows that in control and EDS fibroblasts this protein is not organized in the ECM and is mainly detectable in the cytoplasm (Fig. 1A, g–i). In particular, in EDS cells the amount of intracellular COLLI is higher, compared with control fibroblasts. Furthermore, we analyzed by IF the distribution in EDS cells of the major fibroblast COLL receptor, the αvβ3 integrin. This receptor, which is widely distributed in the plasma membrane of control cells (Fig. 1A, l), is strongly reduced in both EDS cell strains (Fig. 1A, m and n). FACS analysis shows that 91% of control fibroblasts are αvβ3 integrin-positive and their fluorescence intensity (FL1), ranging between 10^1 and 10^3, is higher than that measured in negative controls (minus primary antibody) (Fig. 2). Only 3.3% EDSI and 3.9% EDSIV cells expose in membrane the αvβ3 integrin receptor (FL1 > 10^3) (Fig. 2). An analysis of the distribution of α1-containing integrins, by IF and by FACS analysis with an anti-α1 mAb, disclosed comparable patterns in control and EDS cells (data not shown). Because the α1 subunit has been found only in combination with the β1 subunit (48), this indicates that the α1β1 integrin, acting as a COLL receptor, is not affected in EDS cells.

Similar COLLs and αvβ3 integrin patterns were observed in both EDS strains following a 2-day treatment with ascorbic acid, indicating that the defects observed in these cells do not depend on this cofactor involved in COLLs maturation (Figs. 1A and B and 2, A and B).

**EDS Fibroblasts Lack the FN-ECM and Organize αβ1 Instead of αβ3 Integrin**—In the control and EDS strains reported here, FN-ECM organization and the distribution of FN receptors, the α5β1 and αvβ3 integrins, were also analyzed, in comparison with control fibroblasts. IF analysis, performed with an anti-FN polyclonal Ab, shows that control fibroblasts assemble FN in a fibrillar network overlaying the cells (Fig. 3A, a); whereas EDSI (Fig. 3A, d) and EDSIV (Fig. 3A, g) cells lack a fibrillar FN-ECM, and organize only a few FN fibrils in the extracellular spaces. In both EDS cell types, FN is stored in the cytoplasm. Quantitative evaluation by image analysis of FN detected by IF showed that in control cells FN is mainly assembled in the ECM, whereas in EDS cells FN is mainly stored in the cytoplasm. The distribution and the level of FN in control and EDS cells were also analyzed by Western blotting, performed under non-reducing conditions with the f33 anti-FN mAb, on
complete media, deoxycholate-insoluble and deoxycholate-soluble fractions collected from 2 to 48 h after cell seeding. Fig. 3, B and C show different distribution and levels of FN in the extracellular and in the intracellular compartment of control and EDS fibroblasts. In particular, the level of FN in the culture medium of control fibroblasts is 5–14-fold higher than that evaluated in EDS cells at 48 h after seeding (Fig. 3B, a and C, a). Increasing amounts of FN are detectable in the medium of control fibroblast from 2 to 48 h after seeding, whereas the amount of FN in EDS cells increases from 2 to 16 or 24 h (EDSI and EDSIV cells, respectively) and decreases at 24 and 48 h (Fig. 3B, a and C, a). The deoxycholate-insoluble fraction of control cells contains increasing amounts of FN from 2 to 16 h; after this time, the FN level slightly decreases (Fig. 3B, b and C, b). The deoxycholate-insoluble fraction of both EDS cells contain low levels of FN, which are detectable only at 24 and

![Image of Fig. 3](image-url)

**Fig. 3.** Organization and distribution of FN, αsβ1, and αvβ3 integrins in control and EDS skin fibroblasts. A, control (C), EDSI, and EDSIV cells, grown at confluence, were immunoreacted with polyclonal anti-FN Ab (a, d, and g), anti-αsβ1 (b, e, and h), and anti-αvβ3 (c, f, and i) mAbs. Both EDS fibroblast strains do not organize FN (d and g) in the ECM, express lower levels of αsβ1 (e and h) integrin, compared with control fibroblasts, and organize the αvβ3 integrin on the whole cell surface (f and i). Scale bar, 6 μm. B, Western blotting analysis with the f33 anti-FN mAb, performed under non-reducing conditions, of FN released in the culture medium (a), or present either in the deoxycholate-insoluble (b) or in the deoxycholate-soluble fraction (c) of control (C), EDSI, and EDSIV cells. Equal amounts of proteins from all fibroblasts cultures fractions were analyzed. C, quantitative evaluation by image analysis of the FN bands reported in B. ND, not detectable; FD, fold decrease obtained by normalizing the IODs at 48 h over the highest values measured in control cells; FI, fold increase obtained by normalizing the IODs at 48 h over the values measured in control cells. D, organization of FN in the deoxycholate-insoluble fraction of control and EDS fibroblasts, detected by IF with the f33 anti-FN mAb. E, number of αvβ3 integrin patches, counted on the acquired images at the fluorescence microscope (A) in control, EDSI, and EDSIV cells. The number of αvβ3 integrin patches are averages obtained in 50 cells for each strain. Fp, fold increase obtained normalizing the number of αvβ3 integrin patches counted in EDS cells on the number of patches counted in control cells. The cell areas reported for each strain are average numbers measured in 50 cells. FP, fold increase obtained by normalizing the cell area of EDS cells on the area of control cells.
48 h after seeding (Fig. 3B, b and C, b). These data are in agreement with the IF reported in Figs. 3A and B and are confirmed by the IF analysis of the FN present in the deoxycholate-insoluble fraction of the three cell strains (Fig. 3D).

Finally, the levels of FN present in the deoxycholate-soluble fraction of control cells decreases as a function of the time after seeding, whereas it increases, from 2 to 48 h, in both EDS cells (Fig. 3B, c and C, c). At 48 h the amount of FN in EDS cells is about 3- and 14-fold higher than in control cells. In the EDS deoxycholate-soluble fractions degradation of FN to a different extent also occurs (Fig. 3B, c). Taken altogether, these data confirm the accumulation of FN detected in EDS cells by IF (Fig. 3A) and also show a reduction of FN in the extracellular compartment of these cells, either in the soluble form, present in the culture medium, or in the insoluble form organized in the ECM.

The distribution of the most abundant FNR expressed by cultured fibroblasts, the \( \alpha_5\beta_1 \) integrin, analyzed by IF with an anti-\( \alpha_5\beta_1 \) FNR mAb, that recognizes both subunits, shows that in control fibroblasts (Fig. 3A, b), these integrin patches are distributed over the whole cell surface either in focal or in fibrillar adhesions (7). On the contrary, EDS cells (Fig. 3A, e and h) show rare \( \alpha_5\beta_1 \) integrin patches, preferentially distributed in focal adhesions and at the boundary between cells, where few FN fibrils are organized (Fig. 3A, d and g). IF analysis of \( \alpha_5\beta_1 \) integrin FNR distribution in control fibroblasts shows rare dot-like patches localized at focal adhesion sites (Fig. 3A, c) and large integrin patches either in the focal or in the fibrillar adhesions in EDSI and EDSIV cells (Fig. 3A, f and i). The cytoplasmic/nuclear storage of \( \alpha_5\beta_1 \) integrin detected in control fibroblasts is reduced or absent in EDS cells. Fig. 3E shows that the average number of \( \alpha_5\beta_1 \) integrin patches organized per cell by control fibroblasts is \( 7 \pm 2 \). This number increases 31–47-fold in EDS cells. Western blotting analysis, performed with specific Abs against \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \) integrins expressed by control and EDS fibroblasts, shows that while the \( \alpha_5\beta_1 \) integrin is 2.3–2.4-fold higher in control cells, compared with EDS fibroblasts, \( \alpha_5\beta_3 \) integrin is 2.8-fold higher in EDS cells, compared with control fibroblasts (Fig. 4). These data are in agreement with those obtained by IF analysis (Fig. 3A).

The evaluation of \( \alpha_5\beta_1 \) integrin by FACS analysis confirms its reduction in EDS cells; indeed, only 2% of EDSI and 4% of EDSIV cells show a FL1 ranging between \( 10^3 \) and \( 10^4 \), while 98% of control fibroblasts are positive for this integrin (peak at \( 10^5 \)) (Fig. 2, A and B). In parallel with the \( \alpha_5\beta_1 \) integrin low levels, EDS cells show high \( \alpha_5\beta_3 \) FNR levels, compared with control fibroblasts which are almost negative for this integrin (Fig. 2, A and B). The FACS analysis also shows that the reduction of \( \alpha_5\beta_1 \) integrin in EDS fibroblasts is paralleled by an increase of \( \alpha_5\beta_3 \) integrin patches, compared with control fibroblasts. The levels of these integrins detected by FACS and IF analyses are not identical, because of the different and specific Abs used in each assay for the same receptor.

A comparable disorganization of COLL- and FN-ECM, as well as the peculiar distribution of their integrin receptors reported above, is detectable in other EDSI and EDSIV strains thus suggesting that this is a common feature of EDS cells carrying mutations in COLLV and COLLIll genes (Figs. 3A–E and 4).

\( \alpha_5\beta_1 \) Integrin Is an FN and a VN Receptor in EDS Fibroblasts—Both EDS cell strains, with reduced \( \alpha_5\beta_1 \) integrin levels, show adhesion, proliferation, and migration properties comparable with those exhibited by control fibroblasts (Fig. 5). These results suggest that the \( \alpha_5\beta_1 \) integrin receptor expressed by EDS could be involved in cell adhesion, growth, and migration.

To verify whether \( \alpha_5\beta_1 \) integrin, expressed by EDS-FN cells, is an FNR or a VN receptor, EDSI (Fig. 6) and EDSIV (not shown) cells were seeded on uncoated or FN- or VN-coated coverslips and, after 60 min, immunoreacted with anti-\( \alpha_5\beta_1 \) mAb. Polyslysin coating completely inhibits the adhesion and spreading of both cell types (Fig. 6, d and h). Both control and EDS cells adhere and spread more efficiently in the presence of FN and VN than in their absence. Control fibroblasts organize \( \alpha_5\beta_1 \) integrin patches mainly in the focal adhesions when seeded either on FN or VN (Fig. 6, b and c), indicating that in these cells the \( \alpha_5\beta_3 \) integrin is involved in FN and VN adhesion. In EDS fibroblasts, \( \alpha_5\beta_1 \) integrin is abundantly organized by cells seeded on uncoated and FN-coated coverslips (Fig. 6, e and f). In particular, in the presence of FN, \( \alpha_5\beta_3 \) integrin is distributed on the whole lower cell surface (Fig. 6, f), while in the presence of VN it is localized only in focal adhesions (Fig. 6, g), confirmed by confocal microscopy (not shown). Similar results were obtained for EDSIV fibroblasts. Therefore, \( \alpha_5\beta_1 \) integrin organized by EDS cells is either an FN or a VN receptor: VN binding involves patches localized in focal adhesions, while FN binding mainly involves integrin patches distributed on the whole lower cell surface.

In order to verify whether the \( \alpha_5\beta_1 \) integrin patches organized in the fibrillar adhesions of EDS cells (Fig. 3A, f and i) are FNRs, EDSI cells were treated for 48 h with increasing amounts of purified human pFN (from 1 to 10 \( \mu \)g/ml). Starting from 2.5 \( \mu \)g/ml, the treatment induced the formation of a fibrillar FN-ECM (not shown). Therefore, both EDS cell strains were treated with 5 \( \mu \)g/ml pFN. Exogenous pFN induces in EDSI and EDSIV cells the organization of a fibrillar ECM network, comparable with that assembled by control fibroblasts (Figs. 7 and 3A, a). However, the treatment does not induce in EDS cells the \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \) integrin clustering and the \( \alpha_5\beta_3 \) integrin patch disorganization observed in control fibroblasts (Figs. 3A and 1A), as also detected by FACS analysis. Treatment of EDSI and EDSIV cells with pFN also fails to restore the organization of COLLV and COLLIll in the ECM. In control fibroblasts, treatment with pFN induces the organization of a thicker FN-ECM, the enhancement of \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \) integrins and the organization of COLLIll, but not of COLLV, in the ECM (Fig. 7). Taken altogether, these data indicate that in EDS cells the \( \alpha_5\beta_3 \) integrin forming the fibrillar adhesions is an FNR capable of organizing the exogenous FN. Moreover, these data show that the FN–\( \alpha_5\beta_3 \) binding is not sufficient to trigger \( \alpha_5\beta_1 \) integrin patch organization (Figs. 5–7).

**Purified COLL Treatment Restores a Control Phenotype in EDS Fibroblasts**—Western blotting analysis with specific anti-
were grown in medium containing human purified COLLV or COLLIII. Immunoreaction with polyclonal anti-COLLV Ab and anti-α2β1 integrin mAb was performed on EDSI cells, treated with increasing amounts of purified COLLV. Fig. 8, A and B shows that, starting from 2.5 μg/ml, COLLV induced in EDSI-defective cells a COLLV-ECM, which increased with COLLV concentration. The organization of the COLLV-ECM was paralleled by the enhancement of α2β1 integrin receptor in the plasma membrane of the treated cells. In particular, 10 μg/ml COLLV induced an 11-fold enhancement of the α2β1 integrin level, as evaluated by image analysis of the IF signals. Treatment with COLLV also induced in EDSI cells a reduction of the cell surface to values comparable to those of control fibroblasts and a more elongated phenotype, compared with untreated cells (Fig. 8B). Therefore, EDSI- and EDSIV-defective cells were treated with 5 μg/ml COLLV or COLLIII, respectively. As already shown in Fig. 8, EDSI cells, after COLLV treatment, were capable of organizing this molecule in the extracellular spaces in a network (Fig. 9A, b) similar to that observed in control cells grown without and with COLLV (Figs. 1A, a and 9A, a). Likewise, treatment of EDSIV cells with COLLIII leads to the organization of this protein into an ECM (Fig. 9A, d) comparable to that observed in control fibroblasts without and with COLLIII treatment (Figs. 1A, d and 9A, c). Control fibroblasts in the presence of COLLV or COLLIII showed a slight increase of COLLV- and COLLIII-ECM (Fig. 9A, a and c and 9B), if compared with basal conditions (Figs. 1A, a and d and 1B). Treatment of EDS cells with COLLV and COLLIII also induced the organization of α2β1 integrin patches to levels comparable with those observed in control fibroblasts treated with the purified molecules (Fig. 9A, c–h). These data were confirmed by FACS analysis showing that 97% of EDSI and EDSIV cells treated with COLLV and COLLIII, respectively, organized levels of α2β1 integrin (Fig. 10) comparable to those measured in untreated control fibroblasts (Fig. 2). In EDS cells the level of membrane-bound α2β1 integrin receptor is regulated in the same manner by both COLLV and COLLIII ligands: the receptor clustering is up-regulated in the presence of an organized COLLV-ECM and down-regulated in its absence.

COLLV treatment also induces in EDS cells the FN-ECM reorganization (Fig. 9A, j and l and 9B). Western blotting analysis with the 933 anti-FN mAb shows that this event is associated with the presence of enhanced FN levels in the culture medium of EDS fibroblasts, which are comparable to those measured in control cells (Fig. 11). In parallel with the increase of extracellular FN, both EDS cell strains after COLLV treatment showed a strong reduction of intracellular FN (Fig. 11).

COLLV or COLLIII treatment induces in EDS cells not only the organization of the FN-ECM but also an α2β1 integrin switch. In particular, the α2β1 patches in EDSI and EDSIV COLLV-treated cells are disorganized (Fig. 9A, r and t) and their number per cell decreases from 225 and 334, in EDSI and EDSIV, respectively (Fig. 3E) to 10 in both EDS cells, after COLLV treatment (not shown). These values are comparable to those measured in control fibroblasts (Fig. 3E). At the same time, COLLV treatment induced in EDS cells the formation of numerous α2β1 integrin patches distributed over the whole cell surface (Fig. 9A, n and p). FACS analysis of these FN-Rs in EDS-COLLV-treated cells showed that they reach levels (Fig. 10) similar to those observed in control fibroblasts (Fig. 2). In conclusion, the addition in the culture medium of EDS cells of purified COLLV induces an ECM and an integrin profile comparable to those of control fibroblasts, probably acting on the up-regulation of the α2β1 integrin receptor organization (Figs. 8 and 9 A and B, 10, and 11).
Perturbation of COLls and Functional Blocking of Their Receptor Induce an EDS Phenotype in Control Fibroblasts

To address further the role of COLLV and COLLIII in the organization of FN and in the induction of the EDS phenotype, the effects of polyclonal Abs directed against COLLV and COLLIII in control fibroblasts, organizing either COLLS or FN in the ECM, were investigated. Confluent control cells were treated with antibodies against COLLV or COLLIII blocking the sites of polymerization of these proteins. Both treatments induced in control cells an EDS phenotype, i.e. strong reduction of COLLS in the ECM, disorganization of the FN network, reduction of /H9251/5/H9252/1 integrin patches, organization of /H9251/2/H9252/3 patches and decrease of /H9251/2/H9252/1 COLL receptor (Fig. 12). In particular, anti-COLLV Ab inhibits the organization of COLLV and COLLIII into the ECM, whereas anti-COLLIII Ab inhibits COLLIII, but not COLL assembly; again indicating that COLL is necessary in cultured skin fibroblasts for COLLIII deposition but not vice versa.

Similar results were obtained in adherent control fibroblasts after functional blocking with a mAb directed to the /H9251/2 cytoplasmic tail integrin (46) (Fig. 13). Indeed, the lack of functional /H9251/2 integrin does not affect the adhesive properties of control fibroblasts and induces the /H9251/2/H9252/3 integrin switch, which is not observed after treatment of the cells with a polyclonal anti- /H9251/2 cytoplasmic tail integrin Ab (Fig. 13, + anti- /H9251/2). These results demonstrate that in EDSI and EDSIV cells the ECM defect depends on the lack of organized COLLS in the ECM and that COLL disorganization modulates the level of its receptor. This event, in turn, induces the /H9251/5/H9252/1/ and /H9251/2/H9252/3 switch (Figs. 12 and 13).

DISCUSSION

In this work we studied the effect of mutations in COL5A1 and COL3A1 genes, leading in vivo to classical (EDSI) and vascular (EDSIV) EDS, on cultured skin fibroblast phenotypes. In particular, in these cells we studied the organization of COLLS and FN into the ECM, the distribution of their integrin receptors, and the adhesive and growth features in comparison with skin fibroblasts derived from a healthy donor. Control fibroblasts organize an ECM composed mainly of FN, but also containing COLLS and COLL fibrils and rare COL1 fibrils. EDS cells, carrying mutations in COL5A1 and COL3A1 genes, do not organize the aberrant proteins into the ECM, although they synthesize these molecules that are stored as intracellular deposits and partly secreted in the culture medium. In the absence of organized extracellular protein, the cytoplasmic...
storage of COLIII molecules is a common feature of in vitro grown skin fibroblasts derived from EDSIV patients, who prob-
ably carry different mutations in the COL3A1 gene that influence protein export (49, 50). The aberrant COLIII and COLIV proteins secreted by EDSIV and EDSI cells are not capable of organizing into fibrils detectable by IF analysis. The COL5A1 mutation present in EDSI cells not only reduces COLIII organ-
ization into the ECM but also affects the assembly of COL-
LIII, showing that a missense mutation in a gene that influ-
ences by their levels in the culture medium. Therefore, the assembly into the ECM of skin fibroblasts of structural proteins such as COLIII, COLIV, and FN is influ-
enced by their levels in the culture medium.

ECM components are known to modulate integrin expression (51); in particular, αβ1 integrin is up-regulated by FN and fibrin and down-regulated by COL in endothelial cells (37), and αβ1, αβ3, and αβ5 integrins are highly induced by platelet-
derived growth factor when dermal fibroblasts are in a COLL
gel (52). Thus, the integrin-ECM interaction is reciprocal: inte-
grins mediate cell attachment to the matrix, and the matrix, at least to a degree, controls the expression and activity of inte-
grins (30). Therefore, EDS fibroblasts showing alterations in the ECM organization are a cellular model for the study of COL and FN integrin receptor modulation, consequent to COL and FN variation in the extracellular environment. We demonstrate that defective COL assembly in EDS cells, or inhibition of COL deposition by antibody perturbation in control fibroblasts, induces modifications of the FN integrin recep-
tor repertoire: the decrease of αβ1 and αβ3 integrin receptors and the organization of αβ5 FNR in the cell surface. The primary event consequent to COLIV or COLIII defect in the ECM of cultured fibroblasts, is the reduction of the COL receptor, the αβ3 integrin, in the plasma membrane, as dem-
onstrated by IF and FACS analysis and by αβ3 integrin func-
tional blocking with a specific antibody competing with its ligand(s), which induces the αβ3 integrin switch in control fibroblasts. On the other hand, treatment of EDS cells with purified COLIII and COLIV molecules induces the organiza-
tion of αβ3 integrin in this work we observed that treatment of EDS cells with the COLL molecule they fail to secrete, as a consequence of specific mutations, not only restores the COLL-ECM, but also induces the enhancement of FN in the culture medium and its organization into the ECM. Therefore, the assembly into the ECM of skin fibroblasts of structural proteins such as COLIII, COLIV, and FN is influ-
enced by their levels in the culture medium.
Addition of purified FN into the culture medium of EDS cells induces the formation of an FN-ECM but fails to restore α5β1 integrin patches, suggesting that αvβ3-FN complexes do not trigger α1 integrin recruitment in the absence of a COLL molecule organized into the ECM. Taken altogether, these data reinforce the role of α1 integrin in the control of FNR recruitment and show that FNR switch is regulated not by FN present in the ECM but rather by an α1-transduced signal. Therefore, the absence of an integrin ligand not only affects its receptor organization but also triggers a cascade of events involving modulation of receptors having different ligand specificities. Although the intracellular mechanism by which the modulation of FNRs by αvβ3 integrin occurs has not been yet investigated, the data reported here indicate the existence of functional cooperation between these integrins: αvβ3 integrin in the absence of its ligand exerts inhibition of α5β1 and activation of αvβ3 integrin organization in skin fibroblasts, underlining the existence of cross-talk events between integrins already reported in different cell models (40, 53–56).

With respect to the αvβ3 integrin organized by EDS cells, this receptor supports cell adhesion and in vitro growth in the absence of either α5β1 or α5β1 integrin. Whereas in vivo the αvβ3 integrin is typically expressed by fibroblasts migrating into the provisional matrix of FN and fibrinogen during wound healing (36, 37), in in vitro cultured fibroblasts, this integrin is a minor FNR mainly organized in the focal adhesions (7). In EDS cells, αvβ3 patches are organized not only in focal but also in fibrillar adhesions (7, 57) distributed over the whole cell surface. This is a peculiar phenotypic trait of all EDS fibroblast types and therefore, together with the lack of the FN-ECM, it represents a marker of skin fibroblasts derived from EDS patients. Adhesion assay of EDS fibroblasts to FN and VN has shown that these cells engage αvβ3 integrin in focal adhesions for binding to either FN or VN and that the largest amount of αvβ3 integrin on the lower cell surface binds to FN. When the cells are maintained in complete medium, these αvβ3 integrin patches are distributed in the fibrillar adhesions, which are localized on the whole cell surface, and are capable of supporting the assembly of exogenous FN. However, in the absence of

![Image of a graph showing the effect of COLL V and COLL III treatment on the cell surface distribution of αvβ3, α5β1, and α5β1 integrin receptor in EDS skin fibroblasts, as detected by FACS analysis. EDSI and EDSIV cells were collected and immunoreacted with saturating concentrations of anti-α5β1, anti-αvβ3, and α2β1 FACS grade mAbs, prior to fluorescence flow cytometry analysis. The fluorescence intensities of the different antibodies, displayed as histograms in A, were subtracted from that of the negative control (F-Ab). B, percentage of positive cells evaluated in each sample.]
high levels of FN in the medium, these αβ3 integrin patches could, in EDS FN-ECM-deficient cells, be involved in cellular processes other than cell adhesion and spreading. Indeed, it is known that both αβ3 and αβ1 integrins can act synergistically with the epidermal growth factor receptor and transduce survival and proliferation signals from the ECM inside the cell (54, 58, 59). In EDS cells defective for the ECM and deprived of its survival signals (5, 60, 61), the αβ3 integrin clustered in fibrillar adhesions might play an important role in the control of cell growth and survival.

The fact that the αβ3 alternative FNR does not trigger FN organization into the ECM in EDS cells is probably due to the reduced amount of FN present in the culture medium of these cells, as compared with control fibroblasts. This hypothesis is supported both by the biochemical analysis of extracellular FN levels and by the exogenous FN assembly capability exhibited by EDS-αβ3+ cells. FN bound to αβ3 integrin is organized into the ECM even in the absence of a COL-ECM, supporting the notion that deposition of FN into the ECM is independent and precedes COLL assembly (3, 27, 62). The FN-ECM organized by αβ3 integrin is not sufficient to revert the EDS fibroblasts phenotype since it does not induce the up-regulation of αβ1 integrin and the consequent organization of COLL into the ECM. These data show the existence of a hierarchy of events regulating αβ1, αβ3, and αβ1 integrins in a coordinated and directional manner: secreted COL associates with αβ1 integrin and up-regulates its organization in plasma membrane; COLL receptor recruits αβ1 FNR with the resulting disorganization of αβ1 integrin; FN is secreted; the FN-ECM is assembled; and the COL-ECM is assembled.

Because αβ3 and αβ1 integrins on fibroblast plasma membrane are required in different and specific stages in wound healing (36, 38), our data give new insights into the understanding of the defective wound healing often observed in EDS patients and suggest the use of purified COLls for restoring a normal wound-healing process.

Acknowledgments—We thank S. F. Retta and E. Tanghetti for helpful discussions and F. Fappani for technical assistance.

REFERENCES
1. Aumaillé, M., and Geyraud, B. (1998) J. Mol. Med. 76, 253–265
2. Gumbiner, B. M. (1996) Cell 84, 343–357
3. McDonald, J. A. (1988) J. Cell Sci. 91, 207–218
4. Giancotti, F. G. (1997) Curr. Opin. Cell Biol. 9, 691–700
Human Fibroblasts with Mutations in COL5A1 and COL3A1 Genes Do Not Organize Collagens and Fibronectin in the Extracellular Matrix, Down-regulate $\alpha_2\beta_1$ Integrin, and Recruit $\alpha_4\beta_3$ Instead of $\alpha_5\beta_1$ Integrin

Nicoletta Zoppi, Rita Gardella, Anne De Paepe, Sergio Barlati and Marina Colombi

*J. Biol. Chem.* 2004, 279:18157-18168.
doi: 10.1074/jbc.M312609200 originally published online February 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312609200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 19 of which can be accessed free at http://www.jbc.org/content/279/18/18157.full.html#ref-list-1