The Fibronectin Domain ED-A Is Crucial for Myofibroblastic Phenotype Induction by Transforming Growth Factor-β1

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Abstract. Transforming growth factor-β1 (TGFβ1), a major promoter of myofibroblast differentiation, induces α-smooth muscle (sn) actin, modulates the expression of adhesive receptors, and enhances the synthesis of extracellular matrix (ECM) molecules including ED-A fibronectin (FN), an isoform de novo expressed during wound healing and fibrotic changes. We report here that ED-A FN deposition precedes α-SM actin expression by fibroblasts during granulation tissue evolution in vivo and after TGFβ1 stimulation in vitro. Moreover, there is a correlation between in vitro expression of α-SM actin and ED-A FN in different fibroblastic populations. Seeding fibroblasts on ED-A FN does not per se elicit α-SM actin expression; however, incubation of fibroblasts with the anti-ED-A monoclonal antibody IST-9 specifically blocks the TGFβ1-triggered enhancement of α-SM actin and collagen type I, but not that of plasminogen activator inhibitor-1 mRNA. Interestingly, the same inhibiting action is exerted by the soluble recombinant domain ED-A, but neither of these inhibitory agents alter FN matrix assembly. Our findings indicate that ED-A–containing polymerized FN is necessary for the induction of the myofibroblastic phenotype by TGFβ1 and identify a hitherto unknown mechanism of cytokine-determined gene stimulation based on the generation of an ECM-derived permissive outside in signaling, under the control of the cytokine itself.

Key words: fibroblast • α-SM actin • extracellular matrix • wound healing • fibrosis

Acquisition of smooth muscle (SM)1 cell features by fibroblastic cells is observed during morphogenetic processes, wound healing, organ fibrosis, and stroma reaction to epithelial cancer (for review see Grinnell, 1994; Desmoulière and Gabbiani, 1996). α-SM actin–expressing myofibroblasts have long been recognized as suppliers of the driving force for granulation tissue contraction (Gabbiani et al., 1972), a mandatory function for an efficient and rapid wound closure. Moreover, these cells are involved in the pathogenesis of several fibrotic diseases, being responsible for tissue retraction and overproduction of extracellular matrix (ECM) components, such as collagen type I (Zhang et al., 1994). During development and under normal conditions, myofibroblasts are accountable for the generation of the structural and functional complexity of fundamental physiological units such as the glomerulus (Soriani, 1994) and the lung alveolar sack (Boström et al., 1996). In addition, myofibroblasts are typical components of the stroma reaction to epithelial tumors where they secrete proteolytic enzymes and growth factors which may activate cancer cell invasive behavior (for review see Rønnov-Jessen et al., 1996). The role of factors regulating the generation of the myofibroblastic phenotype remains largely unknown. Transforming growth factor-β1 (TGFβ1) is the prototype of a large family of cytokines that control cell proliferation, differentiation, motility, and apoptosis, exerting their functions both during embryogenesis, in terms of pattern formation and tissue specification, and in the adult organism, where they orchestrate complex phenomena such as inflammation, tissue repair, and neoplastic transformation (Roberts and Sporn, 1993; Kingsley, 1994; Massagué et al., 1997). It is well accepted that TGFβ1, which is known to stimulate
α-SM actin synthesis by fibroblasts (Desmoulière et al., 1993; Rønnow-Jessen and Petersen, 1993), upregulates fibrillar collagen and fibronectin (FN) expression (Ignotz and Massagué, 1986; Roberts et al., 1986).

FN, a 440-kD dimeric glycoprotein widely distributed in plasma and in ECM, is expressed at high levels in healing wounds (Kurkinen et al., 1980). Each FN subunit is formed by a series of repeating homologous modules and contains binding sites for cell surface receptors and for other ECM components. FN polymorphism is due to alternative splicing of the type III segments ED-A, ED-B, and IIICS. Recently, a novel splicing variant lacking the IIICS region and the segments I-10 and III-15 has been characterized (MacLeod et al., 1996). Two of these alternative spliced segments, namely ED-A and ED-B, are included in the so-called cellular FN (Hynes, 1990; Kosmehl et al., 1996; MacLeod et al., 1996). Previous in situ hybridization studies have demonstrated that granulation tissue fibroblasts show a FN splicing pattern consisting of ED-A and ED-B domains, similar to that found in the embryo (ffrench-Constant et al., 1989). In vitro, TGFβ1 increases total FN levels by preferentially promoting accumulation of the ED-A FN isoform (Balza et al., 1988; Borsi et al., 1990; Kocher et al., 1990). For this reason we hypothesized that ED-A FN, interacting with a not yet characterized cell surface receptor, could transduce signals initiated by TGFβ1 and/or synergize with them, behaving as a crucial intermediary for the induction of myofibroblastic features, such as α-SM actin and collagen type I expression. Moreover, it has been previously suggested that ED-A FN modulates hepatic stellate cells to α-SM-actin-expressing myofibroblast-like cells (Jarnagin et al., 1994).

It is well known that integrin-mediated adhesion to ECM regulates transmission of activated growth factor receptor tyrosine kinases and that convergence of integrin and growth factor-dependent pathways is required for the proper stimulation of gene expression, cell growth and differentiation (Clarke and Brugge, 1995; Schwartz, 1997; Sladepf and Hunter, 1998). The TGFβ serine/threonine kinase receptors signal from cell membrane to the nucleus mainly through the SMAD family of signal transducers (Heldin et al., 1997). However, it is not yet known whether and how ECM can influence TGFβ effects on target cells. Here we provide a molecular dissection of the ECM-generated pathway that needs to be activated for the induction of the myofibroblastic phenotype by TGFβ.

### Materials and Methods

#### Antibodies

We used an affinity-purified fibronectin polyclonal antibody recognizing both cellular and plasma FN (Sigma Chemical Co., St. Louis, MO) and three mouse IgG1 mAbs selectively raised against different domains of FN isoforms (Borsi et al., 1987; Carnemolla et al., 1987, 1989, 1992): (a) IST-4, to the fifth FN type III domain shared by cellular and plasma FN; (b) IST-9, against the ED-A FN type III domain of cellular FN; and (c) BC-1, recognizing a cryptic epitope within the seventh FN type III domain, which is unmasked only when the ED-B domain is included in the cellular FN molecule. Anti-α-SM-1, an IgG2a mAb, against α-SM actin (Skalli et al., 1986), and DIA-900, an IgG1 mAb, against the 6× His tag (Dianova, Hamburg, Germany), were also used. For control purposes, irrelevant antibodies of the same isotypes were used.

#### Purification of FN and Production of Recombinant ED-A Domain

Plasma (ED-A −, ED-B −) and cellular (ED-A +, ED-B +) FNs were purified from human plasma and from the conditioned medium of the SV-40-transformed embryonic human lung WI-38-VA cell line as previously reported (Zardi et al., 1987). The presence of the absence of ED-A and ED-B in purified FNs was further verified by Western blotting with IST-9 and BC-1 mAbs (Borsi et al., 1987; Carnemolla et al., 1987, 1989).

The 270-bp cDNA sequence coding for the complete amino acid sequence of the ED-A domain (Kornblith et al., 1984) was generated by PCR amplification starting from the full-length cellular FN cDNA clone pFH111 (gift of F.E. Baralle, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy) and using Pwo Pyrococcus woesei DNA polymerase (Boehringer Mannheim, Mannheim, Germany) and the following primers: (a) 5′-CTCGATACCAACATTGATCGCCCTAAA-3′, which covers a FN sequence from base 5084 to base 5101 and includes the underlined BamHI restriction site, and (b) 5′-CTCGATACCAATAGCT-GTGGACTGGGT-3′, which covers a FN sequence from base 5342 to base 5359 and includes the underlined BamHI restriction site. PCR product was isolated, digested with BamHI restriction enzymes, and then cloned in the pQE-12 expression vector with a 3× 6× His tag (QIAGEN, Inc., Santa Clarita, CA). Escherchia coli were transformed with this construct and the 6× His COOH-terminal-tagged recombinant ED-A (rED-A) protein was purified using a Ni-NTA resin column (QIAGEN Inc.) according to the manufacturer. 6× His-tagged rED-A was then dialyzed against PBS and sterilized through a 0.22-μm filter. After filtration, protein concentration was estimated by absorbance at a 280-nm wavelength. Protein purity and size were verified by Coomassie blue staining following SDS-PAGE on a 10% polyacrylamide gel. Immunological protein reactivity was investigated by Western blotting with mAb IST-9.

#### In Vivo Experimental Procedures

Excisional wound granulation tissue was generated as previously described (Darby et al., 1990). In brief, on day 0, eight-week-old female Wistar rats were anaesthetized and a 2 × 2-cm skin wound was made on the middorsal surface. Granulation tissue samples were collected at 4, 7, and 12 days after wounding. All procedures involving animals were reviewed and approved by the Animal Care Committee at the University of Geneva. These procedures conformed the guidelines as established in the Guide for the Care and Use of Laboratory Animals and Public Health Service Policy on Human Care and Use of Laboratory Animals.

#### Double Indirect Immunofluorescence and Confocal Laser Scanning Microscopy Analysis

Tissue samples were embedded in OCT 4583 (Miles Scientific, Naperville, IL) and snap frozen in precooled liquid isopentane. 4-μm serial sections were fixed for 5 min in acetone at −20°C, air dried for 2 h at room temperature, sequentially incubated with anti-α-SM-1, revealed by a TRITC-tagged goat anti-mouse IgG2a (Jackson ImmunoResearch Labs, Inc., West Grove, PA), and then with IST-9, followed by a dichrotaizinyl amino fluorescein-labeled goat anti-mouse IgG1 (Jackson ImmunoResearch Labs, Inc.). For the qualitative FN matrix assembly assay, after in vitro blocking experiments with the IST-9 mAb or the rED-A fragment (see below), cultured fibroblastic cells were rinsed in PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton-X 100 for 5 min at room temperature, rinsed in PBS, and then stained in immunofluorescence. The primary monoclonal antibodies used were the affinity-purified rabbit polyclonal anti-FN antibody (Sigma Chemical Co) anti-FN antibody (Sigma Chemical Co) or combined with DIA-900 (Dianova), and then revealed by a TRITC-tagged goat anti-rabbit and a dichrotaizinyl amino fluorescein-labeled goat anti-mouse (both from Jackson ImmunoResearch Labs, Inc.), respectively.

Specimens were observed with a confocal laser scan fluorescence inverted microscope (model LMR 410; Carl Zeiss, Oberkochen, Germany) equipped with two lasers used simultaneously: (a) a helium laser (excitation wavelength at 543 nm) and (b) an argon-neon laser (excitation wavelength at 488 nm). The appropriate combination of filters was used to separate excitation and emission spectra. The objective used was an immersion oil plan-neofluar 63/1.4. Images of 512 × 512 pixels were stored on an erasable optical disk (Sony Corp., Tokyo, Japan) and then printed with a
Cell Culture and Treatment

Passage 5 human fibroblasts obtained from explants of breast skin, palmar fascia, or Dupuytren’s nodules were plated on Petri dishes containing Eagle’s minimum essential medium (MEM; Gibco BRL, Basel, Switzerland) supplemented with Monomed (a defined serum-free medium containing insulin, transferrin, sodium selenite, 2-mercaptoethanol, 2-mercaptoethanol, sodium pyruvate, glutamine, and a BSA-oleic acid complex; Commonwealth Serum Laboratories, Melbourne, Australia), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Cell density was ~1.5 × 10^6 cells/cm². They were maintained at 37°C in a humid atmosphere of 5% CO₂ and 95% air. Medium was removed 24 h after plating and fibroblasts were incubated for 4 d in MEM plus Monomed alone or containing 10 ng/ml of TGFβ1 (gift of A. Roberts, National Institutes of Health, Bethesda, MD, and purchased from Sigma Chemical Co.), or TGFβ2 (gift of A. Cox, Novartis, Basel, Switzerland). Passage 5 rat fibroblasts obtained from explants of subcutaneous tissue, lung, and dermis were plated on Petri dishes (1.5 × 10^6 cells/cm²) containing MEM (Gibco BRL) supplemented with Monomed (Commonwealth Serum Laboratories) and were cultured at 37°C in a humid atmosphere of 5% CO₂ and 95% air for 4 d.

In blocking experiments with mAbs, 50, 150, or 300 μg of each anti-FN mAb were diluted in 1 ml of 2% gelatin (Sigma Chemical Co.) and then coated onto 6-cm Petri dishes; coatings of the same volume of 2% gelatin alone or containing equal amounts of irrelevant mAbs were used as controls. In blocking experiments with rED-A domain 50, 150, or 300 μg of this fragment were diluted in 1 ml of 2% gelatin (Sigma Chemical Co.) and then coated onto 6-cm Petri dishes. Cells were then plated on pre-coated Petri dishes containing MEM supplemented with Monomed (Commonwealth Serum Laboratories). Cell density and culture conditions were the same as above. Medium was removed 24 h after plating and fibroblasts were incubated for 24 h (extraction of total RNA) or for 3 d (extraction of proteins) in MEM plus Monomed alone or containing 10 ng/ml of TGFβ1 or TGFβ2.

The effects of cell adhesion on plasma and cellular FN were investigated as follows. 6-cm Petri dishes were coated with increasing concentrations of plasma FN or cellular FN (25, 50, and 100 μg/ml in PBS, pH 7.4). Proteins were allowed to bind overnight at 4°C. In some experiments the Petri dishes were rinsed and blocked for 2 h at 37°C with 5% heat-denatured BSA (RIA grade; Sigma Chemical Co.) in PBS, pH 7.4. In another set of experiments, the blocking step was omitted. Passage 5 human fibroblasts were resuspended in MEM (Gibco BRL) supplemented with Monomed (Commonwealth Serum Laboratories), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine, and then plated (1.5 × 10⁶ cells/cm²) on Petri dishes precoated with plasma FN or cellular FN. They were maintained at 37°C in a humid atmosphere of 5% CO₂ and 95% air for 1–4 d in MEM plus Monomed. All experiments were repeated at least five times and results were similar with all tested fibroblasts.

Western Blot Analysis

Cells were harvested and then extracted, or directly extracted on the dish with a buffer containing 1% SDS (Bio-Rad Laboratories AG, Glattbrugg, Switzerland), 1% dithiothreitol (Fluka Chemie AG, Buchs, Switzerland), 1 mM PMSF, 1 mM N-p-tosyl-L-arginine methyl ester (Sigma Chemical Co.) in 0.4 M Tris-HCl, pH 6.8, immediately sonicated, boiled for 5 min, and then centrifuged at 10,000 g for 20 min (model 5415C, Eppendorf Scientific Inc., Hamburg, Germany). Protein content was determined according to Bradford (1976). Equal amounts of total proteins (15 μg for actin analysis and 50 μg for FN analysis) were fractionated by SDS-PAGE in acrylamide gels (5–20% gradient for actin analysis and 6% for FN analysis) and transferred to nitrocellulose filters (0.45 μm; Schleicher & Schuell, Dassel, Germany) as previously described (Serini and Gabbiani, 1996). Filters were then probed with mAbs IST-9, BC-1, anti-α-SM-1, or the affinity-purified rabbit polyclonal anti-FN antibody (Sigma Chemical Co.). The secondary antibodies were either goat anti-mouse IgG or a goat anti-rabbit IgG both conjugated with alkaline phosphatase (Promega Corp., Madison, WI). Specific binding was detected by the Prolab Western Blot AP system (Prolab Corp.).

Northern Blot Analysis

Total RNA was isolated from cultured cells by TRI REAGENT (Molecular Research Center Inc., Cincinnati, OH), according to the manufacturer’s instructions. 25 μg of total RNA per lane were denatured by glyoxal/DMSO treatment, separated by electrophoresis on a 1% agarose gel, and then transferred overnight onto an Electran nylon membrane (BDH, Poole, UK). RNA was immobilized on membrane by cross-linking in a Stratalinker UV light box (Stratagene, La Jolla, CA). To verify correct loading and transfer, filters were stained with 0.04% methylene blue in 0.5 M Na-acetate. Filters were then processed for hybridization with three different probes: (a) a 120-bp α-SM actin cDNA derived from the rat α-SM actin gene, (b) a 500-bp fragment of the human α-SM actin mRNA in one band at 1.7 kb (prepared in our laboratory by P. Neuville and T. Christen), (b) a 1600-bp rat α1(I) collagen cDNA recognizing the human α1(I) collagen mRNA in two typical bands, one at 5.8 kb and the other one at 4.7 kb (Genovese et al., 1984), and (c) a 600-bp bovine plasminogen activator inhibitor type 1 (PAI-1) cDNA (gift of M.S. Pepper, Department of Morphology, University of Geneva, Switzerland) recognizing the human PAI-1 mRNA in two typical bands, one at 2.6 kb and the other one at 3.6 kb (Cicila et al., 1989). Probes were labeled by random priming using the MEGAPRIME DNA labeling system (RPN 1606, Amersham, Little Chalfont, UK). Prehybridization and hybridization were performed for 4 and 16 h, respectively, at 55°C in 5× standard saline citrate, 5× Denhardt’s solution, 0.1% SDS, and 400 μg/ml denatured salmon sperm DNA. After hybridization, filters were washed twice for 15 min at 55°C in 5× standard saline citrate and 0.1% SDS. Northern blots were then exposed on Kodak film at ~70°C (X-Omat SO-282).

Quantitation and Statistical Analysis

For quantitation, membranes and films corresponding to each Western and Northern blot experiment were scanned with an Arcus II scanner (Agfa, Mortsel, Belgium) and analyzed with the ImageQuant Software Version 3.3 (Molecular Dynamics, Sunnyvale, CA) obtaining the sum of the pixel values of band areas, as previously described (Bochaton-Piallat et al., 1998). Depending on the experiment, densitometric analysis results were presented as fold increase, percentage of the corresponding control, or percentage of induction inhibition (see Results) and expressed as arithmetical mean of all experiments performed ± SEM. For statistical evaluation, results were analyzed with Student’s t-test.

Results

ED-A FN Deposition Precedes α-SM Actin Expression during Granulation Tissue Evolution

To assess the potential role of ED-A FN as an in vivo inducer of α-SM actin, first we have investigated both spatial and temporal relationships between ED-A FN and α-SM actin expression in a rat excisional model of wound repair. As previously reported (ffrench-Constant et al., 1989) ED-A FN was not present in fibroblasts of normal dermis under our conditions (data not shown). Fibroblastic cells containing cytoplasmic actin but not α-SM actin (Fig. 1, a and b) were abundant within the 4-d-old granulation tissue. At this time, ED-A FN was already expressed in huge amounts around them (Fig. 1 b). Only after this early ED-A FN deposition did α-SM actin start accumulating, evident around the seventh day (Fig. 1 c), and reached a maximal peak at the twelfth day (Fig. 1 d). Hence, during wound repair ED-A FN appearance precedes α-SM actin expression by fibroblastic cells.

Levels of ED-A FN and α-SM Actin Expression Are Related in Different Fibroblastic Populations; ED-A Precedes α-SM Actin Induction by TGFβ1

Although cultured fibroblastic cells from different origins display a roughly uniform morphology, they are heterogeneous in terms of growth, gene expression, and cell behavior (for review see Sappino et al., 1990). Indeed, when grown in vitro, fibroblasts from diverse organs can express
quantitative changes of ED-A FN expressed by fibroblastic cells (Fig. 2) and was 11.3-fold higher in lung fibroblasts. The content was similar in subcutaneous and dermal fibroblasts (data not shown), which is as effective as TGFβ1 itself, such as ED-A FN. Indeed, continue fibroblast stimulation with TGFβ1 caused a fivefold ED-A FN increase within the first 24 h and a further increase (6.5-fold) after 48–72 h (Fig. 3). Therefore, during TGFβ1 treatment of cultured fibroblastic cells, the rise of ED-A FN precedes and then parallels α-SM actin increase. All together, these data are compatible with a role for ED-A FN as intermediary between α-SM actin and its positive regulator TGFβ1.

**TGFβ1 Induction of Myofibroblastic Phenotype Requires a Permissive ED-A FN–derived Outside In Signaling**

To directly investigate the role of ED-A FN as intermediary between α-SM actin and TGFβ1, we stimulated with TGFβ1 fibroblastic cells plated on gelatin containing specific blocking mAbs raised against different type III domains of FN (Borsi et al., 1987; Carnemolla et al., 1989, 1992). When examined after 72 h of TGFβ1 treatment (densitometric analysis values being expressed as percentages of the corresponding control), fibroblasts seeded on gelatin showed the expected α-SM actin increase (217 ± 32%) compared with control cells. Plating fibroblasts on gelatin containing IST-9, an IgG1 against the ED-A domain of cellular FN, led to a complete inhibition of α-SM actin induction (77 ± 13%, P < 0.001), whereas neither BC-1 (an IgG1 against the ED-B containing FN isoform), nor IST-4 (an IgG1 against the fifth type III domain of both cellular and plasma FN) were active in this regard (220 ± 29% and 221 ± 26%, respectively; Fig. 4). Similar results were obtained by stimulating cells with TGFβ2 (data not shown), which is as effective as TGFβ1 in upregulating α-SM actin both in vivo and in vitro (Serini and Gabbiani, 1996). The action of IST-9 was dose dependent (50–300 μg/ml; Fig. 5). Densitometric analysis revealed that the percentage of inhibition of α-SM actin induction by TGFβ1 was 25 ± 5% for 50 μg/ml, 59 ± 8% for 150 μg/ml, and 96 ± 15% for 300 μg/ml. It is known that TGFβ secreted and proteolytically activated by cultured fibroblasts induces a limited myofibroblastic differentiation (Masur...
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Figure 3. Time course analysis of α-sm actin and ed-a fn expression modulation by tgfβ1 in cultured human subcutaneous fibroblasts. Fibroblasts were incubated with 10 ng/ml of tgfβ1 and total proteins were extracted after different time of continuous stimulation. Immunoblotting after SDS-PAGE shows that tgfβ1 induces a clear-cut α-sm actin increase only after 72 h of treatment, whereas ed-a fn response to tgfβ1 precedes and then parallels α-sm actin increase. Tracks were loaded with equal amounts of total proteins. These data are the means of five independent experiments; the SEMs, which are not represented in the figure, were always lower than 5% of the values.

et al., 1996); seeding cells on gelatin containing only ist-9 resulted in a slight lowering of the basal α-sm actin expression levels (data not shown). As previously described tgfβ1 is able to induce the insertion within fn not only of the ed-a, but also of the ed-b domain (balza et al., 1988). indeed, Western blot analysis revealed that treatment of fibroblasts with tgfβ1 induced an increase of both ed-a (3.5 ± 0.3-fold) and ed-b (7.3 ± 0.5-fold) FN isoforms (fig. 6). This, together with our blocking experiment data, further confirms the role played by ed-a fn during myofibroblast formation.

We then tested whether tgfβ-regulated genes other than α-sm actin are dependent on the ed-a fn-driven signaling. First, we selected collagen type I because its production represents a hallmark of myofibroblastic transition and a key pathogenetic event in the progression of fibrotic diseases (border and noble, 1994; zhang et al., 1994). Northern blot analysis revealed that as expected, ist-9 treatment inhibited the tgfβ1-induced increase of α-sm actin at the mrna level by 68 ± 12% (p < 0.001; fig. 7); moreover, ist-9 mAb inhibited by 95 ± 17% the tgfβ1 stimulation of collagen type I mrna (p < 0.001; fig. 7). Hence, collagen type I mrna induction by tgfβ requires a functionally active ed-a domain within the cellular fn molecule, similar to α-sm actin. The next gene analyzed was the pAI-1 which plays a crucial role both in the regulation of extracellular matrix-degrading enzymes and in the production of active tgfβ1 (lund et al., 1987; keski-oja et al., 1988). Blocking the ed-a domain with ist-9 mAb did not counteract significantly the increase of pAI-1 mrna level induced by tgfβ1 (fig. 7). Thus, tgfβ1 regulation of pAI-1 expression differs from that of the two main myofibroblastic markers, α-sm actin and collagen type I.

To investigate whether ed-a fn is not only necessary for tgfβ1 activity on fibroblasts, but also sufficient to cause their modulation to α-sm actin expressing myofibroblasts, cells were plated on petri dishes precoated with increasing amounts of ed-a-negative plasma fn or ed-a-containing cellular fn (refer to materials and methods). 1–4 d after plating, no changes in α-sm actin expression were noted at any dose used (data not shown). Hence, ed-a fn does not directly stimulate the conversion of cultured fibroblasts to myofibroblasts. Next, we studied the influence of soluble ed-a fn on fibroblast modulation into α-sm actin–expressing myofibroblasts. For this purpose we used the isolated human rED-A domain (refer to materials and methods). Cells were plated on gelatin containing carrier solution or rED-A domain and stimulated with tgfβ1. After 72 h of tgfβ1 treatment, fibroblasts seeded on gelatin-containing carrier solution showed the usual upregulation in α-sm actin expression compared with control cells (fig. 8). Plating fibroblasts on
gelatin-containing rED-A domain resulted in a slight decrease (15 ± 3%, $P < 0.001$) of α-SM actin basal expression levels and in a clear-cut inhibition (61 ± 5%, $P < 0.001$) of α-SM actin induction by TGF-β1 (Fig. 8). The effects exerted by the rED-A domain mimicked the results obtained using the mAb IST-9 (data not shown; refer to Fig. 4), suggesting that these two approaches affect the same biological mechanism. These results can be interpreted in different ways. One is that the exogenous ED-A domain interferes with cellular FN matrix assembly. Indeed, FN matrix assembly can be disrupted using FN fragments containing critical domains (e.g., III-1 domain or α5β1-binding domain) or antibodies against these domains (McDonald, 1994). Moreover, TGF-β1 is known to increase the assembly of FN by human fibroblasts (Allen-Hoffmann et al., 1988). To test this hypothesis, we monitored TGF-β1-induced FN matrix assembly by cultured fibroblasts in the presence of IST-9 mAb or rED-A fragment (as described above) by means of double immunofluorescence staining, using the polyclonal FN antibody, IST-9 mAb, or the antibody against the 6× His tag of rED-A. As expected, TGF-β1 increased the expression and assembly of cellular FN when compared with control (Fig. 9, a and b); however, neither IST-9 mAb (Fig. 9 c) nor rED-A (data not shown) blocked basal and TGF-β1-stimulated FN matrix expression and assembly (Fig. 9, a and b). Interestingly, whereas IST-9 mAb colocalized with FN fibrils within the assembled matrix, rED-A did not, as revealed by a monoclonal directed against its 6× His tag (data not shown). These results indicate that the inhibition of the TGF-β1 induction of the myofibroblastic phenotype by IST-9 mAb and rED-A domain is not due to an inhibition or a perturbation of FN matrix assembly. Our data are compatible with the possibility that IST-9 mAb acts by preventing the ED-A domain to interact with a hypothetical receptor. Soluble rED-A domain would compete for the binding of the same receptor. In any event, our results suggest that in order to be permissive for the action of TGF-β1, the ED-A domain should be incorporated within the assembled and polymerized FN molecule.

**Discussion**

TGF-β1 is presently considered as the main inducer of the myofibroblastic phenotype, being able to upregulate α-SM actin as well as collagen expression in fibroblasts both in vitro and in vivo (Border and Ruoslahti, 1992; Sporn and Roberts, 1992; Desmoulière et al., 1993; Rønnov-Jessen and Petersen, 1993; Zhang et al., 1994). Many data point to TGF-β as a key cytokine in controlling tissue repair, and disregulation of its production may be a cause of tissue fibrosis (Border and Ruoslahti, 1992; Sporn and Roberts, 1992; Border and Noble, 1994). When compared with other cytokines, a distinctive feature of TGF-β is the ability to control cell adhesion and migration by modulating the adhesion molecule repertoire (Zambruno et al., 1995) as well as the synthesis of ECM components such as FN and collagen (Ignotz and Massagué, 1986; Roberts et al., 1986). Furthermore, expression of TGF-β1 gene has been shown to be influenced by ECM molecules (Strüelli et al., 1986), suggesting a feedback loop in vivo. However, the mechanisms by which ECM influences TGF-β effects on target cells are not yet fully characterized.

We demonstrate here that ED-A FN deposition precedes α-SM actin expression both in vivo, during granulation tissue evolution, and in vitro, during TGF-β1 fibroblast stimulation. Moreover, the degree of myofibroblastic differentiation exhibited by fibroblasts cultured from different organs is proportional to the different amounts of ED-A FN they produce. Furthermore, selectively blocking the ED-A domain of cellular FN by IST-9 mAb inhibits α-SM actin and collagen type I mRNA induction by TGF-β1 in cultured fibroblasts. In contrast, TGF-β1 upregulation of PAI-1 is not influenced by ED-A FN, indicating that the PAI-1 gene is regulated differently than α-SM actin and collagen type I. Interestingly, it has been shown that the increase of collagen type I and actin mRNA induced by TGF-β1 is dependent on protein synthesis, whereas the induction of PAI-1 transcript is not (Lund et al., 1987; Keskioja et al., 1988; Penttinen et al., 1988). Hence, the
synthesis of an intermediary protein such as ED-A FN is necessary for the stimulation by TGFβ1 of at least some morphofunctional genes, i.e., α-SM actin and collagen type I. Thus, it appears that TGFβ1 regulates gene expression through different mechanisms, possibly according to the biological functions exerted by the corresponding proteins.

Hautmann and colleagues (1997) have identified a TGFβ response element in the α-SM actin promoter that drives the stimulation of α-SM actin gene expression in concert with two CArG elements in rat sn cells. We demonstrate here that, at least in fibroblastic cells, the presence of functional ED-A FN is mandatory for α-SM actin induction by TGFβ. The ED-A FN signaling is necessary but not sufficient for α-SM actin–positive regulation by TGFβ. Taken together, these observations suggest that TGFβ activation of α-SM actin expression results from the cooperation of two signal transduction pathways raised respectively by TGFβ and ED-A FN.

The observation that treatment of fibroblasts with the soluble rED-A domain inhibits TGFβ1 induction of the myofibroblastic phenotype without interfering with FN assembly (similar to treatment with IST-9 mAb) allows to hypothesize the existence of an hitherto unknown specific receptor interacting with the ED-A domain. It is worth noting that a TGFβ-dependent morphogenic process, i.e., the cellular condensation event that occurs during chondrogenesis (Leonard et al., 1991), has been recently shown to be spatiotemporally correlated to and to depend upon the ED-A domain insertion in cellular FN; moreover, this stepwise formationally sensitive manner. In this respect, two recent reports (Shrivastava et al., 1997; Vogel et al., 1997) outline an intriguing new paradigm for ECM signaling: the receptor tyrosine kinases DDR1 and DDR2 bind to and are activated by collagen in a conformation-dependent way. It is proposed that signals generated by the activation of these receptors act in concert with signals generated by binding of ECM molecules to classical integrins. Further work along these lines may help in identifying the hypothetical ED-A cell-surface receptor.

Unlike Jarnagin et al. (1994) and Van De Water et al. (Van De Water, L., C. Reimer, L. Plantefaber, R.O. Hynes, and J.H. Peters. 1996. Abstract from Wound Repair in Context, Keystone Symposium, Taos, NM), we were unable to induce α-SM actin expression by simply plating cells on ED-A–containing cellular FN. This discrepancy may be related to the different cell types (hepatic stellate cells versus fibroblasts) or alternatively to the different sources of FN the authors used in their assay system, e.g., endothelial cell-derived ECM and commercial FNs. Indeed, an increasing number of cytokines, including TGFβ, have been found associated with the ECM proteins and both latent and active form of TGFβ have been found to bind cellular FN of many cell types, endothelial cells included (Taipale and Keski-Oja, 1997). Moreover, many commercial sources of FN contain TGFβ activity (Fava et al., 1987). Our data show that ED-A FN is necessary, but not sufficient, to induce myofibroblastic differentiation and that it exerts a permissive effect on TGFβ activity.

It is well accepted that ED-A FN is synthesized by mesenchymal cells which are driven toward an α-SM actin–positive phenotype by TGFβ in many physiological and pathological settings, such as wound healing (Gabbiani et al.,...
1972; Darby et al., 1990), Dupuytren’s disease (Berndt et al., 1995), organ fibrosis (Schürch et al., 1992), developing aorta (Glukhova et al., 1990), arterial intimal thickening (Glukhova et al., 1989), and stroma reaction to epithelial cancers (Pujuguet et al., 1996). Our results support that in such different systems ED-A FN acts as a necessary ECM molecule that allows TGFβ to induce SM differentiation.

Pathological deposition within tissues of ECM components results in fibrosis, which may alter irreversibly the function of the involved organ. Recently it has been demonstrated that α-SM actin–expressing myofibroblasts are the main collagen type I synthesizing cells during fibrosis (Zhang et al., 1994). Blocking TGFβ1 with antibodies or with decorin was therapeutic in many models of fibrotic disease (Border and Noble, 1994) and, where investigated, was associated to a significant attenuation of ED-A FN expression in rat arterial smooth muscle cells during fibrosis (Border and Noble, 1994). ED-A FN, which was associated to a significant attenuation of ED-A FN expression in rat arterial smooth muscle cells during fibrosis (Border and Noble, 1994), decreases the probability that its receptor can be unfavorable, causing autoimmune-like disease (Shull et al., 1992; Kulkarni et al., 1993) and leading to malignant transformation (Marx, 1995). ED-A FN could be considered as a potential target for therapy since: (a) it is an extracellular, easily reachable molecule; and (b) in contrast to TGFβ1, it drops to low levels in adult tissues (Kornblith et al., 1996) decreasing the probability that its blocking results in side effects. Thus, the outside in signal (Kornblihtt et al., 1996) decreasing the probability that its receptor can be unfavorable, causing autoimmune-like disease (Shull et al., 1992; Kulkarni et al., 1993) and leading to malignant transformation (Marx, 1995). ED-A FN could be considered as a potential target for therapy since: (a) it is an extracellular, easily reachable molecule; and (b) in contrast to TGFβ1, it drops to low levels in adult tissues (Kornblith et al., 1996) decreasing the probability that its blocking results in side effects. Thus, the outside in signal decreasing the probability that its receptor can be unfavorable, causing autoimmune-like disease (Shull et al., 1992; Kulkarni et al., 1993) and leading to malignant transformation (Marx, 1995). ED-A FN could be considered as a potential target for therapy since: (a) it is an extracellular, easily reachable molecule; and (b) in contrast to TGFβ1, it drops to low levels in adult tissues (Kornblith et al., 1996) decreasing the probability that its blocking results in side effects. Thus, the outside in signal decreasing the probability that its receptor can be unfavorable, causing autoimmune-like disease (Shull et al., 1992; Kulkarni et al., 1993) and leading to malignant transformation (Marx, 1995). ED-A FN could be considered as a potential target for therapy since: (a) it is an extracellular, easily reachable molecule; and (b) in contrast to TGFβ1, it drops to low levels in adult tissues (Kornblith et al., 1996) decreasing the probability that its blocking results in side effects. Thus, the outside in signal decreasing the probability that its receptor can be unfavorable, causing autoimmune-like disease (Shull et al., 1992; Kulkarni et al., 1993) and leading to malignant transformation (Marx, 1995). ED-A FN could be considered as a potential target for therapy since: (a) it is an extracellular, easily reachable molecule; and (b) in contrast to TGFβ1, it drops to low levels in adult tissues (Kornblith et al., 1996) decreasing the probability that its blocking results in side effects. Thus, the outside in signal decreasing the probability that its receptor can be unfavorable, causing autoimmune-like disease (Shull et al., 1992; Kulkarni et al., 1993) and leading to malignant transformation (Marx, 1995). ED-A FN could be considered as a potential target for therapy since: (a) it is an extracellular, easily reachable molecule; and (b) in contrast to TGFβ1, it drops to low levels in adult tissues (Kornblith et al., 1996) decreasing the probability that its blocking results in side effects. Thus, the outside in signal decreasing the probability that its receptor can be unfavorable, causing autoimmune-like disease (Shull et al., 1992; Kulkarni et al., 1993) and leading to malignant transformation (Marx, 1995).
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