Alternative Splicing of Endothelial Cell Fibronectin mRNA in the IIICS Region

Functional Significance

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Transforming growth factor-β_1 (TGF-β_1) is thought to play a role in modulating vascular cell function in vivo. In vitro, it decreases endothelial cell proliferation and migration. We postulated that these biologic activities could be mediated through TGF-β_1 modulation of specific gene expression. Therefore we differentially screened a human umbilical vein endothelial cell cDNA library with cDNAs prepared from both untreated and TGF-β_1-treated bovine aortic endothelial cells. Using this technique, we isolated many TGF-β_1-induced cDNA clones. Sequence analysis of these cDNAs showed that many of them corresponded to alternatively spliced fibronectin mRNAs. These fibronectin clones all contained the extradomain I (ED I) but three different forms of the type III connecting segment (IIICS). These different fibronectin cDNAs were expressed in bacteria and the recombinant proteins used to study the effects of IIICS alternative splicing on cell attachment, spreading, and migration in bovine aortic endothelial and smooth muscle cells and B16F10 melanoma cells. The results of these experiments show that attachment and spreading of bovine aortic endothelial and smooth muscle cells depend primarily on the presence of the Arg-Gly-Asp-Ser (RGDS) sequence in the recombinant fibronectin proteins. However attachment and spreading of bovine aortic endothelial cells are modulated by alternative splicing in the IIICS region. Specifically splicing of the IIICS region decreases spreading and increases migration rates of the endothelial cells. On the contrary, using a cell line (B16F10 melanoma cells) that is known not to require the RGDS sequence for adhesion confirmed previous findings that B16F10 melanoma cells do not require the presence of the RGDS sequence for attachment and spreading. Indeed B16F10 cells were able to attach and spread on two recombinant proteins that did not contain the RGDS sequence. However attachment and spreading of B16F10 were dramatically inhibited when a 75-base pair DNA fragment was removed from the 5' end of the IIICS region. These results suggest that various regions of the fibronectin molecule may be able to interact with different cell populations to promote cell attachment and spreading, and that alternative splicing may modulate this process. (Am J Pathol 1990, 137:1509–1524)

The endothelium is a metabolically active monolayer of cells lining the internal lumina of the vascular system. One of its main functions is to maintain homeostasis in the artery wall. Dysfunction or injury of the endothelial monolayer is thought to be a key event in the development of arteriosclerosis.[1,2] Recent studies suggest that the composition of the extracellular matrix, as well as the release of growth factors by platelets or blood monocytes at sites of injury, might perturb the efficiency of endothelial regeneration.[1,3]

Transforming growth factor-β_1 (TGF-β_1) is a panregulin present in large amounts in platelets and has been shown to modulate replication, differentiation, and migration of endothelial cells and other cell types.[4-7] Recent reports show that TGF-β_1 upregulates expression of fibronectin, type I collagen, as well as integrin molecules in fibroblasts and in migrating endothelial cells.[3,8-11]

Fibronectin is a high–molecular-weight glycoprotein found in plasma, at the cell surface, and in the extracellular matrix.[12,13] It is involved in a variety of biologic functions such as cell attachment, spreading, and migration. Although fibronectin mRNA is a product of a single gene,

it can be alternatively spliced in at least three regions, thus generating up to 20 different fibronectin molecules from a single gene in the human and at least 12 different fibronectin isoforms in bovine tissues.[14-16] The biologic significance of this alternative splicing is still not completely known. However recent reports suggest that alternatively spliced portions of the molecule may be involved in modulating cell attachment, migration, and spreading.[17-19]

To better understand the mechanism of action of TGF-β_1 and to determine which genes are induced by TGF-β_1 in endothelial cells, we used differential cDNA library screening techniques on TGF-β_1-treated and untreated cultured bovine aortic endothelial cells (BAECs).

Among the cDNA molecules modulated by TGF-β_1 in BAECs, three groups of fibronectin cDNA clones were identified, which correspond to the products of alternative splicing of the IIICS region of fibronectin mRNA.

Six recombinant proteins were produced from these clones and used to investigate the significance of alternative splicing in the IIICS region. Our results suggest that alternative splicing in the fibronectin IIICS region encodes proteins that have different capacities to modulate cell attachment, spreading, and migration in vitro.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells were harvested and grown as previously described[20] on bacteriologic petri dishes (Falcon Labware Oxnard, CA) or Nunc trays (Nunc, Roskilde, Denmark) coated with type I collagen.[21]

Bovine aortic smooth muscle cells (BASMC) were isolated and grown as previously described.[22]

B16-F10 murine melanoma cells (provided by Dr. I. J. Fidler) were cultured as previously described.[23]

Matrix Proteins

Calf dermal type I collagen and human plasma fibronectin were prepared as previously described.[20,24]

Growth Factors

Transforming growth factor-beta$_1$ (prepared as described by Assoian[25]) was a gift from Drs. Michael Sporn and Anita Roberts, Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, MD. It was added to the culture media at a concentration of 0.5 ng/ml.

RNA Extraction

Cultured cells were scraped from petri dishes using a rubber policeman in a solution containing 4.5 mol/l (molar) guanidine thiocyanate, 50 mmol/l (millimolar) ethylene diamine tetraacetate (EDTA), 25 mmol/l sodium citrate, 0.1 mol/l 2-β-mercaptoethanol, and 2% sodium-N-laurylsarcosine, and further homogenized using a syringe with a 25-gauge needle. RNA was purified by ultracentrifugation through a cushion of 5.7 mol/l CsCl, as previously described,[26] and the RNA pellet was recovered as previously described.[26] Poly A+ RNA was purified using an oligo-dT cellulose column (Collaborative Research, Lexington, MA) as described by Aviv and Leder.[27]

Synthesis of cDNAs and Differential cDNA Library Screening

Three micrograms of polyadenylated RNA were transcribed with 15 units of AMV Reverse Transcriptase (Promega, Madison, WI) in 25 μl of 40 mmol/l TRIS-HCl (pH 8.3), 80 mmol/l NaCl, 6 mmol/l MgCl2, 5 mmol/l DTT, 0.5 mmol/l of each dNTP, containing 1.5 μg oligo-dT12-18 and 75 μCi of α32P-dCTP (400 Ci/mmol) (Amersham, Arlington Heights, IL) at 44°C for 45 minutes.

The mRNA-cDNA hybrids then were converted into double-stranded cDNAs by adding 75 μl of 40 mmol/l TRIS-HCl (pH 7.4), 10 mmol/l MgCl2, 80 mmol/l NaCl containing 2 units of RNAse H (Promega) and 25 units of DNA polymerase I (Promega), and subsequently incubated at 12°C and 18°C for 60 minutes each.

Incorporation of radioactivity was determined after chromatography of the cDNAs through a G-50 Sephadex (Pharmacia, Piscataway, NJ) spin column. Before hybridization, double-stranded cDNAs were denatured.

Two sister replicas from a human umbilical vein endothelial (HUVEC) cDNA library[28] (provided by Dr. Tucker Collins, Department of Pathology, Brigham and Women's Hospital, Boston, MA) were hybridized with the cDNAs from untreated or TGF-β_1-treated BAECs.

Phage DNA Preparation

Phage DNA minipreparations were performed for each selected clone according to Maniatis et al.[29]

Southern Analysis

After digestion with EcoRI, phage DNA from the selected clones were run on two similar agarose gels together with appropriate-size markers. The gels then were transfered after denaturation–neutralization according to Southern[30]

to nitrocellulose BA-85 (Schleicher and Schuell Inc., Keene, NH). The filters were baked for 2 hours under vacuum at 80°C, prehybridized in 50% formamide, 2.5× Denhardt's solution, 4× sterile sodium citrate (SSC) buffer (SSC: 0.15 mol/l NaCl, 0.015 mol/l Na-Citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) and 100 μg/ml denatured salmon sperm DNA for 4 hours at 42°C, and hybridized in the same solution overnight with equal amounts of radioactivity (about 5 × 10^6 cpm) of cDNAs from either untreated or TGF-β_1–treated BAECs. The filters then were washed for 30 minutes at room temperature in 2× SSC, 0.1% SDS, and then for 1 hour at 60°C in 1× SSC, 0.1% SDS. They were exposed to X-Omat AR films (Kodak, Rochester, NY) between intensifying screens at −70°C for 1 to 7 days.

Northern Analysis

RNAs (5 μg per lane) were denatured with formaldehyde and electrophoresed in 1% agarose formaldehyde gels containing 0.5 μg/ml ethidium bromide, examined under ultraviolet light and transfered to Biodyne filters (Pall Filter Co., Glen Cove, NY). After blotting, filters were baked for 2 hours at 80°C under vacuum.

Northern blots were prehybridized for 4 hours at 42°C in 50% formamide, 2.5× Denhardt's solution, 4× SSC, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA.

Hybridizations using cDNA fibronectin probes isolated from the differential library screening were carried out under the same conditions with ^{32}P-labeled cDNA probes using a random priming labeling kit from Boehringer-Mannheim (Mannheim, FRG). The Northern blots were washed at room temperature for 60 minutes in 2× SSC and 0.1% SDS and subsequently at 65°C in 1× SSC and 0.1% SDS for another 60 minutes. The filters then were exposed to X-Omat AR films at −70°C between intensifying screens.

DNA Sequencing

After subcloning into PGEM-4Z plasmids (Promega), the selected cDNA clones were sequenced using the method of Sanger from both 5′-and 3′-ends, using SP6 and T7 RNA polymerase primers and the GemSeq sequencing kit (Promega). Sequencing libraries using the deletion method for internal sequencing were generated for several cDNA clones using the Erase-a-Base kit (Promega).

Polymerase Chain Reaction

Primers

Oligonucleotide primers (24-mers complementary to sequences defining the 5′ ends of the complementary

template strands of the type III domains flanking the IIICS region: 3′-TCGCTCGGGGACTAACCTTCCTTT and 3′-GTTCTTCGAGAGAGAGTCTGTTGG) were synthesized on an Applied Biosystems Model 380A DNA synthesizer (Foster City, CA) and purified using an oligonucleotide purification cartridge (Applied Biosystems, Foster City, CA).

Reverse Transcriptase Reaction

500 μl microcentrifuge tubes containing 1 μg of each BAEC RNA, 0.025 mmol/l of each dNTP, and 100 pmoles of complementary oligomer (3′-GTTCTTCGAGAGA-GAGTCTGTTGG) in a total of 30 μl of water were incubated at 65°C for 5 minutes. After this 10 μl of 5× GeneAmp polymerase chain reaction (PCR) buffer, 1 unit/μl RNAsin, and 200 units of Moloney MuLV reverse transcriptase were added, yielding total volumes of 53 μl. The tubes then were incubated at 37°C for 1 hour.

Polymerase Chain Reaction

Twelve-microliter aliquots of the reverse transcriptase reaction mixtures, 60 μl of water, 10 μl of 10× PCR buffer, 1 mmol/l of each dNTP in 16 μl of water, and 1 μl each (66 ng) of the upstream and downstream primers were added to 500-μl microcentrifuge tubes. The tubes were incubated at 95°C for 5 minutes and then overlaid with 50 μl of mineral oil. The polymerase chain reactions were carried out in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 35 cycles, each consisting of 2 minutes at 65°C, 2 minutes at 72°C, and 90 seconds at 94°C.

Analysis of PCR Products

Ten-microliter aliquots of the PCR reaction products were analyzed using electrophoresis on Ethidium Bromide containing 5% acrylamide gels and 2% agarose gels. Products were visualized using ultraviolet light and base pair lengths were determined by comparison of mobilities with known high– and low–molecular-weight standards (high MW standards: Hind III digest of Lambda DNA; low MW standards: Hae III digest of ϕX174 virion DNA, New England BioLabs, Beverly, MA).

Fusion Protein Production

For fusion protein production, DNA fragments from the different fibronectin cDNA clones were subcloned into the pGEX-3X plasmid, which is a glutathione S-transferase expression vector containing an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible tac promoter[31] (Amrad Corporation Ltd., Melbourne, Australia). *Escherichia coli*

JM101 were transformed with the recombinant plasmids and fusion protein production was induced with 1 mmol/l IPTG. The three smaller fusion proteins were affinity purified using a glutathione-agarose column (Sigma Chemical Co., St Louis, MO), as previously described.[31] The three larger fusion proteins were purified using a glutathione-agarose column (Sigma Chemical Co.), as previously described,[31] followed by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 6% gels and electroelution of the protein bands (using an ISCO electroelution cup and an NH_4HCO_3 buffer; 5 mmol/l NH_4HCO_3, 0.05% SDS, 5 mmol/l EDTA, 0.5 mmol/l DFP, 0.1% β-mercaptoethanol, pH = 8.2) visualized by incubation of the gels in cold 1.0 mol/l KCl.

Attachment and Cell-spreading Assays

Thirty-five millimeter bacteriologic plastic petri dishes (Falcon Labware, Oxnard, CA) were coated overnight at 4°C with human plasma fibronectin, glutathione S-transferase, and fusion proteins at a concentration of 12.5 μg/ml in 0.1 mol/l carbonate coating buffer, pH = 9.0, as previously described.[20] The dishes then were washed with PBS and incubated for 45 minutes at room temperature with 1% heat-inactivated bovine serum albumin (BSA) to block sites of nonspecific cell attachment.[17] One milliliter of cells at 2×10^4 cells/ml in Dulbecco's minimal essential medium supplemented with 1% BSA were allowed to attach and spread for a period of 4 hours. The cells then were fixed and stained at 1- and 4-hour time points after gentle washing with PBS containing Ca^{++} and Mg^{++}. Attached cells were counted on multiple (six) random fields per petri dish using a projection microscope. The results were expressed as percentage of cell attachment to the different fusion proteins with respect to cell attachment to fibronectin. Cell attachment to glutathione S-transferase alone was minimal (3%) and was subtracted from all other values.

The degree of cell spreading was assessed by measuring cell size by planimetry on multiply photographed randomly selected fields and expressed as percentage of cell spreading on the different fusion proteins with respect to cell spreading on fibronectin.

Statistical analyses were performed on a Macintosh Plus computer (Apple Computer, Inc., Cupertino, CA), using the Statworks program (Cricket Software, Philadelphia, PA) as previously described.[7]

Cell Migration Assays

Stimulus for BAEC or BASMC migration was accomplished by release from contact inhibition of confluent cultures using a Teflon fence assay, as previously described.[32] Briefly cells were plated in the center wells of the fences and allowed to attach and become confluent. At this time the fences were removed and radial migration rates were calculated from determinations of increases in area covered (mm^2). Migration rates were determined from area measurements using a graphics tablet and a Macintosh Plus computer, as described.[3,32] Migration rates on the different fusion proteins were expressed with respect to the migration rate of cells on fibronectin. Statistical analyses were performed on a Macintosh Plus computer using the Statworks program, as previously described.[7]

Indirect Immunofluorescence

Cells were allowed to attach for 4 hours on bacteriologic plastic petri dishes coated with either fibronectin or S10 or L10 fusion proteins. They were fixed with paraformaldehyde-lysine-periodate fixative (PLP), permeabilized with 0.1% Triton X-100, and labeled with a polyclonal antibody to a β1 integrin cytoplasmic domain 10-amino acid synthetic peptide WDTGENPIYK (anti-β1/10P) provided by Dr. C. Buck (Wistar Institute, Philadelphia, PA).[11] Rhodamine-conjugated goat anti-rabbit secondary antibody were from Cappel Laboratories (Cochranville, PA).

Results

Differential cDNA Library Screening and Isolation of Endothelial Cell Fibronectin cDNA Clones

Transforming growth factor-β_1–modulated cDNA clones initially were selected on the basis of colony hybridization (Figure 1) and confirmed by hybridization according to Southern,[30] with radiolabeled cDNA from untreated and TGF-β_1–treated BAEC (Figure 2). The EcoRI inserts from modulated clones then were subcloned into pGEM-4Z (Promega) and subjected to restriction map analysis. Several of the induced cDNA clones showed similar but not identical restriction maps. DNA sequencing analysis showed that they were fibronectin cDNA clones (Figure 3),[33] all of which contained the extradomain I (ED I), when they extended far enough in the 5′ direction. Variations in the restriction map pattern of these fibronectin clones were due to three different patterns of alternative splicing in the connecting segment III (IIICS) (Figure 3): a complete IIICS for HECFn10 and HECFn27; a 93-bp splicing on the 3′-end of the IIICS for HECFn14; or a combination of a 93-bp deletion on the 3′-end and a 75-bp deletion on the 5′ end of IIICS (HECFn54) (Figure 3).

Northern blot analysis of RNA extracted from TGF-β_1–treated and untreated BAECs using fibronectin cDNA probes (HECFn27, HECFn14, and HECFn54) isolated from
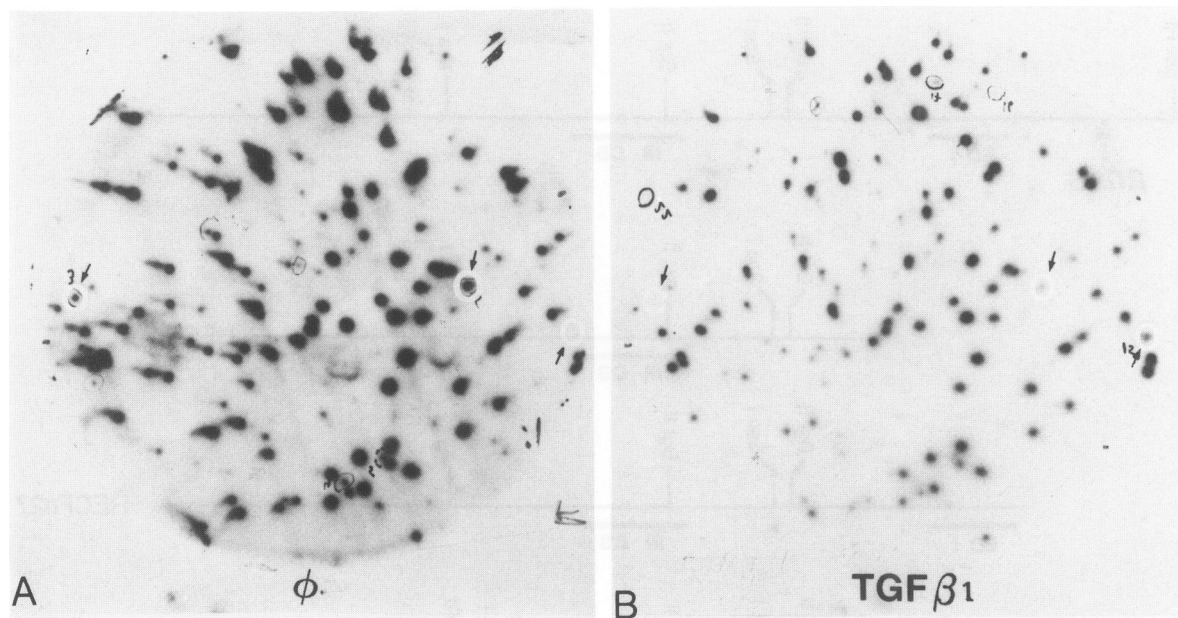
Figure 1. *Screening of the endothelial cell cDNA library by differential hybridization using radiolabeled cDNAs from untreated* (A) *or TGF-β₁-treated* (B) *BAECs. Examples of upregulated or downregulated cDNA clones are indicated (small arrows). The plate screening is preliminary in the screening process for modulated clones because it is very difficult to transfer the same amount of phage DNA on both replicas. At this point, the clones are largely preselected, and it is only at the Southern blot level that we can definitively decide if a cDNA clone is modulated.*

the differential library screening (Figure 3) confirmed an induction of fibronectin mRNAs. This induction (illustrated in Figure 4 using clone HECFn27) was observed to be transient with a maximum obtained after a 24-hour incubation period with TGF-β_1 when Northern analyses were performed after 0 hours, 5 hours (not shown), 24 hours, and 48 hours of TGF-β_1 treatment (Figure 4).

In an attempt to address the question whether changes in alternative splicing in the IIICS region occur after TGF-

β_1 treatment, we performed PCR reactions using RNAs from untreated, 24-hour TGF-β_1-treated and 48-hour TGF-β_1-treated BAEC cultures as templates and 24-mer primers that flank the IIICS region. Analyses of the 5% acrylamide and 3% agarose gel electrophoreses revealed the presence of a 315-bp fragment and a 192-bp fragment in the untreated samples consistent with the presence of a 93-bp splicing on the 3' end of the IIICS and a combination of a 93-bp deletion on the 3' end and a 75-bp

Figure 2. *Southern blot analysis of selected cDNA clones after digestion with EcoRI, hybridized with radiolabeled cDNAs from untreated* (A) *or TGF-β₁-treated* (B) *BAECs. The first lane shows an example of non-modulated clone as a control. The other clones are upregulated by TGF-β₁ treatment. Clones 9, 10, 14, 27, 29, and 54 have been determined to be fibronectin clones.*
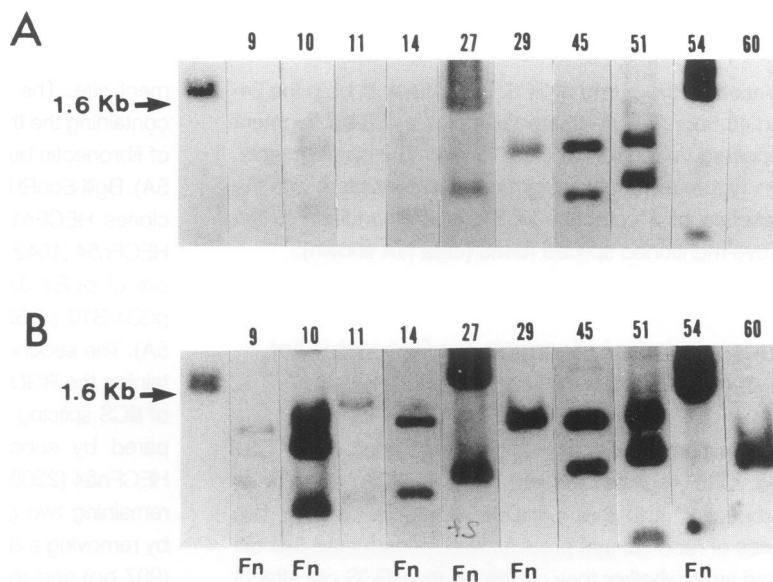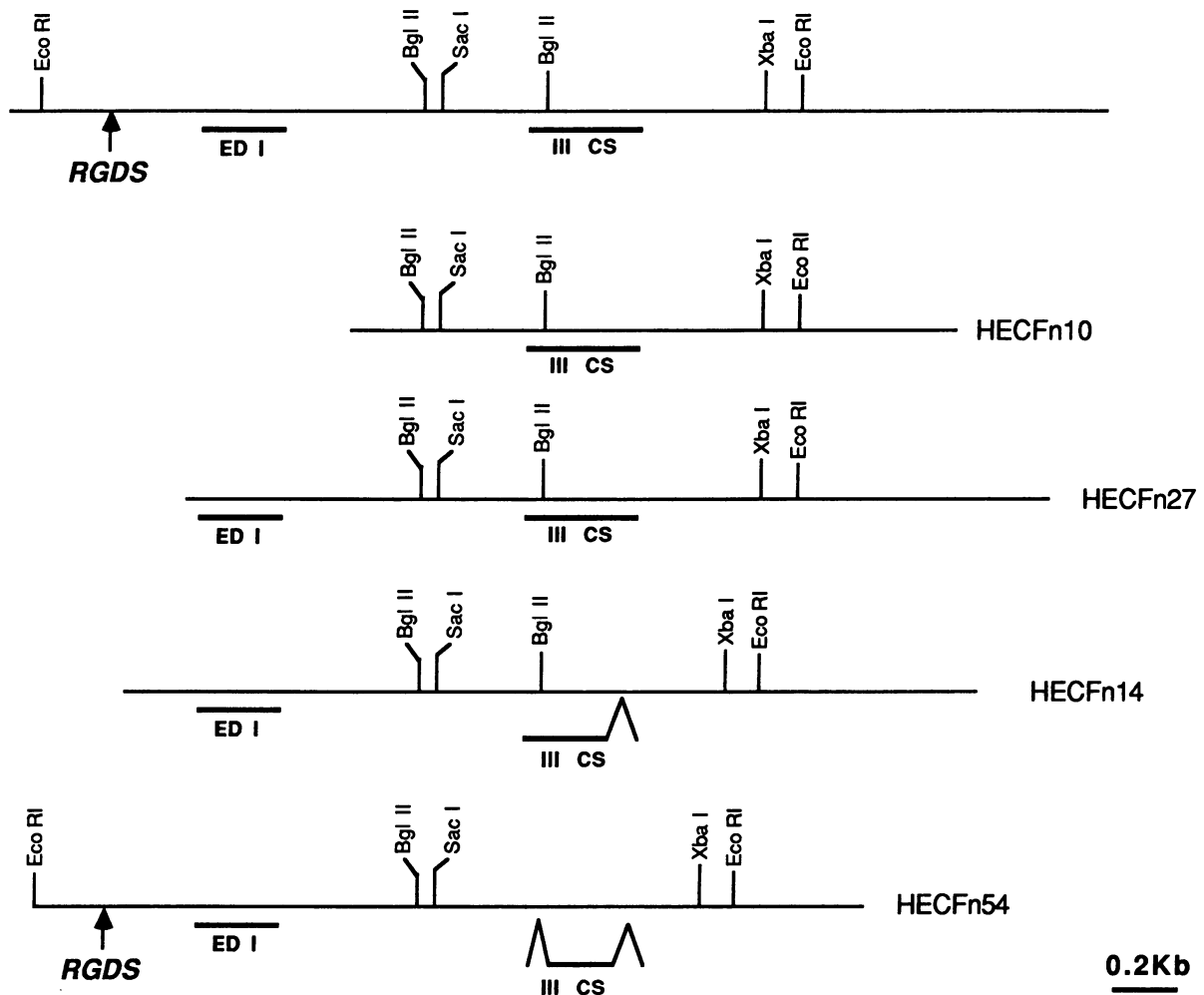
Figure 3. *Restriction map analysis of typical human cell surface fibronectin cDNA clones showing the positions of the RGDS sequence, the extra domain I (ED I), and the type III connecting segment (IIICS), and of the fibronectin cDNA clones isolated from the endothelial cell library. HECFn10 and HECFn27 contain the complete IIICS. In addition, HECFn27, which extends further in the 5' direction, contains the ED I. HECFn14 shows 93 bp deletion in the 3' region of the IIICS and contains ED I. HECFn54 shows the same 93 bp deletion as HECFn14 and, in addition, a 75-bp deletion in the 5' region of the IIICS. The ED I domain is present in HECFn54.*

deletion on the 5' end of IIICS. In contrast, in both the 24- and 48-hour TGF-β_1-treated samples a 408-bp fragment appeared in addition to the 315- and 192-bp fragments. This higher molecular-weight band is consistent with the presence of a complete IIICS region in addition to the above-mentioned spliced forms (data not shown).

Construction of Plasmids for Recombinant Peptide Production

To investigate possible biologic significance of the CS1 and CS5 regions present in the IIICS domain of fibronectin[34] and their possible alternative splicing, two series of recombinant proteins were constructed that differed as to whether they contained the RGDS cell attach-

ment site. The first series consisted of three plasmids containing the three patterns of splicing in the IIICS region of fibronectin but not the RGDS sequence region (Figure 5A). BglII-EcoRI DNA restriction fragments from the cDNA clones HECFn10 (1210 bp), HECFn14 (1117 bp), and HECFn54 (1042 bp) were subcloned into the BamHI-EcoRI site of pGEX-3X, resulting in the recombinant plasmids pG3X-S10, pG3X-S14, and pG3X-S54, respectively (Figure 5A). The second series consisted of three plasmids containing the RGDS region in addition to the three patterns of IIICS splicing (Figure 5B). Plasmid pG3X-L54 was prepared by subcloning the EcoRI DNA fragment from HECFn54 (2300 bp) into the EcoRI site of pGEX-3X. The remaining two plasmids in this series were constructed by removing a Sac1-Xba1 DNA fragment from pG3X-L54 (907 bp) and replacing it with the Sac1-Xba1 DNA frag-
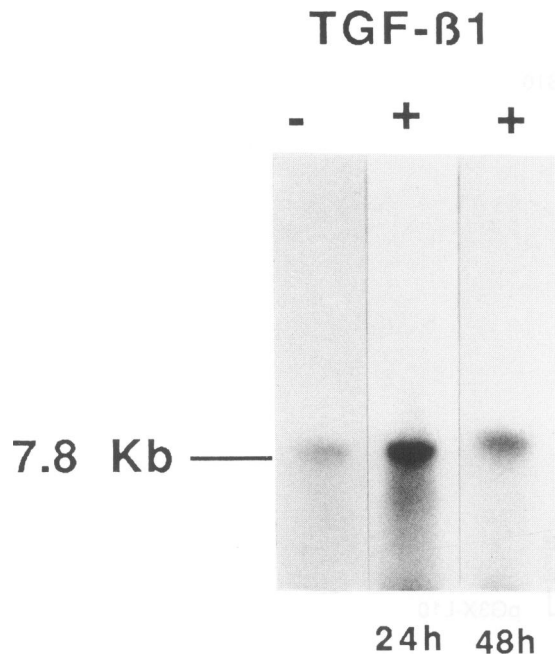
TGF-β1

- + +

7.8 Kb —

24h   48h

Figure 4. *Northern blot analysis of RNA extracted from cultured BAECs untreated or treated with TGF-β₁ for 24 or 48 hours and hybridized with ³²P-labeled fibronectin cDNA probe HECFn27 isolated from the differential library screening described in Figure 3.*

ment from HECFn10 (1075 bp) or HECFn14 (982 bp), resulting in the plasmids pG3X-L10 and pG3X-L14, respectively (Figure 5B).

Escherichia coli JM 101 were transformed with the different recombinant plasmids as well as the pG3X parent plasmid and recombinant fusion protein production induced with IPTG for 4 hours according to Smith and Johnson.[31] Fusion proteins were purified from bacterial lysates using a glutathione-agarose column (Sigma Chemical Co.) followed by preparative electrophoresis and electroelution in the case of the larger fusion proteins, and analyzed on SDS-polyacrylamide gel electrophoresis (Figure 6). All fusion proteins were recognized by a polyclonal antibody to fibronectin, as determined by Western blot analysis (data not shown).

Attachment and Spreading Experiments

Bacteriologic plastic petri dishes were coated with 12.5 µg/ml of either plasma fibronectin or each of the six different fusion proteins, S10, S14, and S54 (−RGDS, +IIICS splicing) or L10, L14, and L54 (+RGDS, +IIICS splicing) (Figure 5). We verified by enzyme-linked immunoassay using an anti-fibronectin antibody that saturation of the bacteriologic plastic petri dishes was obtained at this coating concentration for each individual fusion protein

(Figure 7). We studied attachment and spreading of three cell populations including B16F10 melanoma cells (which have known attachment and spreading characteristics on specific domains of fibronectin residing in the IIICS region),[18,35] BASMCs, and endothelial cells (BAEC), on the different fusion proteins relative to attachment and spreading on fibronectin (Figures 8 and 9). All three cell populations attached and spread well on plasma fibronectin.

B16F10 Melanoma Cells

B16F10 melanoma cells were chosen because of their known attachment to the IIICS region of fibronectin, which provided us with a known 'control' cell population with which to compare the vascular cells. In agreement with previously published data,[18,35] B16F10 melanoma cells attached well on the RGDS minus fusion proteins S10 and S14 (71% and 67%, respectively), but not on S54 (30%) ($P < 0.001$) (Figures 8 and 9). Cell attachments on the RGDS plus fusion proteins L10 and L14 (74% and 75%, respectively) were comparable to that on S10 and S14 and therefore were not potentiated by the presence of the RGDS sequence. Attachments of B16F10 were not significantly different on fusion proteins L54, L10, and L14, but all were higher than on fusion protein S54 (Figures 8 and 9).

No significant changes in B16F10 cell spreading occured on fibronectin, S10, S14, L10, L14, and L54 (100%); however cell spreading was dramatically reduced on S54 (20%) ($P < 0.001$) (Figures 8 and 9), which also correlates with previously published data.[18,35,36]

Bovine Aortic Smooth Muscle Cells

Bovine aortic smooth muscle cell attachment on the RGDS minus fusion proteins did not exceed 35% of that on fibronectin, and BASMC attachment was not significantly higher on S10, which contains the complete IIICS, than on S14 and S54 (Figures 8 and 9). Bovine aortic smooth muscle cell attachment on the fusion proteins containing the RGDS sequence was significantly higher than on the fusion proteins not containing the RGDS sequence. However BASMC attachment was not significantly different on L10, L14, or L54 (120%, 87%, and 80%, respectively) (Figures 8 and 9).

Bovine aortic smooth muscle cell spreading on fusion protein S10 was 55% of that on fibronectin and significantly decreased on fusion peptide S54 (33%) compared to S10 ($P < 0.05$). Bovine aortic smooth muscle cells spread well on all fusion proteins containing the RGDS sequence, with no significant differences noted among L10, L14, and L54 (102%, 90%, and 82%, respectively) (Figures 8 and 9).
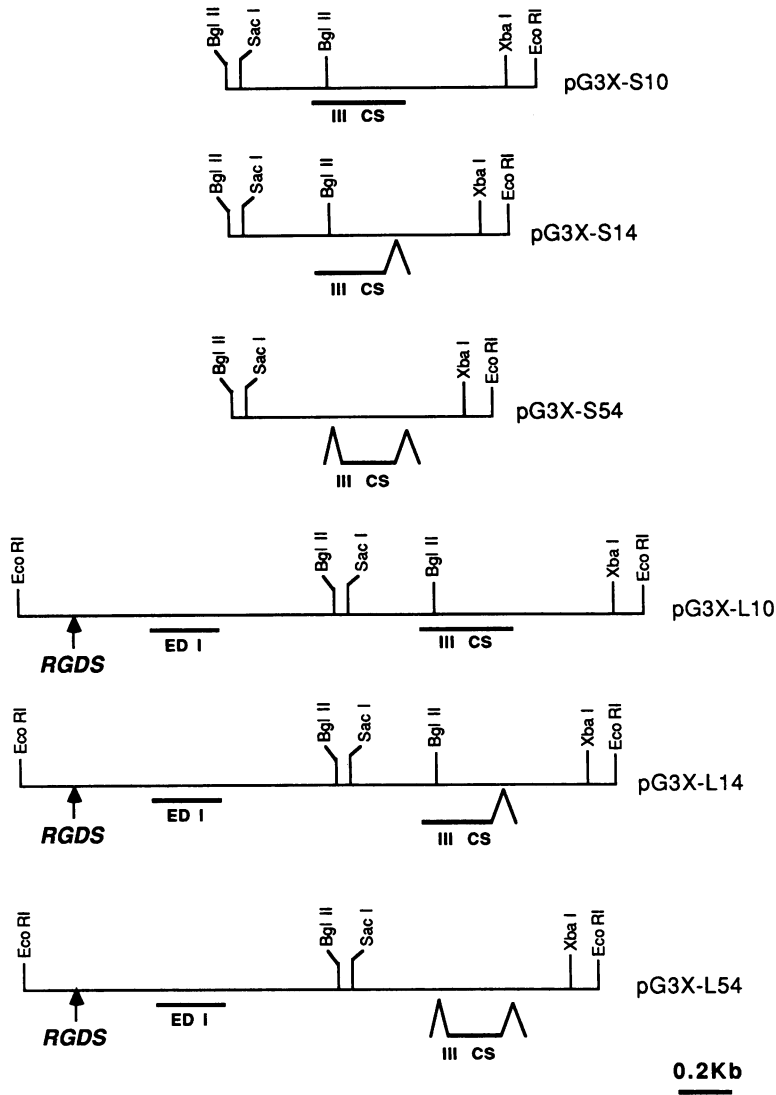
Figure 5. *Restriction map analysis of the DNA fragments subcloned into pG3X plasmid used for glutathione-S-transferase fusion protein production. The positions of the IIICS, ED I, and RGDS sequence are indicated.*

Bovine Aortic Endothelial Cells

Bovine aortic endothelial cell attachment on the fusion proteins S10, S14, and S54 did not exceed 15% of that on fibronectin. Attachment was significantly higher on the fusion proteins L10, L14, and L54 (73%, 57%, and 47%, respectively), which all contain the RGDS sequence (Figure 5), and reached 73% of the attachment on fibronectin when BAECs were seeded on L10. Bovine aortic endothelial cell attachments were significantly decreased on fusion proteins L14 and L54 compared to fusion peptide L10 ($P < 0.05$) (Figures 8 and 9).

Bovine aortic endothelial cell spreading was minimal on the fusion proteins S10, S14, and S54. On the contrary, BAECs spread effectively on the fusion proteins L10 (90%) and L14 (80%) and was significantly reduced on L54 (58%) ($P < 0.001$) (Figures 8 and 9) when compared to spreading on fibronectin.

BAEC and BASMC Migration Experiments

To study the possibility that specific domains residing in the IIICS region might serve as modulators of vascular cell interactions with the fibronectin RGDS domain, migration studies with BAEC and BASMC plated on fibronectin and the fusion proteins containing the RGDS sequence and the three alternatively spliced isoforms of the IIICS domain were performed (Figure 10). In these experiments we used a Teflon fence assay[32] to compare the migration of BAECs and BASMCs on bacteriologic plastic petri dishes coated with type I collagen, fibronectin, or fusion proteins L10, L14, and L54. As shown previously, BAECs exhibit maximal migration rate on type I collagen, while a fibronectin substratum elicits a significantly lower (40%) migration rate. In contrast, BASMC migration rates are modest on a type I collagen substrate but are increased 40% on a fibronectin substrate.[3,7,37] The results
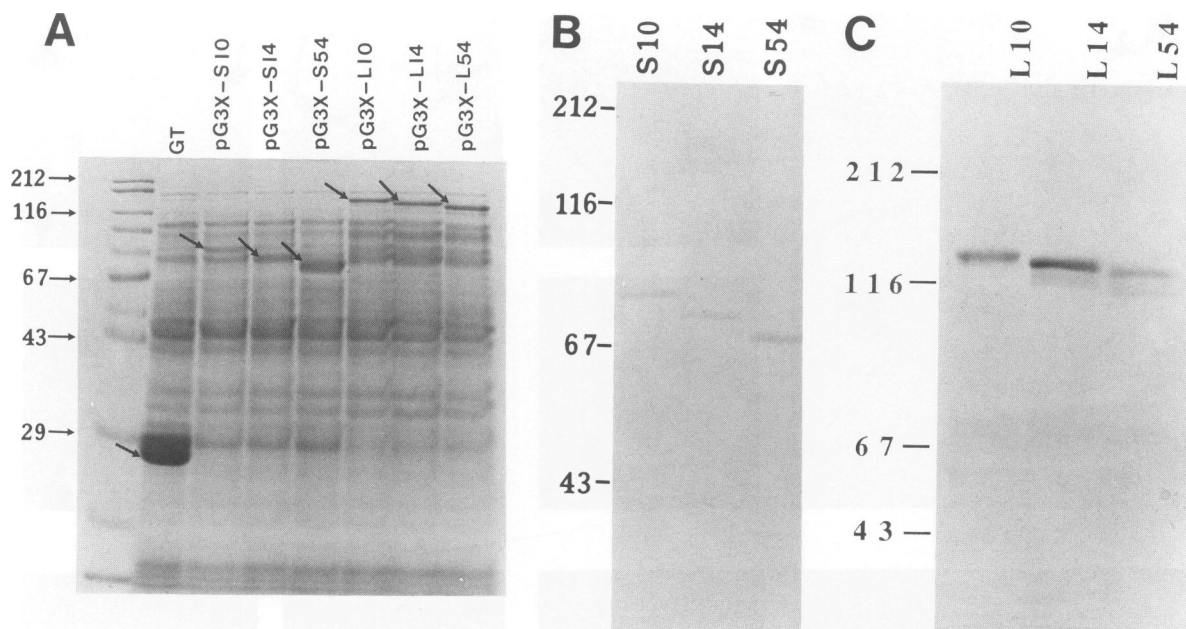
Figure 6. A: *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) of bacterial lysates from* E. coli *JM101 transformed with PG3X, or the PG3X constructs and induced with IPTG. Glutathione S-transferase (GT) and the fusion proteins (S10, S14, S54, L10, L14, L54) are indicated with arrows.* B: *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5%) of the three RGDS minus fusion proteins after affinity purification. The positions of molecular weight standards are indicated.* C: *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (6.0%) of the three RGDS plus fusion proteins after affinity purification and electroelution. The positions of molecular weight standards are indicated.*

illustrated here showed that compared to fibronectin, BAEC migration was significantly decreased on fusion protein L10 (15%, $P < 0.001$) and on L14 (8%, $P < 0.017$) but not on L54. In contrast, BASMC migration was decreased by 30% on the three fusion proteins L10, L14, and L54 compared to fibronectin.

Immunofluorescence Localization of $\beta1$ Integrin

Because β_1 integrins have been shown to be involved in, and are organized during, cell spreading in several cell types, we performed the following experiments to elucidate the roles of the IIICS domain in processes of cell spreading and β_1 integrin organization.[11,36] The distribution of β_1 integrin was studied in the three cell populations (B16F10, BASMC, and BAEC) seeded on fibronectin, L10 (+RDGS, +IIICS), and S10 (−RGDS, +IIICS) fusion protein-coated bacteriologic plastic petri dishes (Figure 11).

When B16F10 were seeded on fibronectin or the L10 fusion protein, β_1-integrin staining was localized to linear structures throughout and at the periphery of the cytoplasm and in the perinuclear and Golgi regions of the cells. This staining pattern was not observed in B16F10 seeded on S10 fusion protein despite the fact that B16F10 were well spread on that substrate (Figure 11a to c).

β_1-integrin staining was localized to linear structures in BASMC seeded on fibronectin and fusion peptide L10 (Figure 11d and e). However the immunofluorescence staining was mostly diffuse and localized in the perinuclear area when BASMC were seeded on fusion peptide S10 (Figure 11d to f), despite the fact that BASMC exhibited moderate spreading on this substrate (Figure 8b and Figure 9).
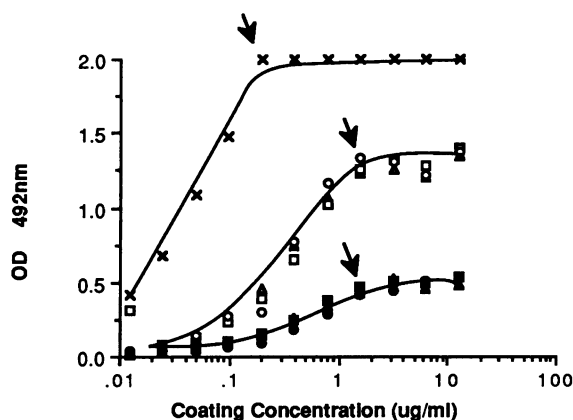

Figure 7. *ELISA assay using an anti-fibronectin antibody in which various concentrations of intact fibronectin (Fn) and fusion proteins have been used to coat 96-well plates. Saturating concentrations of fibronectin (X), the RGDS plus (open circle = S10, open triangle = S14, open square = S54) and the RGDS minus (filled circle = S10, filled triangle = S14, filled square = S54) fusion proteins are indicated with arrows.*
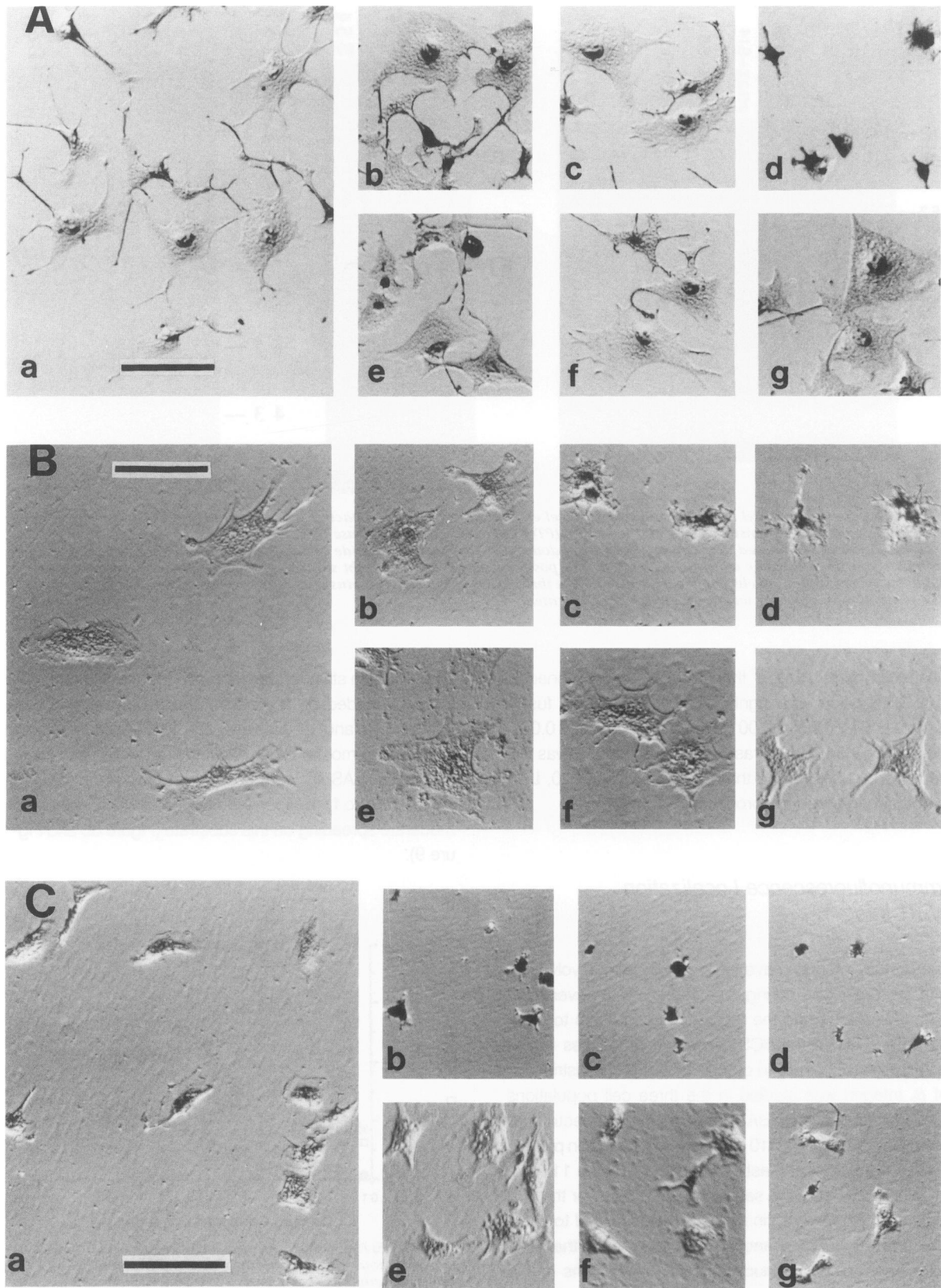
Figure 8. *Phase-contrast microscopy of attachment and spreading of B16F10 melanoma cells* (A), *bovine aortic smooth muscle* (B), *and endothelial* (C) *cells on plasma fibronectin* (a), *S10* (b), *S14* (c), *S54* (d), *L10* (e), *L14* (f), *and L54* (g) *fusion proteins. Bar, 100 μm.*
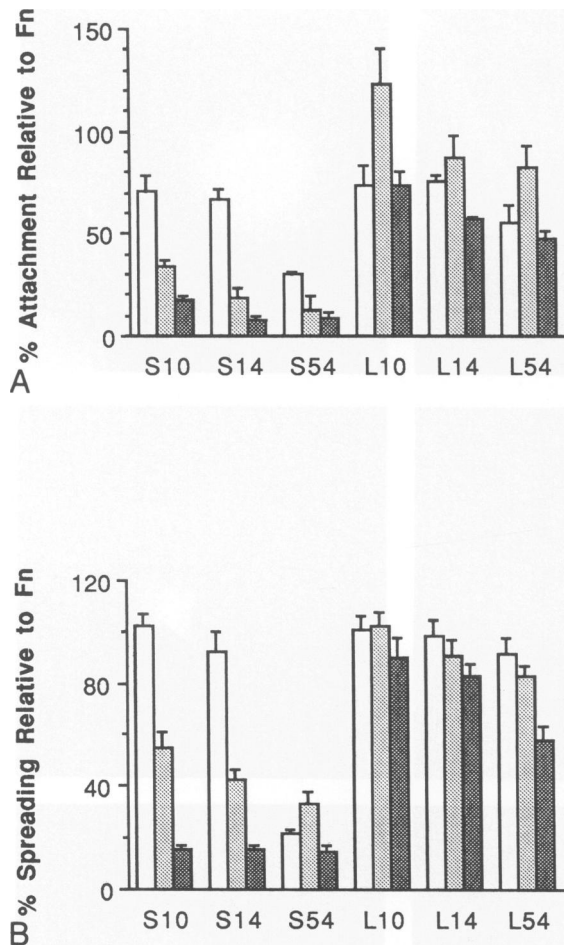
Figure 9. *Attachment and spreading of B16F10 melanoma cells (white boxes), bovine aortic smooth muscle cells (BASMC, lightly shaded boxes), and bovine aortic endothelial cells (BAEC, darkly shaded boxes) relative to fibronectin on bacteriologic petri dishes coated with the different fusion peptides.*

In BAEC seeded on fibronectin or L10 fusion protein, the β_1-integrin staining was localized to linear structures extending toward putative cellular adhesion plaques[11,37] (Figure 11g to i). Diffuse cytoplasmic staining with no particular organization was noted in BAEC seeded on S10 fusion peptide, a substrate that does not support any appreciable BAEC spreading (Figure 8b and c and Figure 9).

Discussion

In this study, we used differential cDNA library screening to isolate cDNA clones corresponding to genes modulated by TGF-β_1 in endothelial cells. The identification of such genes is important because recent data suggested that TGF-β_1 inhibits large-vessel endothelial cell replication and migration *in vitro*[7,38,39] and, in addition, might be respon-

sible for reducing the capacity of endothelial regeneration after experimental injury *in vivo*.[3] Using this method, a total of 30 cDNA clones were selected from a HUVEC library. Preliminary restriction map analysis showed that a third of them displayed similar but not identical restriction maps. DNA sequencing analysis showed that these clones corresponded to alternatively spliced forms of fibronectin mRNA. Alternative splicing of fibronectin mRNA occurs in at least three regions of the molecule defined as ED I and ED II (or ED A and ED B) and IIICS (or variable) region.[14,16,40-47] The EDI and ED II are thought to be spliced out in plasma fibronectin[15] but might be present or absent in cell-surface fibronectin.[12,13] ED II expression has been associated with cell transformation.[48] The third region of alternative splicing, defined as IIICS or variable region, can be completely present, partially spliced out, or totally
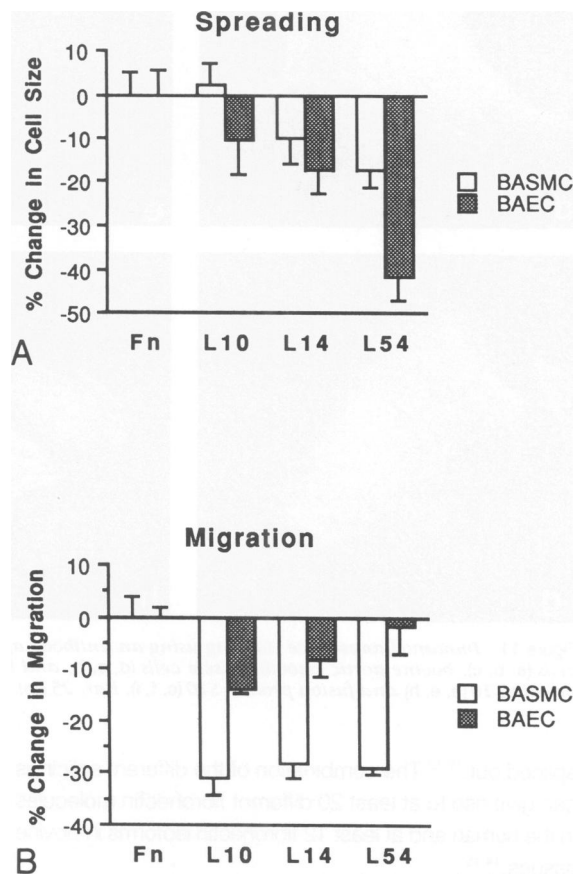

Figure 10. *Endothelial and smooth muscle cell spreading and migration on RGDS plus fusion proteins. This figure illustrates the correlation between vascular cell spreading properties and the migration rates on the RGDS plus fusion proteins L10, L14, and L54 relative to fibronectin. BASMC (white boxes) are noted not to exhibit statistically significant changes in cell size (spreading) when plated on fibronectin (Fn) or the fusion proteins L10, L14, and L54 (A). Similarly their migration rates on all the fusion proteins are essentially the same (B). In contrast, BAEC (shaded boxes) cell sizes show progressive decreases from Fn > L10 > L14 > L54 (A), while BAEC migration increases from L10 < L14 < L54 (B), suggesting an inverse relationship between cell spreading and migration in BAEC.*
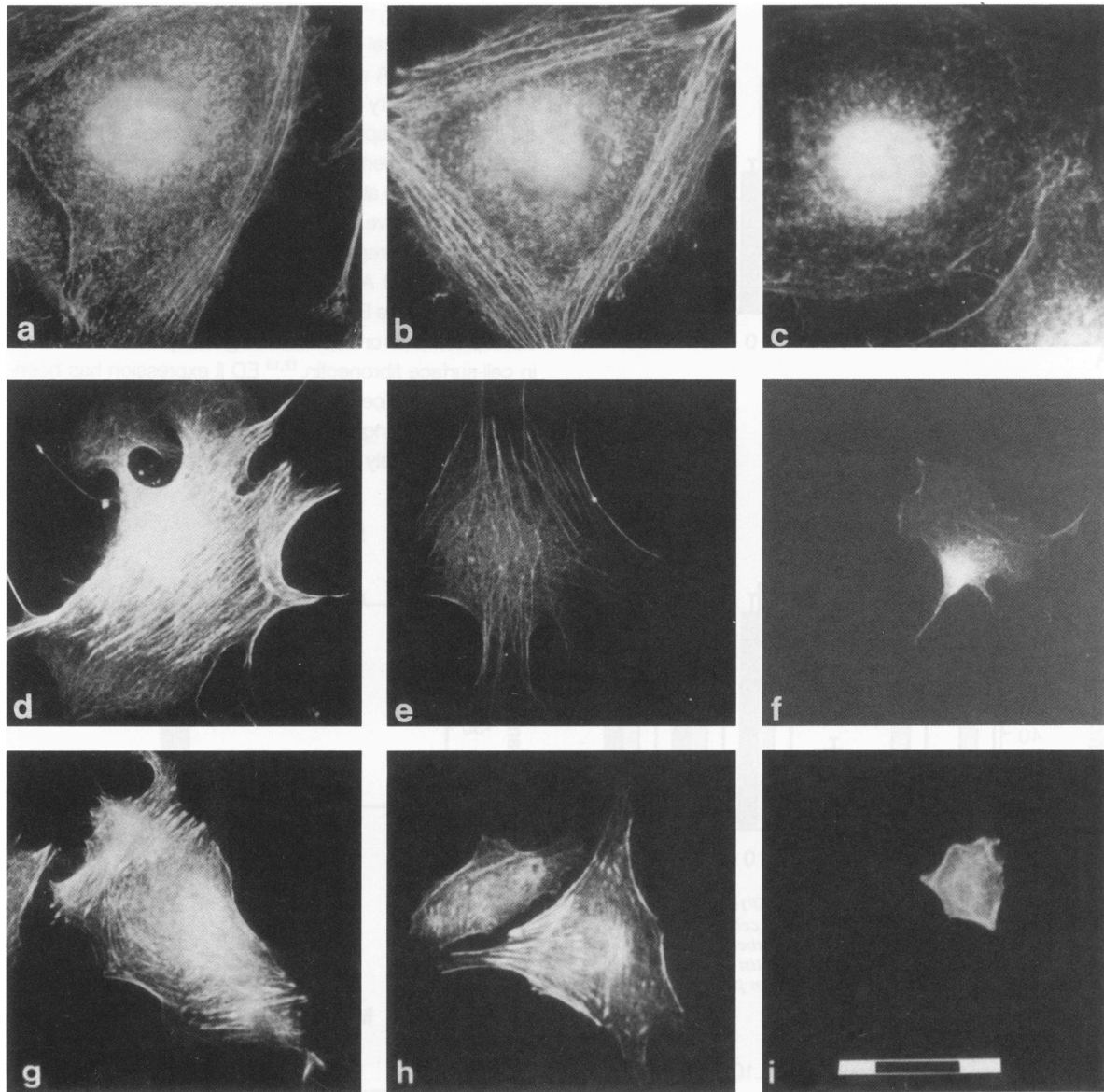
Figure 11. *Immunofluorescence staining using an antibody against the cytoplasmic domain of β-1 integrin of B16F10 melanoma cells (a, b, c), bovine aortic smooth muscle cells (d, e, f), and bovine aortic endothelial cells (g, h, i) on fibronectin (a, d, g), fusion protein L10 (b, e, h) and fusion protein S10 (c, f, i). Bar, 25 μm.*

spliced out.[12,13] The combination of the different splicings can give rise to at least 20 different fibronectin molecules in the human and at least 12 fibronectin isoforms in bovine tissues.[14,15]

The fibronectin cDNA clones that we isolated from the human endothelial cell cDNA library contained the ED I region (Figure 3), known to be expressed only in cell-surface fibronectin, and, in addition, showed three different forms of alternative splicing of the IIICS region. Two forms are typical for cell-surface fibronectin[14,40,41,43–46] and are represented in clones HECFn10 and HECFn27, which contain the complete IIICS region (Figure 3), as well as clone HECFn14, which contains the 93-bp deletion at the

3′ end of the IIICS region (Figure 3), which has been documented in human material and may be present in bovine tissues.[15] The third clone, HECFn54, has a 75-bp splicing at the 5′ end of the IIICS region, in addition to the 93-bp deletion of the 3′ end of the IIICS region, which also has been documented in human material and may be present in bovine tissues[15] (Figure 3). The combination of these two deletions has been described only in plasma fibronectin clones to date and may not be typical for cell-surface fibronectin.[49] Northern blot analyses confirmed the transient induction of fibronectin mRNA by TGF-β_1 in BAEC (Figure 4), which has been previously described for TGF-β_1–treated fibroblasts and endothelial cells.[3,10]

Polymerase chain reaction analyses using the RNAs of untreated and TGF-β_1-treated BAECs as templates and primers flanking the IIICS region demonstrated the presence of Fn mRNA containing the 93-bp deletion at the 3' end of the IIICS region and an isoform containing a 75-bp splicing at the 5' end of the IIICS region in addition to the 93-bp deletion of the 3' end of the IIICS region. TGF-β_1-treated samples contained the above two Fn RNAs as well as an additional isoform of Fn RNA, namely, one containing the complete IIICS region. These data support the notion that TGF-β_1 may elicit its effects on BAEC migration, in part, by modulating the alternative splicing patterns of a particular matrix component, Fn.

During these past 10 years, the role of fibronectin in promoting cell adhesion has been well studied.[12,13] The principal cell attachment site of fibronectin consists of the RGDS sequence and has been localized to one of the type III repeats of the molecule.[50] Mutation or deletion of the RGDS sequence has been shown to reduce spreading of BHK fibroblasts on fibronectin fusion peptides by at least 95%.[35] However several recent reports have suggested that other regions of the fibronectin molecule, such as the IIICS region (namely the CS1 and CS5 domains of IIICS) and the heparin-binding region, might be involved in promoting cell attachment and spreading.[17,18,19,51,52] The biologic significance of alternative splicing in fibronectin might be, therefore, to modulate attachment and spreading of different cell populations by inserting or deleting specific domains that cells can bind to directly (such as the CS1 domain of the IIICS region) or by inserting or deleting specific domains that are capable of modulating cell interactions with the RGDS and/or the heparin-binding domains of the fibronectin molecule.

To investigate this hypothesis and to determine if selected regions of the IIICS domain (CS1 and CS5) could function as modulators of cell-fibronectin adhesion events, we used the fibronectin cDNA clones isolated from a HUVEC library to produce two groups of fusion proteins containing three different patterns of alternative splicing in the IIICS region (Figure 5). The three RGDS minus fusion proteins (S10, S14, and S54) were designed to delineate a potential cell attachment and spread promoting activity in the IIICS region, independent from the RGDS sequence. The RGDS plus fusion proteins (L10, L14, and L54), which extend to the RGDS sequence, were designed to determine if the presence or absence of the CS1 and CS5 regions of the IIICS domain could modulate the activity of the RGDS sequence on vascular cell attachment and spreading.

The results of these experiments (Figures 8 and 9) show that fusion proteins containing the RGDS sequence are substantially more active in promoting attachment and spreading of BAEC and BASMC. Indeed attachment and spreading of BAEC on the RGDS minus fusion proteins

was only minimal and not significantly modulated by alternative splicing of the IIICS region. However significant modulation of attachment and spreading was observed in BAEC on the various RGDS plus fusion proteins, suggesting that alternative splicing of the IIICS region might somehow modulate the activity of the RGDS sequence on this particular cell population. In contrast, BASMC attached and spread more efficiently than BAEC on the RGDS minus fusion peptides, and spreading was significantly decreased when both 3' and 5' ends of the IIICS region were spliced out, suggesting that these regions might influence spreading of BASMC in the absence of the RGDS sequence. In addition, no significant modulation of attachment and spreading of BASMC were obtained on the RGDS plus fusion proteins, suggesting that these regions (CS1 and CS5) of the IIICS domain do not modulate the activity of the RGDS sequence on this particular cell population and have different biologic functions on these two vascular cell populations.

B16F10 melanoma cells clearly attached and spread on fusion proteins that did not contain the RGDS sequence (Figures 8 and 9), as previously described by Humphries et al.[17,18] Comparison of B16F10 attachment and spreading on fusion proteins S10, S14, and S54 showed that the 5' 25-amino acids in the IIICS region (CS1) were essential for attachment and spreading of B16F10. However this 25-amino acid sequence seemed to be less essential for attachment and spreading of B16F10 on the RGDS plus fusion proteins, consistent with the notion that the heparin-binding domain located N terminal to the IIICS region and C terminal to the RGD sequence also is capable of mediating cell attachment and spreading in this cell population.[52] These results correlate with previously reported data by Humphries et al[17,18] attributing a similar biologic effect to the synthetic peptide CS1, which contains part of this 25-amino acid sequence. These data suggest that B16F10 melanoma cells can interact with at least two sites during attachment and spreading on the fibronectin molecule: the heparin binding domain and a sequence localized in the 5' end of the IIICS region (Figures 8 and 9).[17,18,34,52] In addition, Guan and Hynes[19] recently showed that lymphoid cells recognize the CS1 alternatively spliced segment of fibronectin via an $\alpha_4\beta_1$ integrin. Our fusion protein repertoire did not allow us to investigate the biologic effect of alternative splicing of the 5' 75-bp DNA fragment (which contains the CS1 peptide) independently of the 3' 93-bp splicing of the IIICS region (which contains the CS5 peptide).

The results of the migration assays, in conjunction with the spreading data, are consistent with the concept that in the presence of the RGDS domain, particular domains of fibronectin, namely the 3' 93-bp and the 5' 75-bp regions of the IIICS domain can modulate cell spreading and sheet migration of certain vascular cell populations (BAEC) (Fig-

ure 10). Specifically there appears to be an inverse correlation between the ability of the particular fusion proteins to promote BAEC spreading and enhance migration (Figure 10). Indeed fusion protein L10 promoted the most spreading and least migration rate, while L54 promoted the least spreading and highest migration rate in BAEC (Figure 10). In addition to these direct effects on vascular cell migration, specific domains of the IIICS region of fibronectin also may participate in modulating lymphocyte and monocyte adhesion and subsequent infiltration in areas of vascular injury and repair.[19,53,54]

The results of immunofluorescence staining of the three different cell populations using a β_1-integrin antibody (Figure 11) suggest that the RGDS sequence, present in plasma fibronectin and in the L10 fusion protein, is necessary for organization of β_1-integrin receptors but that organization of β_1-integrins is not a prerequisite for cell spreading. Indeed B16F10 melanoma cells spread very well and BASMC spread moderately well on the S10 fusion protein, which does not contain the RGDS sequence, without significant organization of β_1 integrin (Figure 11 c). These results correlate with previously reported data[55] suggesting that β_1 fibronectin receptors (with the exception of $\alpha_4\beta_1$) interact with fibronectin through the RGDS sequence[13,55,56] and that $\alpha 4$, $\alpha 6$, and α_V chains can be associated with several β-chain classes.[57]

Differential cDNA library screening has allowed us to isolate several fibronectin cDNA clones induced by TGF-β_1 in endothelial cells. We have shown that HUVECs produce at least three different forms of fibronectin mRNA, two of which contain alternatively spliced deletions in the IIICS region. Experiments using fusion proteins suggest that the IIICS region contains adhesion promoting and modulating sites that are recognized by selected vascular cell populations and elicit changes in vascular cell behavior that may be important in vascular cell responses to injury.

References

1. Ross R: The pathogenesis of atherosclerosis-An update. N Engl J Med 1986, 314:488–500
2. Munro MJ, Cotran RS: Biology of disease. The pathogenesis of atherosclerosis: Atherogenesis and inflammation. Lab Invest 1988, 58: 249–261
3. Madri JA, Reidy MA, Kocher O, Bell L: Endothelial cell behavior after denudation injury is modulated by transforming growth factor-$\beta 1$ and fibronectin. Lab Invest 1989, 60:755–765
4. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AS: Transforming growth factor type β: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Aca Sci USA 1986, 83:4167–4172

5. Sporn MB, Roberts AB, Wakefield LM, de Crombrugghe B: Some recent advances in the chemistry and biology of transforming growth factor-beta. J Cell Biol 1987, 105:1039–1045
6. Madri JA, Pratt BM, Tucker AM: Phenotypic modulation of endothelial cells by transforming growth factor-β depends upon the composition and organization of the extracellular matrix. J Cell Biol 1988, 106:1375–1384
7. Bell L, Madri JA: Effect of platelet factors on migration of cultured bovine aortic endothelial and smooth muscle cells. Circ Res 1989, 65:1057–1065
8. Ignotz RA, Massague J: Cell adhesion protein receptors as targets for transforming growth factor-β action. Cell 1987, 51:189–197
9. Balza E, Borsi L, Allemanni G, Zardi L: Transforming growth factor β regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. FEBS Lett 1988, 228: 42–44
10. Penttinen RP, Kobayashi S, Borstein P: Transforming growth factor β increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. Proc Natl Acad Sci USA 1988, 85:1105–1108
11. Basson CT, Knowles WJ, Bell L, Albelda SM, Castronovo V, Liotta LA, Madri JA: Spatiotemporal segregation of endothelial cell integrin and nonintegrin extracellular matrix binding proteins during adhesion events. J Cell Biol 1990, 110:789–801
12. Hynes R: Molecular biology of fibronectin. Ann Rev Cell Biol 1985, 1:67–90
13. Ruoslahti E: Fibronectin and its receptors. Ann Rev Biochem 1988, 57:375–413
14. Kornblihtt AR, Umezawa K, Vibe-Pedersen K, Baralle FE: Primary structure of human fibronectin: Differential splicing may generate at least 10 polypeptides from a single gene. EMBO J 1985, 4:1755–1759
15. Skorstengaard K, Jensen MS, Sahl P, Petersen TE, Magnusson S: Complete primary structure of bovine plasma fibronectin. Eur J Biochem 1986, 161:441–453
16. Gutman A, Kornblihtt AR: Identification of a third region of cell-specific alternative splicing in human fibronectin mRNA. Proc Natl Acad Sci USA 1987, 84:7179–7182
17. Humphries MJ, Akiyama SK, Komoriya A, Olden K, Yamada KM: Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. J Cell Biol 1986, 103:2637–2647
18. Humphries MJ, Komoriya A, Akiyama SK, Olden K, Yamada KM: Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. J Biol Chem 1987, 262:6886–6892
19. Guan J-L, Hynes RO: Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha_4\beta_1$. Cell 1990, 60:53–61
20. Yannariello-Brown J, Werer U, Liotta LA, Madri JA: 1 Distribution of a 69-kD laminin-binding protein in aortic and microvascular endothelial cells: Modulation during cell attachment, spreading, and migration. J Cell Biol 988, 106:1773–1786

21. Form DM, Pratt BM, Madri JA: Endothelial cell proliferation during angiogenesis: In vitro modulation by basement membrane components. Lab Invest 1986, 55:521–530

22. Madri JA, Dreyer B, Pitlick FA, Furthmayr H: The collagenous components of the subendothelium. Lab Invest 1980, 43: 303–315

23. Fidler IJ: In vitro studies of cellular-mediated immunostimulation of tumor growth. J Natl Cancer Inst 1973, 50:1307–1312

24. Madri JA, Furthmayr H: Collagen polymorphism in the lung: An immunochemical study of pulmonary fibrosis. Hum Pathol 1980, 11:353–366

25. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB: Transforming growth factor-β in human platelets. J Biol Chem 1983, 258:7155–7180

26. Kocher O, Gabbiani G: Analysis of α-smooth-muscle actin mRNA expression in rat aortic smooth-muscle cells using a specific cDNA probe. Differentiation 1987, 34:201–209

27. Aviv H, Leder P: Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA 1972, 69:1408–1412

28. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GAP, Latt SA, Orkin SA: Human von Willebrand factor (vWF): Isolation of complementary DNA (cDNA) clones and chromosomal localization. Science 1985, 228:1401–1406

29. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, 1982

30. Southern E: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975, 98:503–515

31. Smith DB, Johnson KS: Single step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 1988, 67:31–40

32. Pratt BM, Harris AS, Morrow JS, Madri JA: Mechanism of cytoskeletal regulation: Modulation of aortic endothelial cell spectrin by the extracellular matrix. Am J Pathol 1984, 117: 349–354

33. Bernard MP, Kolbe M, Weil D, Chu M-L: Human cellular fibronectin: Comparison of the carboxyl-terminal portion with rat identifies primary structural domains separated by hypervariable regions. Biochemistry 1985, 24:2698–2705

34. Humphries MJ, Olden K, Yamada KM: A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science 1986, 233:467–470

35. Obara M, Kang MS, Yamada KM: Site-directed mutagenesis of the cell binding domain of human fibronectin: Separable, synergistic sites mediate adhesive function. Cell 1988, 53: 649–657

36. Humphries MJ, Akiyama SK, Komoriya A, Olden K, Yamada KM: Neurite extension of chicken peripheral nervous system neurons on fibronectin: Relative importance of specific adhesion sites in the central cell-binding domain and the alternatively spliced type III connecting segment. J Cell Biol 1988, 106:1289–1297

37. Madri JA, Kocher O, Merwin JR, Bell L, Yannariello-Brown J: The interactions of vascular cells with solid phase (matrix) and soluble factors. J Cardiovasc Pharm 1989, 14(Suppl 6): S70–S75

38. Heimark RL, Twardzik DR, Schwartz SM: Inhibition of endothelial regeneration by type-beta transforming growth factor from platelets. Science 1986, 233:1078–1080

39. Muller G, Behrens J, Nussbaumer U, Bohlen P, Birchmeier W: Inhibitory action of transforming growth factor β on endothelial cells. Proc Natl Acad Sci USA 1987, 84:5600–5604

40. Kornblihtt AR, Vibe-Pedersen K, Baralle FE: Human fibronectin: Molecular cloning evidence for two mRNA species differing by an internal segment coding for a structural domain. EMBO J 1984, 3:221–226

41. Kornblihtt AR, Vibe-Pedersen K, Baralle FE: Human fibronectin: Cell specific alternative splicing generates polypeptide chains differing in the number of internal repeats. Nucl Acids Res 1984, 12:5853–5868

42. Paul JI, Schwarzbauer JE, Tamkun, JW, Hynes RO: Cell-type-specific fibronectin subunits generated by alternative splicing. J Biol Chem 1986, 261:12258–12265

43. Schwarzbauer JE, Tamkun JW, Lemischka IR, Hynes RO: Three different fibronectin mRNAs arise by alternative splicing within the coding region. Cell 1983, 35:421–431

44. Schwarzbauer JE, Paul JI, Hynes RO: On the origin of species of fibronectin. Proc Natl Acad Sci USA 1985, 82:1424–1428

45. Schwarzbauer JE, Mulligan RC, Hynes RO: Efficient and stable expression of recombinant fibronectin polypeptides. Proc Natl Acad Sci USA 1987, 84:754–758

46. Schwarzbauer JE, Patel RS, Fonda D, Hynes RO: Multiple sites of alternative splicing of the rat fibronectin gene transcript. EMBO J 1987, 6:2573–2580

47. Tamkun JW, Schwarzbauer JE, Hynes RO: A single rat fibronectin gene generates three different mRNAs by alternative splicing of a complex exon. Proc Natl Acad Sci USA 1984, 81:5140–5144

48. Zardi L, Carnemolla B, Siri A, Petersen TE, Paolella G, Sebastio G, Baralle FE: Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. EMBO J 1987, 6:2337–2342

49. Sekiguchi K, Klos AM, Kurachi K, Yoshitake S, Hakomori S-I: Human liver fibronectin complementary cDNAs: Identification of two different messenger RNAs possibly encoding the α and β subunits of plasma fibronectin. Biochemistry 1986, 25:4936–4941

50. Pierschbacher MD, Ruoslahti E: Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature (London) 1984, 309:30–33

51. McCarthy JB, Hagen ST, Furcht LT: Human fibronectin contains distinct adhesion- and motility-promoting domains for metastatic melanoma cells. J Cell Biol 1986, 102:179–188

52. McCarthy JB, Skubitz APN, Zhao Q, Yi X-y, Mikelson DJ, Klein DJ, Furcht LT: RGD-independent cell adhesion to the carboxy-terminal heparin-binding fragment of fibronectin involves heparin-dependent and -independent activities. J Cell Biol 1990, 110:777–787

53. Hansson GK, Jonasson L, Seifert PS, Stemme S: Immune mechanisms in atherosclerosis. Arteriosclerosis 1989, 9:567–578

54. Hansson GK, Holm J, Jonasson L: Detection of activated T lymphocytes in the human atherosclerotic plaque. Amer J Pathol 1989, 135:169–175

55. Mould AP, Wheldon LA, Komoriya A, Wayner EA, Yamada KM, Humphries MJ: Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin $\alpha 4\beta 1$. J Biol Chem 1990, 265:4020–4024

56. Buck CA, Horwitz AF: Cell surface receptors for extracellular matrix molecules. Ann Rev Cell Biol 1987, 3:179–205

57. Vogel BE, Tarone G, Giancotti FG, Gailit J, Ruoslahti E: A novel fibronectin receptor with an unexpected subunit composition ($\alpha_v\beta_1$). J Biol Chem 1990, 265:5934–5937