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The Extracellular Matrix Ligands Fibronectin and Tenascin Collaborate in Regulating Collagenase Gene Expression in Fibroblasts

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Tenascin (TN) is a large oligomeric glycoprotein that is present transiently in the extracellular matrix (ECM) of cells and is involved in morphogenetic movements, tissue patterning, and tissue repair. It has multiple domains, both adhesive and anti-adhesive, that interact with cells and with fibronectin (FN) and other ECM macromolecules. We have studied the consequences of the interaction of TN with a FN matrix on gene expression in rabbit synovial fibroblasts. Fibroblasts plated on a mixed substrate of FN and TN, but not on FN alone, upregulated synthesis of four genes: collagenase, stromelysin, the 92-kDa gelatinase, and c-*fos*. Although the fibroblasts spread well on both FN and FN/TN substrates, nuclear c-Fos increased within 1 h only in cells that were plated on FN/TN. TN did not induce the expression of collagenase in cells plated on substrates of type I collagen or vitronectin (VN). Moreover, soluble TN added to cells adhering to a FN substrate or to serum proteins had no effect, suggesting that TN has an effect only in the context of mixed substrates of FN and TN. Collagenase increased within 4 h of plating on a FN/TN substrate and exhibited kinetics similar to those for induction of collagenase gene expression by signaling through the integrin FN receptor. Arg-Gly-Asp peptide ligands that recognize either the FN receptor or the VN receptor and function-perturbing anti-integrin monoclonal antibodies diminished the interaction of fibroblasts with a mixed substrate of FN, TN, and VN, but had no effect on the adhesion of fibroblasts to a substrate of FN and VN, suggesting that both receptors recognize the complex. Anti-TN68, an antibody that recognizes an epitope in the carboxyl-terminal type III repeats involved in the interaction of TN with both FN and cells, blocked the inductive effect of the FN/TN substrate, whereas anti-TNM1, an antibody that recognizes an epitope in the amino-terminal anti-adhesive region of epidermal growth factor-like repeats, had no effect. These data suggest that transient alteration of the composition of ECM by addition of proteins like TN may regulate the expression of genes involved in cell migration, tissue remodeling, and tissue invasion, in regions of tissue undergoing phenotypic changes.

INTRODUCTION

Tenascin (TN)[1] is a large oligomeric glycoprotein in the extracellular matrix (ECM) that is thought to play a role in the morphogenetic movement of cells and the patterning of tissues during development. The structure of

[1] Abbreviations used: BSA, bovine serum albumin; CM, conditioned medium; DME, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; FN, fibronectin; FNR, fibronectin receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDLR, low-density-lipoprotein receptor; LH, lactalbumin hydrolysate; mAb, monoclonal antibody; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RGD, Arg-Gly-Asp; RSF, rabbit synovial fibroblasts; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TN, tenascin; TPA, 12-O-tetradecanoylphorbol-13-acetate; VN, vitronectin; VNR, vitronectin receptor; 120FN, 120-kDa fragment of fibronectin.

TN (also called GMEM, cytotactin, hexabrachion, or J1) (reviewed by Erickson and Bourdon, 1989) is determined by a linear array of four motifs: a cysteine-rich, amino-terminal domain that plays a role in TN arm association; a number of epidermal growth factor (EGF)-like repeats; a series of fibronectin (FN) type III repeats, the number of which is determined by alternative splicing; and a carboxyl-terminal fibrinogen-like globular domain. The cDNA sequence of chicken, mouse, and human TN has shown that TN isoforms are a result of alternative splicing of FN type III repeats (Spring *et al.*, 1989; Nies *et al.*, 1991; Nishi *et al.*, 1991; Siri *et al.*, 1991; Weller *et al.*, 1991). Although the number of alternatively spliced type III repeats varies with species, the type III repeats in similar positions share considerable homology (Gulcher *et al.*, 1991).

In vitro, TN binds to immobilized FN (Chiquet-Ehrismann *et al.*, 1991) and to cell surface proteoglycans (Salmivirta *et al.*, 1991). There is some tissue specificity in the interaction of heparan sulfate proteoglycans and TN, because the integral membrane heparan sulfate proteoglycan syndecan, isolated from toothbud extracts, interacts with immobilized TN in cell-free binding assays, whereas preparations of syndecan isolated from mammary gland do not (Salmivirta *et al.*, 1991). The carbohydrate side chains of heparan sulfate and chondroitin sulfate proteoglycans play a role in the interaction with TN (Murphy-Ullrich *et al.*, 1991; Salmivirta *et al.*, 1991); however, the core protein of cytotactin-binding proteoglycan, which contains chondroitin sulfate proteoglycan, mediates the interaction with TN (Hoffman *et al.*, 1988).

TN expression is seen in two types of tissues. The smallest isoform of TN is expressed in regions of dense connective tissue such as gizzard or myotendinous junctions, where tensile strength is important (Chiquet-Ehrismann *et al.*, 1991). In both adult and embryonic tissues the expression of TN is also seen in regions of tissue in which ECM remodeling, cell division, and cell migrations take place. The temporal and spatial distribution of TN expression in the early embryo (Chiquet-Ehrismann *et al.*, 1986; Prieto *et al.*, 1990), coupled with the observations that TN diminishes cell adhesion and migration (Chiquet-Ehrismann *et al.*, 1986; Friedlander *et al.*, 1988; Halfter *et al.*, 1989; Lotz *et al.*, 1989; Riou *et al.*, 1990), suggest that TN is an important molecule used in directed migrations early in development. The transient expression of specific TN isoforms in the developing nervous system suggests that TN has important functions here as well (Steindler *et al.*, 1989; Prieto *et al.*, 1990). TN may also play a role in condensing tissue, because the expression of TN is upregulated during epithelial mesenchymal induction (Ekblom and Aufderheide, 1989; Salmivirta *et al.*, 1991); the distribution of TN in condensing tissues may also change, because TN localization shifts from a diffuse presence to a perichondral localization during cartilage condensation (Chiquet-Ehrismann *et al.*, 1986). In adults, the expression of TN is more limited; it is present in regions of continuous tissue renewal, such as in the intestinal crypts (Weller *et al.*, 1991), and is strikingly induced in the dermis beneath a wound epithelium after tissue injury (Mackie *et al.*, 1988; Whitby *et al.*, 1991). The transient expression of TN in discrete developmental patterns and its upregulation in regions of repairing tissues suggest an important role for TN in remodeling tissue. However, the contributions of a TN-supplemented matrix to a remodeling cascade may be functionally duplicated by other interactions, because TN-null mice develop normally, with no obvious abnormalities (Saga *et al.*, 1992).

The function of TN in remodeling tissue has not yet been clearly defined. However, a common response of fibroblasts, epithelial cells, neurons, and glia cultured in the presence of TN is a diminution of cell adhesion (for review see Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991). This destabilization of cell adhesion to the ECM could enhance cell motility as suggested by Tucker and McKay (1991). Alternatively, the addition of TN to an ECM could change the structure of the mesenchymal matrix as suggested by Whitby *et al.* (1991) and Ekblom and Aufderheide (1989). Finally, the presence of TN in ECM could directly or indirectly signal cells to alter their expression of genes involved in ECM remodeling. This possibility is supported by similar patterns of distribution of TN and ECM-degrading matrix metalloproteinases (MMPs) in repairing and remodeling tissue (reviewed by Alexander and Werb, 1991). The interaction of FN and TN molecules is clearly documented (Chiquet-Ehrismann *et al.*, 1991), and other studies demonstrate that TN inhibits or perturbs adhesion of cells to intact FN and FN fragments that contain the classical Arg-Gly-Asp (RGD) sequence (Chiquet-Ehrismann *et al.*, 1988; Lotz *et al.*, 1989). In fibroblasts, MMP expression is upregulated in response to stimuli that subtly perturb cell adhesion or alter the actin cytoskeleton, as in cells treated with anti-fibronectin receptor (FNR) antibodies or plated on the 120-kDa chymotryptic fragment of FN (120FN) (Aggeler *et al.*, 1984; Unemori and Werb, 1986; Werb *et al.*, 1989). We therefore investigated whether perturbations in cell behavior generated by addition of TN to a substrate of FN regulate cellular gene expression. As our model system, we chose rabbit fibroblasts, which respond to FN fragments or anti-FNR antibodies by induction of MMP expression, and we investigated the regulation of collagenase as a model gene.

MATERIALS AND METHODS

Cells and Cell Culture

Rabbit synovial fibroblasts (RSF), isolated as described previously (Aggeler *et al.*, 1984), were cultured in Dulbecco's modified Eagle's medium (DME) (Cell Culture Facility, University of California, San

Francisco) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Denver, CO). RSF were used between passages 2 and 10. Early-passage fibroblasts in culture often synthesize and secrete TN into the culture medium. However, immunoblotting of the conditioned medium (CM) harvested from the RSF used in these experiments did not detect TN. RSF were subcultured 48 h before experimental procedures. In experiments, cells were added to ECM-coated wells at a density of 1–2 × 10^5 in DME supplemented with 0.2% lactalbumin hydrolysate (DME-LH); the plates were rotated to ensure even suspension of cells and incubated further at 37°C before analysis of MMP expression.

Antibodies

The anti-collagenase monoclonal antibodies (mAbs) were characterized and used as described (Werb *et al.*, 1989). The anti-stromelysin mAb (SL188.2) was a generous gift of Dr. Scott Wilhelm, Miles Research (West Haven, CT) (Wilhelm *et al.*, 1992). The anti-Fos antibody and matching peptide immunogen were purchased from CRB Biologics (Cambridge, England). Adsorption of the anti-Fos antibody with the peptide immunogen was performed as described by the manufacturer. The anti-TNM1 mAb was first described by Chiquet and Fambrough (1984), and the polyclonal anti-TN antibodies and anti-TN68 mAb were prepared and described by Spring *et al.* (1989). The anti-FNR mAb, BIIG2, was a generous gift of Dr. Caroline Damsky, University of California, San Francisco. The anti-vitronectin receptor (VNR) mAb, LM609, was kindly provided by Dr. David Cheresh, Scripps Clinic and Research Foundation (La Jolla, CA). The anti-$\alpha_2\beta_1$ mAb, P1E6, and the anti-$\alpha_3\beta_1$ mAb, P1B5, were purchased from GIBCO/BRL (Gaithersburg, MD). The mAb directed against the rabbit low-density-lipoprotein receptor (LDLR) was a generous gift of Dr. Thomas Innerarity, Gladstone Foundation Laboratories (San Francisco, CA). The biotinylated and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma (St. Louis, MO), and the Texas Red-labeled streptavidin was purchased from Amersham (Arlington Heights, IL).

Preparation of ECM Substrates

Human plasma FN was purchased from Collaborative Research (Waltham, MA) or Boehringer Mannheim Biochemical (Indianapolis, IN), reconstituted as directed by the manufacturer, and frozen at −70°C in single-use aliquots. The 120-kDa chymotryptic fragment of human plasma FN (120FN) was purchased from Telios Pharmaceuticals (La Jolla, CA), reconstituted as directed, and stored in single-use aliquots at −70°C. Chicken TN was purified as described previously (Chiquet-Ehrismann *et al.*, 1991). The anti-TN mAbs TNM1 and TN68 and anti-TN polyclonal antibodies were used as described by Spring *et al.* (1989). For coating of wells, 24- or 48-well culture dishes (Costar, Cambridge, MA) were incubated with 0.1 or 0.2 ml of 30 μg/ml FN or 120FN in phosphate-buffered saline (PBS) overnight (9–15 h) at 4°C. They were then washed three times with PBS and incubated in 0.2% bovine serum albumin (BSA) in PBS for 30 min at ambient temperature to reduce nonspecific binding to the tissue culture dish. The wells were then washed three times with PBS and used immediately. Mixed substrates of FN and TN were prepared by sequential incubation with ECM proteins in a slight modification of the procedure described by Chiquet-Ehrismann *et al.* (1991). Except where noted otherwise, wells were incubated overnight (9–15 h) at 4°C with 30 μg/ml FN in PBS (in one series of experiments, wells were coated with a solution of 30 μg/ml FN and 10 μg/ml vitronectin [VN] as described for FN alone), washed three times with PBS, blocked with a solution of 0.2% BSA in PBS to prevent nonspecific adhesion to the tissue culture dish, then incubated at ambient temperature for 3 h with 0.1–0.2 ml of a 5–10 μg/ml solution of TN in PBS. This solution was aspirated, and the wells were washed with PBS and used immediately. In some experiments, an additional 45-min incubation with anti-TN mAb (50 μg/well) at ambient temperature was included before the final wash with PBS. Type I collagen-coated wells were prepared by incubating wells in 48-well plates overnight at 4°C with a solution of 50 μg/ml pepsin-treated bovine skin collagen (Vitrogen, Collagen, Palo Alto, CA) in distilled water then washed with PBS, and uncoated sites were blocked with 0.2% BSA in PBS. The blocking solution was aspirated, the wells were washed three times with PBS, and 0.1–0.2 ml of 5–10 μg/ml TN in DME-LH was added to the wells before the addition of cells. Wells were coated with bovine VN or mixtures of VN and TN as described for type I collagen-coated wells, except that wells were incubated overnight with a solution of 10 μg/ml VN (Telios Pharmaceuticals) in PBS.

In several experiments RSF in suspension were preincubated with mAb directed against the $\alpha_5\beta_1$ FNR or the $\alpha_v\beta_3$ VNR or with RGD peptide ligands that discriminate between the FNR and the VNR. The RGD peptides were purchased from GIBCO/BRL, reconstituted as directed by the manufacturer, and used at 0.1, 0.5, or 1 mM. Anti-FNR, anti-VNR, or control anti-LDLR mAbs were used at a final concentration of 25 μg/ml. Because crosslinking of FNR with bivalent anti-FNR IgG induces the expression of collagenase in RSF, anti-FNR Fab fragments were used in place of bivalent anti-FNR antibodies (Werb *et al.*, 1989). Incubation of RSF with intact anti-VNR IgG did not induce the expression of collagenase. Antibodies or peptides were added to RSF suspended in DME-LH and incubated at ambient temperature for 40 min; aliquots of this mixture were then plated on ECM-coated wells.

In another series of experiments, cell surface heparan sulfate proteoglycans were removed from RSF by incubation with heparinase III (Sigma). Two sets of adherent RSF cultures were incubated at 37°C for 2 h in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline containing 2 mM $CaCl_2$ and 5 units/ml heparinase III. One pair of heparinase III-treated and control cultures were then subcultured and plated on ECM-coated wells; whereas the other pair of cultures were washed several times with PBS and incubated further in DME-LH.

Biosynthetic Labeling of Proteins Secreted by RSF

RSF were added to ECM-coated wells and cultured in DME-LH for up to 48 h, after which the CM was removed and saved for later analysis. Cultures of RSF were biosynthetically labeled by incubation with 50–70 μCi/ml [^{35}S]methionine (Express Label, New England Nuclear, Boston, MA) for 3–4 h in methionine-free DME (GIBCO/BRL). Radiolabeled secreted proteins were precipitated from the CM with quinine sulfate and sodium dodecyl sulfate (SDS) or by immunoprecipitation with specific antibodies as described previously (Werb *et al.*, 1989). Precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography (En^{3}Hance, New England Nuclear). Radiolabeled collagenase secreted into the culture supernatants was quantified by scanning autoradiograms with an LKB densitometer and GSXL software (LKB-Pharmacia, Piscataway, NJ).

Analysis of Specific Proteinases

Proteinases in the CM harvested from cell cultures were analyzed by gelatin zymography as described previously (Werb *et al.*, 1989). CM was separated on nonreducing 10% polyacrylamide gels that contained 0.1% gelatin. After electrophoresis the gel was soaked in 2.5% Triton X-100 to remove SDS and incubated for 18 h in substrate buffer (50 mM tris(hydroxymethyl)aminomethane [Tris]-HCl pH 7.5, 5 mM $CaCl_2$). The gels were stained with Coomassie Blue R250 (BioRad, Richmond, CA). Gelatinases appear as a clear zone on a blue background; in reverse prints they appear as a dark band on a clear background.

Alternatively, samples of CM were separated by denaturing SDS-PAGE, and the proteins were transferred to membranes (ImmobilonP, New England Nuclear) (Harlow and Lane, 1989). Nonspecific sites on the membranes were blocked with a solution of 3% BSA in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl), after which the membranes were incubated with anti-collagenase or anti-stromelysin mAb in TBS containing 0.5% Tween-20 (Sigma) for 1–2 h at ambient temperature. Before and after incubation with horseradish peroxidase-conjugated

anti-mouse IgG (Sigma), the membranes were washed three times for 20 min with 0.5% Tween-20 in TBS. Specific bands were detected by enhanced chemiluminescence (Amersham) as described by the manufacturer. For better quantification of collagenase in the CM, serial dilutions of the CM were applied to nitrocellulose membranes by using a slot-blot filtration manifold (Schleicher and Schuell, Keene, NH), and the expression of collagenase was determined by immunoblotting as described above.

RNA Isolation and Analysis

Total cellular RNA was isolated from cultured RSF, and 1-μg aliquots were analyzed by reverse transcription (RT) followed by amplification of specific sequences in the polymerase chain reaction (PCR) as described by Rappolee *et al.* (1989). Synthetic primers used to amplify collagenase cDNA sequences were selected from regions of identity in the rabbit and human cDNA sequences as described previously (Brenner *et al.*, 1989). The collagenase primer pair represented the nucleotides 1154–1174 and 1433–1453 in the rabbit collagenase cDNA and amplified a 300-basepair (bp) fragment of the transcribed cDNA. Amplifications with this primer were performed with 4.0 mM $MgCl_2$ at an annealing temperature of 60°C. Synthetic primers used to amplify cDNA sequences coding for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) span the sequence 3308–3337 and 3649–3672 in the rat cDNA and produce a 241-bp fragment, as described by Rappolee *et al.* (1992). Reactions with this primer were performed with 4.0 mM $MgCl_2$ at an annealing temperature of 60°C. For amplification of the collagenase mRNA, the cDNA was derived from RT of 1 μg RNA from cells plated on FN, 120FN, or FN/TN using random hexamers (Pharmacia, Piscataway, NJ) to prime the first strand synthesis; the RT mixture was diluted to 10^{-3}, 10^{-4}, 10^{-5}, and 5×10^{-6} in the PCR. For amplification of GAPDH mRNA, the cDNA was derived by RT of 1 μg RNA as described above, from cells plated on FN, 120FN, or FN/TN, and the RT mixture was diluted to 5×10^{-2}, 10^{-2}, 10^{-3}, and 5×10^{-4} in the PCR. Control samples contained no template. The products were separated on agarose gels and detected by staining with ethidium bromide. We used scanning densitometry (LKB-Pharmacia) to quantify the level of collagenase and GAPDH products in amplifications from dilutions of RT mixture from RNA harvested from cells plated on FN, 120FN, or FN/TN. The densitometer readings were plotted against the dilutions of the RT mix in the amplification; estimates of concentration were made on the linear portion of the graph.

Immunofluorescence

In experiments analyzing collagenase expression, cells were plated on acid-washed glass coverslips coated with ECM proteins as described above. At the indicated time after plating, coverslips were rinsed in PBS, and cells were fixed for 5 min in 2% paraformaldehyde in PBS. After fixation, cultures were made permeable by incubation in 0.25% Triton X-100 in PBS at ambient temperature for 2 min. Coverslips were rinsed in PBS and blocked for several hours with a solution of 1% BSA in PBS before a 1 h incubation with a cocktail of five mAbs against rabbit collagenase (Werb *et al.*, 1989). Cells were washed, incubated for 1 h with biotinylated goat anti-mouse IgG, rinsed, and incubated for 1 h with Texas Red-streptavidin. The cells were photographed on a Zeiss photomicroscope II (Thornwood, NJ) with phase-contrast and epifluorescence and 25× or 63× water immersion lenses.

The coverslips that were stained with anti-Fos antibodies were fixed with ice-cold 4% paraformaldehyde in PBS and incubated on ice for 30 min then made permeable at ambient temperature with 0.25% Triton X-100 in PBS for 5 min. Nonspecific sites were blocked by incubating coverslip cultures for at least 3 h with 0.2% BSA in PBS. Cells were then incubated with the anti-Fos antibody for 1 h at ambient temperature followed by 10 h at 4°C. Coverslips were washed and then incubated with biotinylated anti-rabbit IgG, washed again with PBS, then incubated with Texas Red-streptavidin. The cells were photographed as described above.

RESULTS

Expression of Secreted Proteins Is Altered in Cells Plated on a Substrate of FN and TN

In previous studies we found that secreted proteins are sensitive indicators of altered phenotype in a variety of cell types, including fibroblasts (Aggeler *et al.*, 1984; Werb *et al.*, 1989; Tremble *et al.*, 1993). The expression of collagenase closely correlates with treatments that alter or disassemble the organized actin cytoskeleton (reviewed by Frisch *et al.*, 1989). These perturbations can be extreme, such as that caused by incubating cells with cytochalasins, or subtle, such as that initiated by incubation of fibroblasts with growth factors or plating on 120FN. Because the addition of TN has been shown to perturb the interaction of cells with FN matrices (Chiquet-Ehrismann *et al.*, 1988; Lotz *et al.*, 1989) and to affect the organization of the actin cytoskeleton in cultured cells (Murphy-Ullrich *et al.*, 1991), we wondered if the addition of TN to a FN matrix would, like 120FN, induce RSF to upregulate the expression of the MMPs. Accordingly, we plated RSF on a substrate of FN, 120FN, or a mixture of FN and TN (FN/TN) and cultured the cells for 24 h. The cultures were then incubated with [^{35}S]methionine for 4 h, and the radiolabeled secreted proteins were analyzed. We noted the induction by FN/TN of proteins migrating near 55 kDa that are characteristic of the proenzyme forms of the MMPs collagenase and stromelysin and that are increased in fibroblasts plated on a substrate of 120FN but not on intact FN (Werb *et al.*, 1989) (Figure 1). Collagenase was specifically immunoprecipitated from the CM harvested from these cultures with an anti-collagenase mAb (Figure 2A). By scanning densitometry, we saw a 2.2-fold increase (±0.5 SD, n = 6, $p < 0.005$, Student's t-test) in collagenase protein secreted by cells

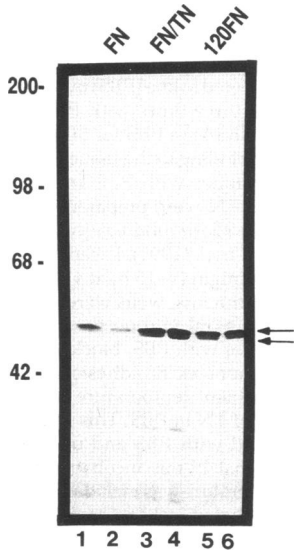

Figure 1. Analysis of proteins secreted by RSF plated on various matrices. Fibroblasts were plated in DME-LH in duplicate wells that were coated with FN (lanes 1 and 2), a mixed substrate of FN/TN (lanes 3 and 4), or on a substrate 120FN (lanes 5 and 6) and incubated for 24 h. The secreted proteins were then biosynthetically labeled by incubating cells with [^{35}S]methionine for 4 h. The radiolabeled secreted proteins were concentrated with quinine sulfate and SDS, and the precipitates were analyzed by SDS-PAGE and fluorography. Procollagenase (CL) migrating at 53 and 57 kDa is indicated by arrows. Molecular weight ($\times 10^{-3}$) markers are indicated at the left.

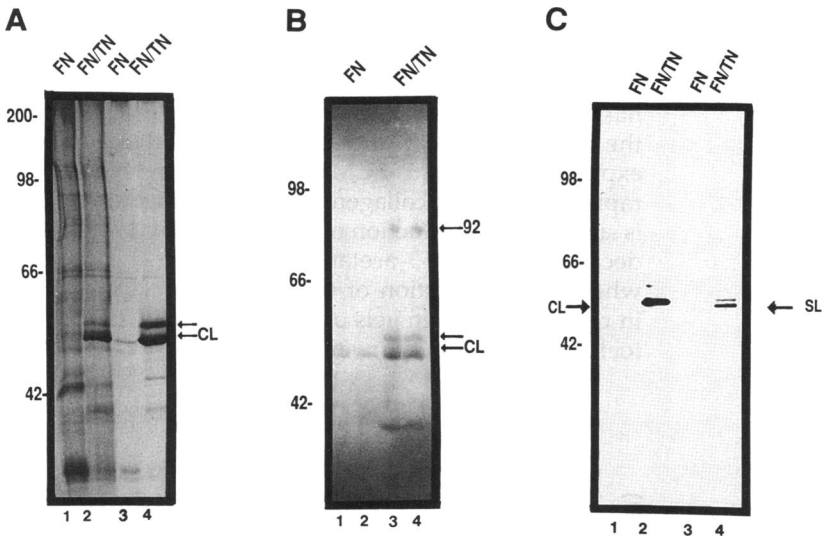

Figure 2. Collagenase and stromelysin are upregulated in cells plated on a mixed substrate of FN/TN. Freshly trypsinized RSF were plated in DME-LH in wells that had been coated with FN or FN/TN and incubated for 24 h. The CM was removed and saved for later analysis, and the cultures were incubated with [^{35}S]methionine for 2 h. (A) Samples of CM containing radiolabeled secreted proteins from cultures plated on FN (lanes 1 and 3) or FN/TN (lanes 2 and 4) were concentrated with quinine sulfate and SDS to show total proteins secreted into the medium (lanes 1 and 2), or proteins secreted into the medium were immunoprecipitated with an anti-collagenase mAb (lanes 3 and 4), and the precipitates were analyzed by SDS-PAGE and fluorography. The doublet of procollagenase (CL) is indicated. (B) The proteinase content of the 40-h CM harvested from cells plated in wells coated with FN or FN/TN was analyzed by gelatin zymography as described in MATERIALS AND METHODS. Proteolytic degradation of gelatin, printed as a negative image, appears as dark bands. Note the induction of gelatinases migrating at 92, 57, and 53 kDa, corresponding to the proenzyme forms of the 92-kDa gelatinase and collagenase. (C) CM harvested from cells plated on FN (lanes 1 and 3) or FN/TN (lanes 2 and 4) was separated by SDS-PAGE under denaturing conditions, and the proteins were transferred to membranes and analyzed by immunoblotting with anti-collagenase (lanes 1 and 2) and anti-stromelysin (SL) (lanes 3 and 4) mAbs. Procollagenase (CL) and prostromelysin (SL) are indicated by arrows. Molecular weight ($\times 10^{-3}$) markers are indicated at the left.

plated on FN/TN when compared with cultures plated on FN alone; pooled data from six experiments, in which two different preparations of TN and RSF cultures derived from several different rabbits, were used.

To characterize the induced secreted proteins further, we analyzed the CM by zymography on gelatin substrate gels. The expression of gelatin-degrading proteinases migrating at 92 kDa and 55 kDa increased in cultures plated on FN/TN (Figure 2B). These gelatinases are the proenzyme forms of the 92-kDa gelatinase (also called type IV collagenase or gelatinase B) and interstitial collagenase, respectively. Immunoblotting procedures confirmed that the expression of collagenase and stromelysin proteins increased in cells plated on FN/TN when compared with cells plated on FN (Figure 2C). In subsequent experiments we used collagenase as a model gene to investigate the mechanisms that underlie the effects of a FN/TN substrate.

The increase in the expression of collagenase protein resulted from an increase in collagenase mRNA. Collagenase mRNA increased fivefold in cells plated on a FN/TN substrate and eightfold in cells plated on 120FN, when compared with cells plated on intact FN (Figure 3). There was a twofold increase in GAPDH mRNA harvested from cells plated on 120FN or FN/TN. Comparing collagenase mRNA with GAPDH mRNA, we saw a net 2.5-fold increase in collagenase expression in cells plated on FN/TN and a fourfold increase in cells plated on 120FN.

We next wished to determine if TN, in the context of ECM proteins other than FN, would alter the pattern of collagenase expression in RSF. We plated cells in DME-LH in wells coated with FN or a mixed substrate of FN/TN or in wells coated with VN or type I collagen, alone, or in the presence of TN. TN did not upregulate the expression of collagenase in cells plated on type I collagen or VN (Figure 4). Moreover, TN did not alter the pattern of collagenase expression when added as a soluble supplement (30 μg/ml) either to fibroblasts in DME-LH adhering to a FN matrix or to fibroblasts in DME-FBS and adhering to serum proteins. In contrast, the expression of collagenase was upregulated in RSF plated on a mixed substrate of FN/TN, prepared by incubating FN-coated wells with solutions of TN that ranged in concentration from 3 μg/ml to 30 μg/ml, compared with RSF plated on FN alone. We were unable to analyze the expression of collagenase in cells plated on TN alone, because they adhered poorly and had a rounded morphology. Because a change in cell shape alone induces collagenase and stromelysin in RSF (Aggeler *et al.*, 1984; Unemori and Werb, 1986), it was not possible to distinguish between a specific effect of TN and the nonspecific effect due to morphologic alteration. We conclude that the effects of TN on the regulation of collagenase in RSF are specific to mixed substrates of FN/TN.

The addition of TN to a FN matrix changes the parameters of the interaction of cultured cells with FN.

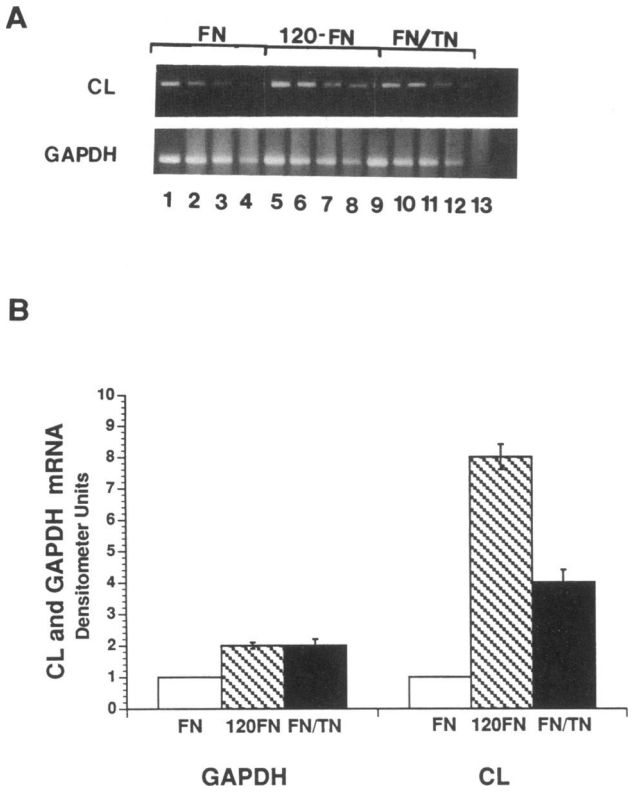

Figure 3. The expression of collagenase mRNA is increased in cells plated on FN/TN. RSF were plated in DME-LH on wells coated with FN, 120FN, or FN/TN and cultured for 30 h. Total cellular RNA was isolated and analyzed by RT-PCR. (A) Sequences in cDNA were amplified with specific primers for collagenase (CL) cDNA and GAPDH cDNA to yield a 300- and a 241-bp fragment, respectively. The products were separated on agarose gels and stained with ethidium bromide. For amplification of the collagenase mRNA the cDNA derived from RT of 1 μg RNA from cells plated on FN (lanes 1–4), 120FN (lanes 5–8), or FN/TN (lanes 9–12) was diluted to 10^{-3}, 10^{-4}, 10^{-5}, and 5×10^{-6}. For amplification of GAPDH mRNA the cDNA derived by RT of 1 μg RNA from cells plated on FN (lanes 1–4), 120FN (lanes 5–8), and FN/TN (lanes 9–12) was diluted to 5×10^{-2}, 10^{-2}, 10^{-3}, and 5×10^{-4}. Lane 13 contains no template. (B) The data were quantified by densitometry and expressed as -fold induction compared with cultures plated on FN.

Although cells attach to the FN/TN matrix, the stabilization or strengthening of adhesion that occurs on a FN matrix is not observed (Lotz *et al.*, 1989). In our experiments RSF attached and spread both on FN matrices and on mixed substrates of FN/TN. Phase-contrast microscopy revealed no differences between them. At 1 and 4.5 h after plating cells on FN/TN, the actin cytoskeletal network, visualized by staining with rhodamine phalloidin, was similar to, although less well established than, that seen in cells plated on FN (Figure 5), and it resembled the actin cytoskeleton in cells plated on 120FN.

Collagenase Expression Increases Within 4 h After Plating on a Substrate of FN/TN

The kinetics of the regulation of collagenase expression has proved to be an important first clue in describing the mechanism that underlies the regulation of gene expression by various ECM substrates. For example, the rapid induction of collagenase in cells plated on 120FN is similar to the induction of collagenase by 12-O-tetradecanoylphorbol-13-acetate (TPA) (Werb *et al.*, 1989), whereas the induction of collagenase in cells cultured in retracted collagen gels or treated with SPARC or cytochalasins is much slower (Unemori and Werb, 1986;

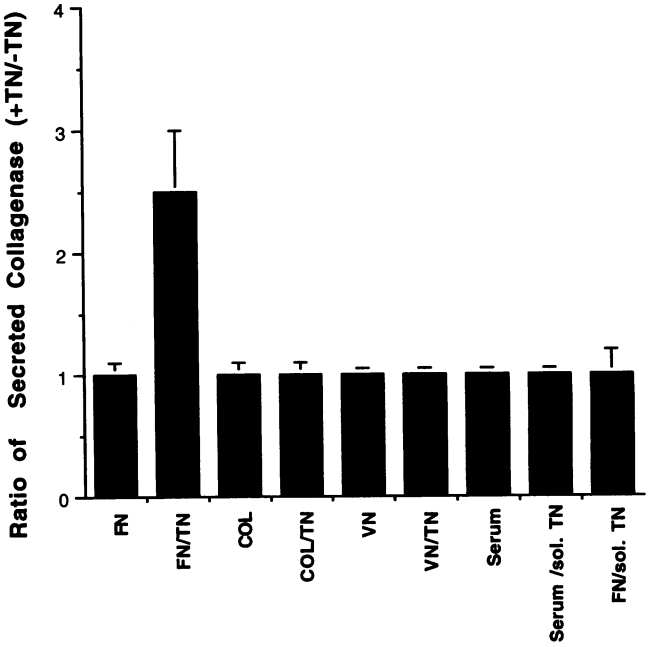

Figure 4. Collagenase expression in cells plated on mixed substrates of collagen and TN, VN and TN, or treated with soluble TN does not differ from that in untreated cultures. Freshly trypsinized RSF were plated in DME-LH in wells coated with FN, FN/TN, collagen type I (COL), collagen type I and TN (COL/TN), VN, or VN and TN (VN/TN). Adherent cells, plated on uncoated wells in DME-10% FBS for 6 h, were washed with DME-LH and further incubated in DME-LH (Serum) or in DME-LH supplemented with 80 μg/ml TN (Serum/sol.TN). RSF were also cultured for 1 h in DME-LH on wells coated with FN and further supplemented with 80 μg/ml TN (FN/sol.TN). The cultures were incubated with [^{35}S]methionine after 30 h in culture, and the radiolabeled secreted proteins were concentrated and analyzed by SDS-PAGE and autoradiography. Collagenase expression was measured by scanning densitometry of the autoradiograms, and the data were compared as the ratio of the amount of collagenase secreted by cultures incubated with TN, to the amount of collagenase secreted by cultures on the same matrix substrate in the absence of TN, which in the graph was normalized to 1. The data for FN, FN/TN, collagen, and collagen/TN are expressed as the range from two separate experiments, each performed in duplicate (range ± SD), and the data for VN, VN/TN, Serum, Serum/sol.TN, and FN/sol.TN are the average of duplicates (±SD) in one experiment. Bars indicate SD.

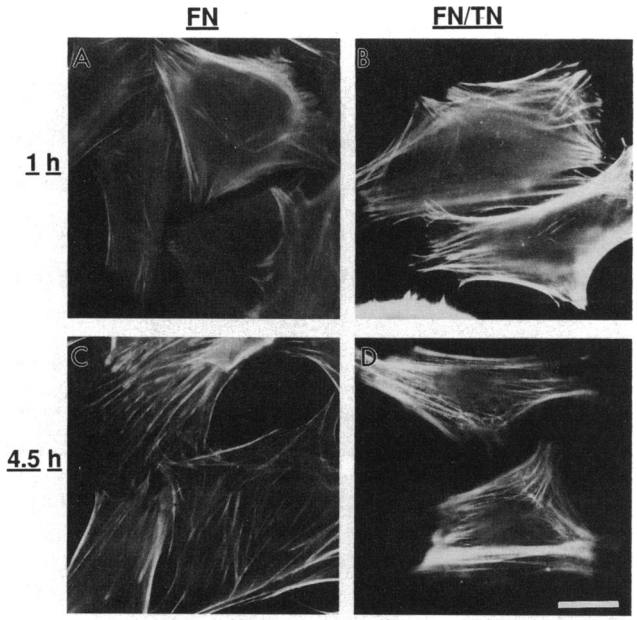

Figure 5. RSF attach and spread on a FN/TN matrix. Freshly trypsinized RSF were plated in DME-LH on coverslips that were coated with FN (A and C) or FN/TN (B and D). At 1 h or 4.5 h after plating, the cultures were fixed, permeabilized, and stained with rhodamine-phalloidin. Bar, 20 μm.

Tremble *et al.*, 1993). We cultured RSF on coverslips coated with FN, 120FN, or FN/TN in DME-LH for 4, 8, or 24 h and then fixed and processed them for immunofluorescent staining of intracellular collagenase. The number of cells expressing collagenase increased by 4 h in cells plated on 120FN or FN/TN and continued to increase for 20 h (Figure 6, A and B). The rapid increase in collagenase expression, detected by immunocytochemistry, in cells plated on a FN/TN substrate paralleled the induction of collagenase that was measured by analysis of [^{35}S]methionine-labeled collagenase secreted by cells plated on 120FN and was similar to the induction of collagenase in cells plated on anti-FNR mAb or with TPA (Werb *et al.*, 1989).

c-Fos Is Expressed in Cells Plated on a Substrate of FN/TN

Induction of collagenase has been linked to binding of AP-1 (which consists of heterodimers of the inducible nuclear proteins c-Fos and c-Jun or homodimers of Jun family members) to the TPA response element in the collagenase promoter. In cells treated with TPA, increases in both the expression and the activation of Fos and Jun family members precede the upregulation of collagenase by phorbol esters (Angel *et al.*, 1988; reviewed by Angel and Karin, 1992). The expression of recorder constructs that contain segments of the colla-

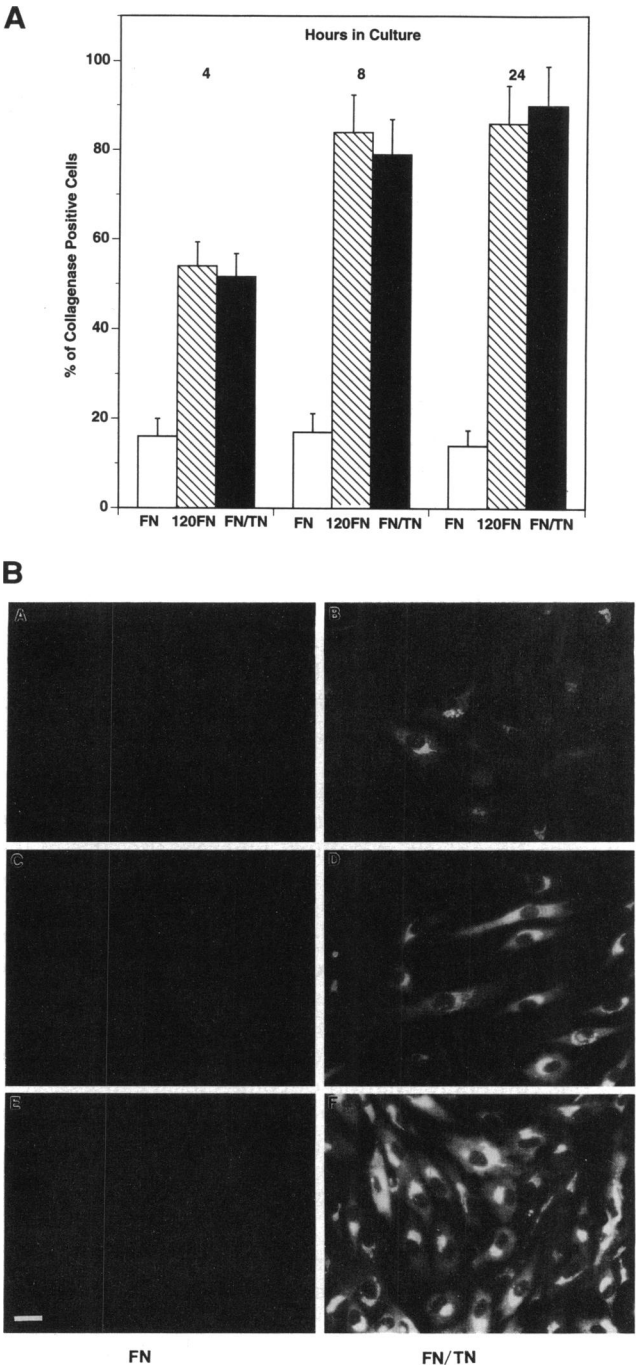

Figure 6. The increase in collagenase expression in cultures plated on FN/TN parallels that is seen in cells plated on 120FN. Freshly trypsinized RSF were plated in DME-LH on glass coverslips coated with FN, 120FN, or FN followed by TN, and cultured for 4, 8, or 24 h. The cultures were fixed and permeabilized, and the intracellular collagenase was detected with an anti-collagenase mAb. (A) The percentage of cells staining for intracellular collagenase was determined by counting ≥50 cells in five microscopic fields and recording the number of cells expressing collagenase. Bars indicate SD. (B) Collagenase expression in cells plated on FN (A, C, and E) or FN/TN (B, D, and F) for 4 h (A and B), 8 h (C and D), or 24 h (E and F). Bar, 20 μm.

genase promoter including the TPA response element is upregulated in cells plated on 120FN when compared with FN (Tremble, Damsky, and Werb, unpublished data) and in cells treated with TPA (Angel *et al.*, 1988; Gutman and Wasylyk, 1990; Auble and Brinckerhoff, 1991). We used immunocytochemistry with an antibody raised against a c-Fos peptide sequence to show a nuclear localization of c-Fos protein in cells plated on a substrate of FN/TN (Figure 7). Expression of c-Fos was scored by recording the number of cells that were stained for nuclear c-Fos in at least four microscopic fields in two experiments. Nuclear c-Fos expression was seen in 91.5% (±9.8% SD) of cells within 1 h of plating on FN/TN, but only in 20% (±16.3% SD) of cells plated on FN. No staining was detected in cells that were incubated with a mixture of anti-Fos antibody and an excess of the peptide immunogen.

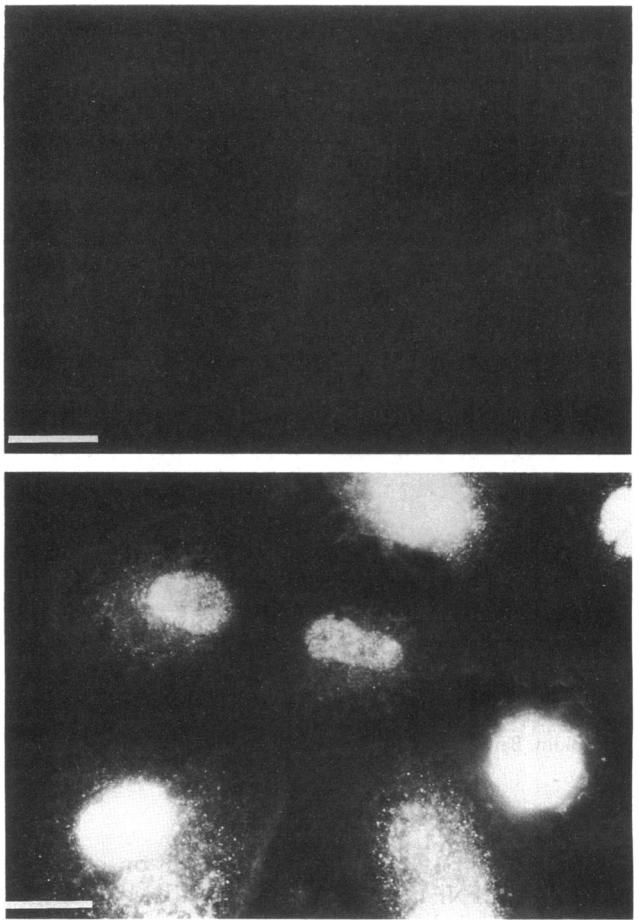

Figure 7. Nuclear Fos is increased in cells plated on FN/TN. RSF were plated in DME-LH on coverslips coated with FN (top) or FN/TN (bottom) and cultured for 1 h. The coverslips were then fixed and stained with an anti-peptide antibody raised against a peptide sequence in c-Fos as described in MATERIALS AND METHODS. As a control, cells were stained with antibody in the presence of an excess of peptide, and these showed no immunofluorescent signal (not shown). Bar, 20 μm.

The Distal Type III Repeats of TN Upregulate Collagenase Expression in RSF Plated on FN/TN

TN contains three defined protein structural domains—EGF-like repeats, FN type III repeats, and a fibrinogen globular domain—that have been well characterized in other proteins (Spring *et al.*, 1989; reviewed by Engel, 1991). Spring *et al.* (1989) defined both adhesive and anti-adhesive regions in the TN arm and showed that an epitope in the EGF-like repeats has an anti-adhesive effect on fibroblasts in culture, whereas the sites in the TN arm that interact with cells map to regions in the type III repeats (Spring *et al.*, 1989; Lochter *et al.*, 1991; Murphy-Ullrich *et al.*, 1991; Zisch *et al.*, 1992). The type III repeats of the TN arm not only are important for the interaction of TN with cells, but also modify the interaction of TN with FN (Chiquet *et al.*, 1991; Chiquet-Ehrismann *et al.*, 1991). To determine which of these regions confers regulation of collagenase expression in RSF plated on FN/TN, we used two anti-TN mAbs, anti-TNM1, which recognizes an epitope in the EGF-like repeats of the TN arm, and anti-TN68, which recognizes an epitope in the FN type III repeats (Spring *et al.*, 1989).

We plated cells on substrates of FN, FN/TN, or FN/TN that had been incubated with the mAbs anti-TNM1, anti-TN68, or a mixture of both anti-TNM1 and anti-TN68, and cultured them in DME-LH for 24 h. Collagenase expression was upregulated in cells plated on FN/TN or on FN/TN that had been incubated with anti-TNM1 (Figure 8). However, when cells were plated on a substrate of FN/TN that had been incubated with anti-TN68, collagenase expression was reduced nearly to the levels seen in cells plated on FN alone. A combination of anti-TNM1 and anti-TN68 was no more effective than anti-TN68 alone in abrogating the regulation of collagenase expression in cells plated on a substrate of FN/TN. Thus, the distal part of the TN molecule containing the FN type III repeats is involved in mediating the changes in gene expression in cells plated on FN/TN matrices.

The Interaction of RSF with FN/TN or 120FN Differs from the Interaction of RSF with FN Alone

Analysis of the regulation of collagenase expression in RSF plated on FN, 120FN, and mixed substrates of FN/TN shows clearly that there are differences between the interaction of RSF with intact FN and with inductive matrices such as FN/TN and 120FN. One striking difference is that matrices of FN/TN or 120FN may not properly coordinate the interaction of integrin and non-integrin (e.g., proteoglycans) receptors for FN (reviewed by Damsky and Werb, 1992). Observations from several investigators suggest that the formation of focal contacts is diminished in some cell types when cells are plated

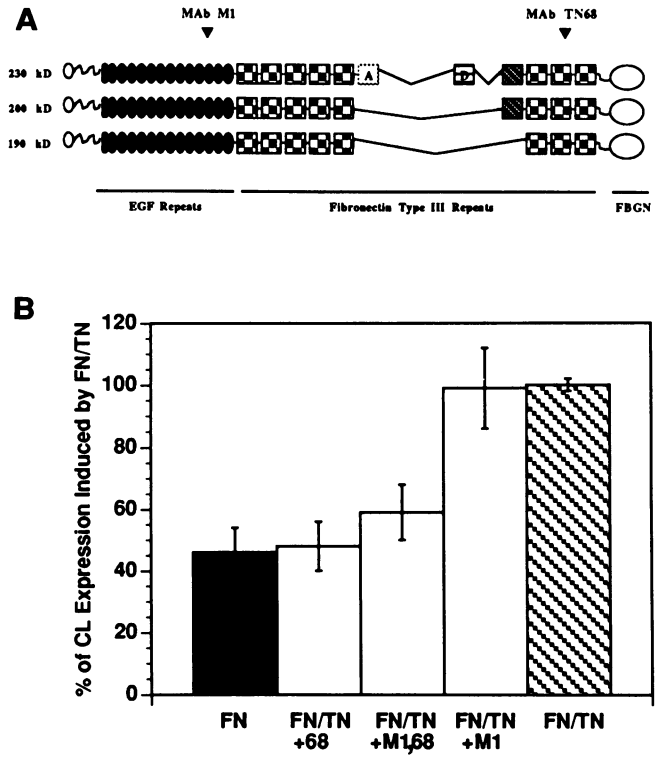

Figure 8. Induction of collagenase expression in fibroblasts plated on FN/TN is diminished by anti-TN68. (A) Structure of the TN isoforms from chicken. Each arm of the TN molecule consists of an amino-terminal globular domain, EGF-like repeats, FN type III repeats, and a carboxyl-terminal globular domain homologous to fibrinogen (FBGN). The epitopes recognized by anti-TNM1 and anti-TN68 are indicated. (B) Induction of collagenase expression in fibroblasts plated on FN/TN and anti-TN68. Fibroblasts were plated in serum-free medium in wells that were coated with FN, FN/TN, or a FN/TN mixture that had been incubated with 250 μg of anti-TN mAbs TNM1 or TN68, or with 250 μg of both antibodies. Cultures were incubated for 24 h; the secreted proteins were biosynthetically labeled by incubation of cells with [^{35}S]methionine, precipitated with quinine sulfate, and analyzed by SDS-PAGE, fluorography, and autoradiography. Collagenase protein secreted by fibroblasts was quantified by scanning densitometry, and the data from two experiments, each performed in duplicate, are expressed as -fold induction. Bars indicate SD.

on 120FN; likewise, disassembly of focal contacts has been observed in cultured cells incubated with soluble TN (Murphy-Ullrich *et al.*, 1991; Woods and Couchman, 1992). In both cases, heparin-binding molecules on the cell surface are thought to play a role in the regulation of focal contacts. The addition of a heparin-binding fragment of FN to the culture medium allows the formation of focal contacts in cells plated on 120FN (Woods and Couchman, 1992). The concurrent addition of exogenous, soluble proteoglycan along with TN prevents the focal contact disassembly observed with TN alone (Murphy-Ullrich *et al.*, 1991).

If the coordinate interaction of cell surface proteoglycans and the integrin receptors for FN with FN substrates plays a role in regulating the expression of collagenase in RSF adhering to FN, we reasoned that if we perturbed the interaction with proteoglycans directly, the expression of collagenase should be increased in cells plated on FN. We removed heparan sulfate from cell surface proteoglycans by incubation of cells with heparinase III before plating RSF in DME-LH on wells coated with FN, 120FN, or FN/TN. Incubation of RSF with heparinase III before plating on FN induced the expression of collagenase in these cells to amounts equivalent to that seen in RSF plated on 120FN or FN/TN (Figure 9). In control cultures, the expression of collagenase increased in cells plated on 120FN, or on FN/TN compared with cells plated on FN. When adherent RSF were incubated with heparinase III the expression of collagenase was the same as that seen in untreated, control cultures. Thus, it is unlikely that heparinase III itself regulates the expression of collagenase in RSF. Rather, these results suggest that the inability to coordinate the interaction of cell surface heparan sulfate proteoglycans and integrin receptors during initial adhesion on FN may be one feature common to the inductive FN-derived matrices.

A second possible mechanism to generate an inductive FN-derived substrate may arise from an alteration in the conformation of the cell-binding domain of 120FN compared with its conformation in intact FN, thereby altering its interaction with integrin receptors

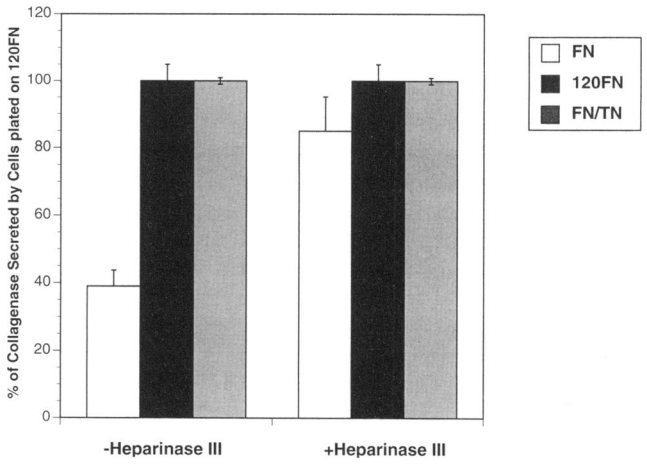

Figure 9. The expression of collagenase in heparinase III-treated RSF cultured on FN approaches that seen in RSF cultured on 120FN or FN/TN. Heparan sulfate was enzymatically hydrolyzed from adherent RSF by incubation of cells with heparinase III (5 units/ml in HEPES-buffered saline containing 2 mM $CaCl_2$) (+Heparinase III) before plating on wells coated with FN, 120FN, or FN/TN. As a control, RSF were incubated in HEPES-buffered saline containing 2 mM $CaCl_2$ alone (−Heparinase III) before plating on ECM-coated wells. The accumulation of secreted collagenase protein was determined by measurement of biosynthetically labeled collagenase protein (two experiments) or by immunoblotting of collagenase in the CM (one experiment). The results were quantified by scanning densitometry. The data from three experiments (mean ± SD) were normalized to collagenase secreted by RSF cultured on 120FN as 100%.

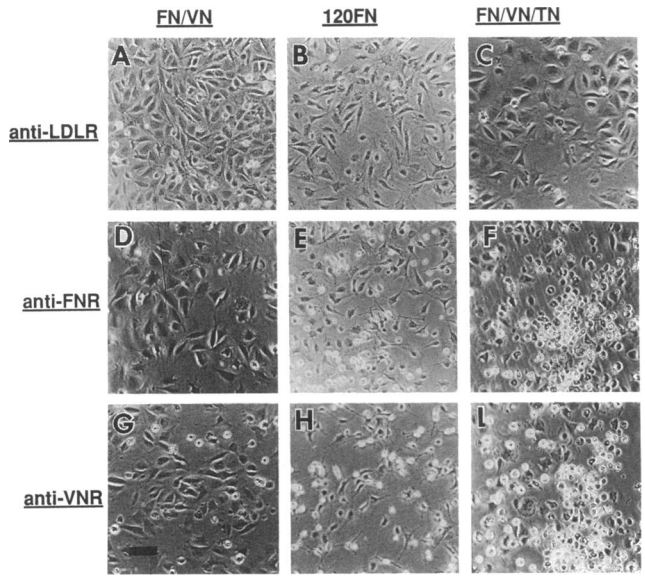

Figure 10. $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins on RSF recognize 120FN and FN/TN substrates. Fibroblasts were preincubated with 25 μg/ml anti-LDLR mAb (A–C), anti-FNR mAb (D–F), or anti-VNR mAb (G–I), then plated on wells coated with FN/VN (A, D, and G), 120FN (B, E, and H), or FN/VN/TN (C, F, and I). RSF were photographed after culture for 45 min. This experiment was repeated 4 times with identical results. Bar, 20 μm.

for FN. In the more complex case of a mixed substrate of FN/TN, it is not clear if the signaling matrix molecule results from an alteration in the conformation of FN, an alteration in the conformation of TN, or a cooperative effect requiring both molecules. To investigate the role of integrins in these interactions, we used function-perturbing anti-integrin mAbs or synthetic RGD peptides that target specific integrin receptors to probe the interaction of RSF with a mixed FN/TN substrate. We preincubated suspended RSF with anti-integrin or control antibodies, or with the integrin-specific peptide ligands before plating cells on wells coated with FN, 120FN, FN/VN, TN-supplemented substrates of FN/TN or FN/VN/TN, or VN alone, and analyzed the cells by phase-contrast microscopy. By including an alternative adhesive substrate (FN or VN) in the mixed substrates, we were able to separately analyze the effects of anti-FNR Fab fragments or anti-VNR antibodies on the interaction of RSF with the substrate.

The addition of anti-FNR Fab directed against the α_5 or GRGDdSP peptides, reagents that interact specifically with the integrin FNR, diminished the adhesion of RSF to substrates of FN/VN/TN and of 120FN but did not affect adhesion of RSF to FN/VN or VN (Table 1, Figure 10). Likewise, molecules that interact with the VNR, either anti-VNR mAb, directed against $\alpha_v\beta_3$, or the cyclic GPenGRGDSPCA peptide, decreased the adhesion of cells to VN, 120FN, and mixed substrates of FN/TN and FN/VN/TN but did not alter the interaction of RSF with a FN substrate. Incubation of RSF with function-perturbing mAbs directed against either the $\alpha_3\beta_1$ or $\alpha_2\beta_1$ integrins, with anti-LDLR mAb, or with GRGESP peptides did not diminish the adhesion of RSF to any of the substrates tested.

Taken together, these results show that both the FNR and the VNR play a role in cell adhesion to the inductive FN ligands, mixed substrates of FN/TN and 120FN. These results do not allow definition of the ECM ligand, however, and it is still unclear if the addition of TN to a FN substrate generates an inductive species owing to changes in the conformation of either FN or TN, or if it results from a combinative effect of FN and TN.

DISCUSSION

In this study we have shown that the addition of TN to a FN substrate forms a matrix that influences the pattern of gene expression in fibroblasts. The expression of c-Fos rapidly increases in cells adhering to a mixed substrate of FN/TN, and this is followed by an increase

Table 1. Agents that target the FNR or the VNR perturb adhesion of RSF to FN/TN or 120FN*

Substrate	anti-FNR	anti-VNR	anti-$\alpha_2\beta_1$	anti-$\alpha_3\beta_1$	anti-LDLR	GRGDdSP	GPenGRGDSPCA	GRGESP
FN	+	−	−	−	−	+	−	−
FN + VN	−	−	−	−	−	−	−	−
FN + TN	+	+	−	−	−	+	+	−
FN + VN + TN	+	+	−	−	−	+	+	−
120FN	+	+	−	−	−	+	+	−
VN	−	+	−	−	−	−	+	−

* Trypsinized RSF were preincubated with anti-integrin mAb or with peptide ligands that preferentially recognize the FNR or VNR before plating on ECM-coated wells. Cells were cultured for 40–60 min then photographed. Adhesion of cells to the substrates was scored by analyzing the cell shape (see Figure 10) with (+) indicating a change from adherent, spread cells to rounded morphology and (−) indicating no effect on cell morphology. The treatments were anti-FNR, 25 μg/ml mAb BIIG2; anti-VNR, 25 μg/ml mAb LM609; anti-$\alpha_2\beta_1$, 1:200 dilution of mAb P1E6 ascites; anti-$\alpha_3\beta_1$, 1:200 dilution of mAb P1B5 ascites; anti-LDLR, 25 μg/ml anti-LDLR mAb; GRGDdSP, 0.5 or 1 mM GRGDdSP peptide, which, when used at this concentration, targets the FNR; GPenGRGDSPCA, 0.1 or 0.2 mM GPenGRGDSPCA peptide, which, when used at this concentration, targets the VNR; GRGESP, 0.1 or 0.5 mM GRGESP peptide, a control peptide.

in metalloproteinase expression, an effect not seen in cell cultures incubated with soluble TN or cultured on FN alone. Moreover, the carboxyl-terminal FN type III repeats in the TN arm function in this interaction. That the integrity of the TN arm near the carboxyl-terminal FN type III repeats, and not the anti-adhesive site mapped to the EGF-like repeats by Spring *et al.* (1989), is necessary for the regulation of collagenase was demonstrated by experiments in which anti-TN68, but not anti-TNM1, blocked the inductive effect of FN/TN matrices. mAb anti-TN68 defines an epitope in the tenth type III repeat that is included in all TN isoforms, is involved in cellular responses to both soluble and substrate-bound TN, and blocks the TN-induced destabilization of cellular adhesion to FN (Chiquet-Ehrismann *et al.*, 1986; Halfter *et al.*, 1989; Spring *et al.*, 1989; Riou *et al.*, 1990).

Cells attach but do not spread on recombinant TN molecules or proteolytic fragments of TN that contain the distal type III repeats (Friedlander *et al.*, 1988; Spring *et al.*, 1989; Prieto *et al.*, 1992), showing that this portion of the TN arm interacts with molecules on the cell surface. Preincubation of cultured cells with recombinant TN species containing only the carboxyl-terminal type III repeats in the TN arm diminishes the adhesion of cells to a FN substrate (Prieto *et al.*, 1992). The interaction of TN with cells and substrate is undoubtedly more complex, because adjacent regions in the alternatively spliced type III repeats alter the interaction of TN with FN substrates (Chiquet-Ehrismann *et al.*, 1991), promote neurite outgrowth (Wehrle and Chiquet, 1990; Lochter *et al.*, 1991; Zisch *et al.*, 1992), and destabilize the adhesion of adherent cultured endothelial cells (Murphy-Ullrich *et al.*, 1991).

Cells interacting with FN/TN matrices behaved like cells adhering to anti-FNR antibodies or to the cell-binding fragment of FN (Werb *et al.*, 1989). There are several possible explanations for how the addition of TN to the FN substrate produces an inductive matrix. One possibility is that TN sterically hinders the accessibility of ECM substrate to the cell surface (Lightner and Erickson, 1990). It is clear from our results that the addition of soluble TN to cultured fibroblasts, adhering either to a FN substrate or largely to VN derived from serum proteins, does not induce collagenase synthesis in these cells. Likewise, collagenase expression in cells plated on substrates of collagen or VN in the presence of TN did not differ from that in cultures plated on collagen or VN alone. This is informative, because it suggests that TN alone is not the inductive molecule but rather that the combination of FN and TN increases collagenase expression in RSF. If a strictly steric mechanism were operating, TN would also induce the expression of collagenase on collagen or VN, and anti-TN mAbs would augment, not inhibit, the increase in collagenase expression in cells plated on FN/TN. Alternatively, the induction of collagenase in fibroblasts plated on a mixture of FN and TN could result from a specific interaction of a mixed substrate of FN/TN with the cell surface. TN disrupts adhesion to FN and FN fragments that have the cell-binding domain containing RGD (Chiquet-Ehrismann *et al.*, 1986) and decreases cellular adhesion to FN only in cells in which RGD also perturbs cellular adhesion to FN (Chiquet-Ehrismann *et al.*, 1988). The fact that MMP expression is upregulated with similar kinetics in cells plated on substrates of 120FN or FN/TN and that the formation of focal adhesions in cells is diminished in cells plated on 120FN (Woods and Couchman, 1992) and in adherent cells treated with TN (Murphy-Ullrich *et al.*, 1991) suggests that there may be similarities in the interaction of cells with FN/TN and the interaction of cells with the large cell-binding fragment of FN, 120FN. The implication that the carboxyl-terminal type III repeats in the TN arm are a necessary component of an inductive matrix also supports this idea, because the alternatively spliced and carboxyl-terminal type III repeats modulate the interaction of TN with FN, and TN fragments that contain the carboxyl-terminal type III repeats (isolated by chromatography on anti-TN68) bind to a heparin affinity column (Chiquet *et al.*, 1991). The observation that both FN/TN and 120FN engage both the FNR and the VNR serves to consolidate the idea that there are cell interactions common to both inductive ligands.

But how does FN/TN exert its inductive effect? FN, like TN, is a large, multidomain molecule in the ECM that interacts with other ECM molecules and is recognized by several classes of cell surface receptors (reviewed by Hynes, 1992). Intact FN in the matrix is recognized by integrin receptors, a matrix assembly receptor, and cell surface proteoglycans, whereas 120FN, which contains the cell-binding domain of FN, interacts primarily with integrin receptors (reviewed by Damsky and Werb, 1992). It is clear that cells plated on 120FN or treated with anti-FNR antibody upregulate the expression of collagenase (Werb *et al.*, 1989). Like FN, both of these ligands interact with the integrin FNR, but unlike FN, these ligands do not contain domains that can interact with cell surface proteoglycans. It may be that the coordinate interaction of several classes of cell surface adhesion receptors (e.g., integrins and proteoglycans) with FN is necessary to restrict expression of MMP (reviewed by Damsky and Werb, 1992). This hypothesis is supported by data that show that focal contact formation is compromised in cells plated on 120FN (Woods and Couchman, 1992) and in cells treated with TN (Murphy-Ullrich *et al.*, 1991). Furthermore, focal contact formation in cells plated on 120FN or in the presence of TN is stabilized by treatments that appear to allow an interaction with cell surface proteoglycans (Murphy-Ullrich *et al.*, 1991; Woods and Couchman, 1992). Taken together, these observations support the idea that TN, in the context of a FN-rich matrix, might interact with cell surface proteoglycans,

thereby disrupting a bipartite interaction of cells with FN. Information could also be transduced through the integrin FNR, which may now sense intact FN to be like 120FN, or through a cooperative interaction of a TN-dependent receptor in concert with the integrin FNR. Proteoglycans on the cell surface that interact with TN have been identified (Hoffman *et al.*, 1988; Salmivirta *et al.*, 1991). The observation that the expression of collagenase in RSF plated on a substrate of intact FN is equal to that of cells plated on 120FN or FN/TN when we directly perturb the interaction of cell surface proteoglycans with the FN substrate supports and strengthens this hypothesis.

Recently, the $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin receptors on endothelial cells have been shown to mediate the adhesion of endothelial cells to a TN substrate (Joshi *et al.*, 1993; Sriramarao *et al.*, 1993). The VNR recognizes the RGD site in the third type III repeat of TN, and it is suggested that the epitope on TN recognized by $\alpha_2\beta_1$ may be in the carboxyl-terminal fibrinogen-like domain of TN (Joshi *et al.*, 1993). Endothelial cells and fibroblasts adhere to, but do not spread on, a substrate of intact TN (Joshi *et al.*, 1993; Sriramarao *et al.*, 1993 and references therein). Because cells remain round, the regulation of metalloproteinase expression of RSF caused by adhesion to a TN substrate cannot be separated from that caused by an alteration in cell shape (Aggeler *et al.*, 1984).

However, the observation that TN disrupts the adhesion of cultured cells to a FN substrate only in cell types in which adhesion to FN is destabilized by RGD peptides (Chiquet-Ehrismann *et al.*, 1988) suggests that integrin-based adhesion to a substrate of FN/TN may well differ from adhesion to FN. In a similar manner, the dynamics of the interaction of integrins with the RGD-containing fragments of FN differ from those observed on intact FN (Ginsberg *et al.*, 1987; Obara *et al.*, 1988). Our data suggest that both the $\alpha_5\beta_1$ FNR and the $\alpha_v\beta_3$ VNR play a role in adhesion to 120FN or FN/TN substrates, because anti-FNR Fab fragments, anti-VNR antibodies, and peptide ligands that recognize either the FNR or the VNR perturb the interaction of cells with the FN/VN/TN substrate. Addition of function-perturbing mAbs against the $\alpha_2\beta_1$ or $\alpha_3\beta_1$ integrins of GRGESP peptides did not diminish the adhesion of fibroblasts to FN/VN/TN substrates. Thus, there are likely to be a number of similar parameters in the interaction of RSF with matrices of 120FN or FN/TN that play a role in initiating the expression of MMPs. Although interesting, our results cannot discriminate between the effects of perturbing the coordinate recognition of intact FN by multiple receptors for ECM and the simpler explanation of an alteration of integrin interactions with matrix. Furthermore, although integrin receptors interact differently with a TN-augmented FN substrate, we cannot determine if this difference could be in response to a conformational change in either FN or TN as a result of the mixed substrate of FN/TN, or in response to an effect requiring epitopes on both FN and TN.

The initiation of an ECM remodeling cascade in response to specific cues in the matrix that are determined by ECM composition and, conversely, the regulation of matrix and tissue structure by proteolysis are likely to be significant in wound healing and/or tissue morphogenesis. For example, MMPs and their inhibitors play a significant role in the expansion, maturation, and resorption of the mouse mammary gland (Talhouk *et al.*, 1992). Controlled proteolysis may also be necessary for the migration of cells seen in both normal developmental processes and the metastatic spread of tumor cells. Likewise, the migration of cultured embryonic mesenchymal cells through basement membrane-like matrices requires a net increase in the proteolytic activity of these cells (Alexander and Werb, 1992). The three MMPs that are induced in cells plated on FN/TN can act in concert to degrade a wide range of tissue matrices. The 92-kDa gelatinase degrades type IV collagen and denatured interstitial collagen, collagenase degrades native fibrillar collagens, and stromelysin degrades a wide range of matrix molecules, including types I and IV collagen, FN, and proteoglycans (reviewed by Alexander and Werb, 1991). Extrapolating, we suggest that the transient expression of TN may also trigger localized expression of MMP to remove and remodel matrices that are no longer functional, or to facilitate cell migration.

In vitro, two examples have been described in which alterations in the FN/TN ratio are seen in conjunction with remodeling tissue or migrating cells. Gatchalian *et al.* (1989) have shown that TN accumulates around denervated synaptic sites and that a specific population of fibroblasts proliferate in perisynaptic spaces. They further have shown that the TN/FN ratio synthesized by fibroblasts enzymatically dissociated from denervated muscle end plate is increased when compared with fibroblasts from innervated muscle. Together with cytokines released locally by macrophages, perturbations in adhesion caused by the interaction of cells with an altered FN/TN substrate may also play a role in the remodeling of the perisynaptic site. In healing skin wounds, TN accumulates in granulation tissue and in the dermis beneath the migrating epidermis (Mackie *et al.*, 1988; Whitby *et al.*, 1991). Studies comparing the closure and healing of fetal wounds, which heal with little or no scar formation, with that of adult wounds, which do scar, show that although the localization of TN immunostaining is similar in fetal and adult wounds, both the upregulation of TN expression and its disappearance are more rapid in embryonic tissues (Mackie *et al.*, 1988; Whitby *et al.*, 1991; Whitby and Ferguson, 1991). FN isoforms expressed beneath the wounded tissue also revert to include isoforms expressed during development; ffrench-Constant *et al.* (1989) have shown that the expression of FN isoforms containing alterna-

tively spliced type III repeats, characteristic of embryonic matrices, is upregulated beneath the migrating wound epithelium. In these two situations, it is tempting to hypothesize that signals generated through alterations in cellular adhesion to the ECM by changes in FN/TN ratios regulate the ECM remodeling phenotype by altering the expression of metalloproteinases.

Recently, targeted disruption of the TN gene by homologous recombination in embryonic stem cells has allowed the derivation of mutant mice that do not express TN (Saga *et al.*, 1992). Although these mice have no gross abnormalities, analysis of tissue structure at higher resolution may reveal differences between wild-type and TN-null mice. It is also possible that alterations in phenotype will be observed in TN-null mice under specific, challenging conditions, such as in repair of wounded tissue. These results, however, question the significance of TN during development and morphogenesis; it may be that developmental cues provided by other molecules in the ECM (which may or may not be TN analogs) can functionally replace the developmental cues normally provided by TN, and that manipulation of more than one molecule will be necessary to characterize a TN-null mouse. Several molecules with structural similarities to TN have been described: undulin, which has similarities to FN and TN (Just *et al.*, 1991), and TN-MHC and restrictin, which more closely resemble TN (Matsumoto *et al.*, 1992; Norenberg *et al.*, 1992). A study of the expression of specific TN isoforms, or genetically altered TN isoforms in a TN-null background, may be informative as to the role of TN during developmental interactions, whereas a null phenotype is not informative. There also are species differences in TN structure and distribution that may be significant in generation of phenotype. For example, chicken TN (used in these experiments) and human TN have an RGD sequence in one of the type III repeats, whereas mouse TN does not contain this sequence. Likewise, the pattern of MMP expression differs between species; collagenase is the dominant MMP expressed in human and rabbit fibroblasts, whereas mouse fibroblasts secrete little collagenase. Information gleaned through characterization of TN structure-function relationships in vitro, identifying domains in the TN molecule that interact with cells or with the ECM (Friedlander *et al.*, 1988; Spring *et al.*, 1989; Murphy-Ullrich *et al.*, 1991) and the consequences of these interactions (Lochter *et al.*, 1991; Wehrle and Chiquet, 1990; present study) will help to clarify the role of TN in developing and remodeling tissues and will facilitate analysis of the effects of TN in vivo.

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