

Collagen and Collagenase Gene Expression in Three-dimensional Collagen Lattices Are Differentially Regulated by $\alpha 1\beta 1$ and $\alpha 2\beta 1$ Integrins

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Abstract. The reorganization of extracellular matrix (ECM) is an important function in many biological and pathophysiological processes. Culture of fibroblasts in a three-dimensional collagenous environment represents a suitable system to study the underlying mechanisms resulting from cell–ECM interaction, which leads to reprogramming of fibroblast biosynthetic capacity. The aim of this study was to identify receptors that transduce ECM signals into cellular events, resulting in reprogramming of connective tissue metabolism. Our data demonstrate that in human skin fibroblasts $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins are the major receptors responsible for regulating ECM remodeling: $\alpha 1\beta 1$ mediates the signals inducing downregulation of collagen gene expression, whereas the $\alpha 2\beta 1$ integrin mediates induction of collagenase (MMP-1). Applying mAb directed against different integrin subunits resulted in triggering the heterodimeric receptors and enhancing the normal bio-

chemical response to receptor ligation.

Different signal transduction inhibitors were tested for their influence on gel contraction, expression of $\alpha 1(I)$ collagen and MMP-1 in fibroblasts within collagen gels. Ortho-vanadate and herbimycin A displayed no significant effect on any of these three processes. In contrast, genistein reduced lattice contraction, and completely inhibited induction of MMP-1, whereas type I collagen down-regulation was unaltered. Calphostin C inhibited only lattice contraction. Taken together, these data indicate a role of tyrosine-specific protein kinases in mediating gel contraction and induction of MMP-1, as well as an involvement of protein kinase C in the contraction process. The data presented here indicate that different signaling pathways exist leading to the three events discussed here, and that these pathways do not per se depend upon each other.

REMODELING of extracellular matrix (ECM)¹ is an important event during many biological and pathophysiological processes, as different as organomorphogenesis during embryonal development, wound healing, tumor invasion and metastasis, and fibrosis. All these processes require the disassembly of old and deposition of new ECM components, processes that have to be tightly regulated spatially and temporally. Cell–ECM interactions play a major role in this complex network that regulates the steady state and guarantees connective tissue integrity (for review see Grinnell, 1994).

A well-characterized in vitro system representing the in vivo situation during ECM remodeling in connective tissues

better than conventional monolayer cell culture systems are contracting three-dimensional hydrated collagen matrices, populated by fibroblasts (Bell et al., 1979). When fibroblasts are provided with a three-dimensional matrix consisting mainly of type I collagen, the cells adhere to the collagen fibers and contract the initially loose network to a dense tissuelike structure. This process is accompanied by a fundamental reprogramming of fibroblast morphology and metabolism, resulting in down regulation of type I collagen and induction of matrix-degrading proteases (Nusgens et al., 1984; Unemori and Werb, 1986; Mauch et al., 1988, 1989). In particular, induction of interstitial collagenase (matrix metalloproteinase-1 [MMP-1]) is controlled at the transcriptional level, whereas collagen synthesis is regulated by transcriptional (Mauch et al., 1989) as well as by posttranscriptional mechanisms (Eckes et al., 1993).

Integrins are well known as the major cell membrane receptors, which mediate a molecular dialogue between cells and their extracellular matrix (for review see Hynes, 1992). They constitute a family of at least 21 distinct molecules,

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; MMP-1, matrix metalloproteinase-1; PKC, protein kinase C; VLA, very late antigen.

which are heterodimeric transmembrane cell surface receptors, assembled by the noncovalent association of one α with one β subunit. Both subunits possess a large extracellular domain, a membrane-spanning region and a short cytoplasmic domain. The extracellular domains of one α and one β chain combine to form the binding region for ECM proteins. The cytoplasmic domains bind to intracellular proteins including talin, vinculin, and α -actinin, thereby linking the extracellular environment with the cell interior (Horwitz et al., 1986; Burridge et al., 1988; Otey et al., 1990). The exact nature of how integrins transduce information from three-dimensional contact to matrix proteins into cellular events, leading to remodeling of the ECM, however, remains to be elucidated.

Phosphorylation of proteins is a common mechanism in signal transduction (Ulrich and Schlessinger, 1990; Glenney, 1992) and could recently be shown to be part of signal cascades downstream of integrin receptors (reviewed in Juliano and Haskill, 1993; Schaller and Parsons, 1993). A new intracellular tyrosine kinase, focal adhesion kinase, was identified as a downstream target of integrin signaling in platelets (Lipfert et al., 1992; Golden et al., 1990), carcinoma cells (Kornberg et al., 1991), and rodent fibroblasts (Guan et al., 1991; Hanks et al., 1992). The observation that the focal adhesion kinase pp125^{FAK} is also activated by phosphorylation in response to contact of fibroblasts to collagen in a three-dimensional as well as in a two-dimensional system (Röckel and Krieg, 1994) suggests that activation is mediated by integrins capable of binding collagen. Binding to native type I collagen by integrins involves mainly α 1 β 1 (very late antigen [VLA]-1) and α 2 β 1 (VLA-2) (Wayner and Carter, 1987; Belkin et al., 1990; Kirchofer et al. 1990; Elices et al., 1989). Cultured fibroblasts from healthy human donors express all of these integrin chains (Schiro et al., 1991). Whether integrin-mediated phosphorylation relates to changes in gene expression which are induced by adhesion or contact to a three-dimensional extracellular matrix environment remains to be determined.

Perturbation studies using antibodies blocking β 1 integrin showed that this interferes with gel contraction (Gullberg et al., 1990). Klein et al. (1991) proposed α 2 β 1 to be the receptor responsible for the contraction process. Their data were corroborated by transfection experiments introducing full-length α 2 cDNA into rhabdomyosarcoma cells (Schiro et al., 1991). Other recent studies demonstrated that among several external factors certain mAb directed to integrin subunits are able to modulate the function of β 1 integrins (for review see Hynes, 1992). The proposed mechanism of action of these mAb is to induce a conformational change in the receptors that modulates the affinity and/or avidity for their ligands. For example, Werb and co-workers (1989) could achieve induction of MMP-1 by ligation of the fibronectin receptor with mAb in rabbit synovial fibroblasts in conventional monolayer cultures.

The aim of this study was to identify receptors involved in regulating collagen metabolism during extracellular matrix remodeling in contracting collagen lattices. We here present evidence that α 1 β 1 integrin is the receptor responsible for down-regulation of collagen I synthesis in fibroblasts cultured within contracting collagen lattices, whereas α 2 β 1 integrin mediates the induction of MMP-1 in this system. In addition, we show that protein phosphorylation is

involved in the further transduction of signals by these integrin heterodimers.

Materials and Methods

Cell Culture

Cultures of primary human fibroblasts were established by outgrowth from skin biopsies of healthy volunteers. Cells were maintained in DME, supplemented with 10% FCS, 50 μ g/ml sodium ascorbate, 300 μ g/ml glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and grown in the moist atmosphere of a CO₂ incubator (5% CO₂) at 37°C. Cells were passaged by trypsinization (0.1% trypsin, 0.02% EDTA in PBS; Boehringer Mannheim Corp., Indianapolis, IN) and were used at a confluent stage in passages 5–9.

Preparation of Collagen Gels

Native porcine collagen (I) was purchased from Deutsche Gelatine Fabrik Stoesch AG (Eberbach, Germany), and purified by dialysis against 0.01 M phosphate buffer, pH 7.2, and redissolved at 3 mg/ml in sterile 0.1% acetic acid. Three-dimensional collagen lattices were prepared as previously described (Mauch et al., 1989; Eckes et al., 1993). In brief: cells were harvested by trypsinization, collected by centrifugation, and resuspended in DME/10% FCS. 10⁷ cells in 3 ml DME/10% FCS were added as last component to a collagen-gel mix consisting of 13.8 ml of 1.76 \times DME, 9 ml collagen type I [3 mg/ml] in 0.1% acetic acid, 1.5 ml 0.1 N NaOH, and 2.7 ml FCS (150-mm diameter, 30-ml vol).

Contraction proceeded for 4–48 h, as indicated. Rates of gel contraction were monitored by determining remaining surface area.

Antibodies

mAb 4B4 is directed against the human β 1-integrin chain (Morimoto et al., 1985) and inhibits cell adhesion to type I collagen (Shimizu et al., 1990); mAb TS2/7 binds to the α 1 chain of integrin α 1 β 1 (VLA-1) and is not inhibiting (Hemler et al., 1985); whereas mAb 1B3.1 recognizes a different epitope within the α 1 chain of human integrin α 1 β 1 (VLA-1) (Bank et al., 1989, 1991, 1994) and inhibits attachment of fibroblasts and keratinocytes to type I collagen (Lange et al., 1994), and of T cells to type IV collagen, but not to type I (Bank et al., 1994), mAb P1E6 detects the α 2-chain of human α 2 β 1 integrins (VLA-2) and inhibits adhesion to type I collagen (Wayner et al., 1988); mAb 5E8 also detects the α 2-chain of human α 2 β 1 integrins (VLA-2) and inhibits adhesion to type I collagen (Chen et al., 1991); mAb P1B5 is specific for the α 3-chain of human α 3 β 1 integrin (Wayner et al., 1987); mAb 1280 recognizes the human HLA-ABC antigens, specifically the HLA-B2M complex. mAb Ts2/7 were purchased from Dianova GmbH (Hamburg, Germany), mAb P1E6 and mAb P1B5 from Telios Pharmaceutical, Inc. (San Diego, CA), mAb 1280 from Chemicon International, Inc. (Temecula, CA), mAb 4B4 from Coulter Corp. (Hialeah, FL), affinity-purified goat anti-mouse IgA from Sigma Chemie GmbH (Deisenhoten, Germany), affinity purified rabbit anti-mouse IgG from Dianova GmbH, mAb 5E8 was kindly provided by Drs. Bankert and Chen (Roswell Park Cancer Institute, Buffalo, NY).

Incubation with Antibodies

Cells were detached from confluent monolayer cultures by trypsinization, collected by centrifugation, and resuspended in 3 ml DME/10% FCS (1 \times 10⁷ cells). After addition of antibodies, cells were preincubated for 15 min at 37°C in a CO₂ incubator and then seeded as last component into collagen gels or cultured as monolayers as described above. For clustering experiments, cells were incubated at 37°C in a CO₂ incubator with a 10-fold excess of the appropriate secondary antibody in 3 ml DME for further 15 min before seeding.

Coating of Petri Dishes

For planar collagen type I coating, the porcine collagen preparation used to cast lattices was diluted to a final concentration of 30 μ g/ml. This solution was added to plastic dishes at a final concentration of 6 μ g/cm² and distributed by rocking for 2 h at 4°C. Coated dishes were allowed to dry and rinsed with PBS before use.

When indicated, tissue culture dishes were coated with 20 μ g/ml of

anti-integrin mAb in PBS for 2 h at 4°C, blocked with 1% BSA in PBS for 2 h, immediately followed by plating cells (density: 5×10^5 /150-mm petri dish) for 24 h in the absence of serum.

Incubation with Signal Transduction Inhibitors

Genistein (4',5,7-trihydroxyisoflavone; Biomol, Hamburg, Germany), a potent inhibitor of tyrosine-specific kinases (Akiyama et al., 1987) with an

$IC_{50} = 15 \mu\text{M}$ for protein kinase C (PKC) (Geissler et al., 1990), was used at final concentrations of 3, 30, and 300 μM . Herbimycin A (Gibco Biocult, Eggenheim, Germany) also a potent inhibitor of tyrosine-specific kinases with an $IC_{50} = 0.5\text{--}875 \text{ nM}$ (Uehara et al., 1986; Hunter, 1989), was used at final concentrations of 90 nM, 900 nM, and 9 μM ; calphostin C (Biomol), a highly selective inhibitor of PKC with an $IC_{50} = 0.05 \mu\text{M}$ (Bruns et al., 1991), was used at final concentrations of 10, 50, 100, 500, and 1,000 nM and dissolved in DMSO. Sodium orthovanadate (Sigma

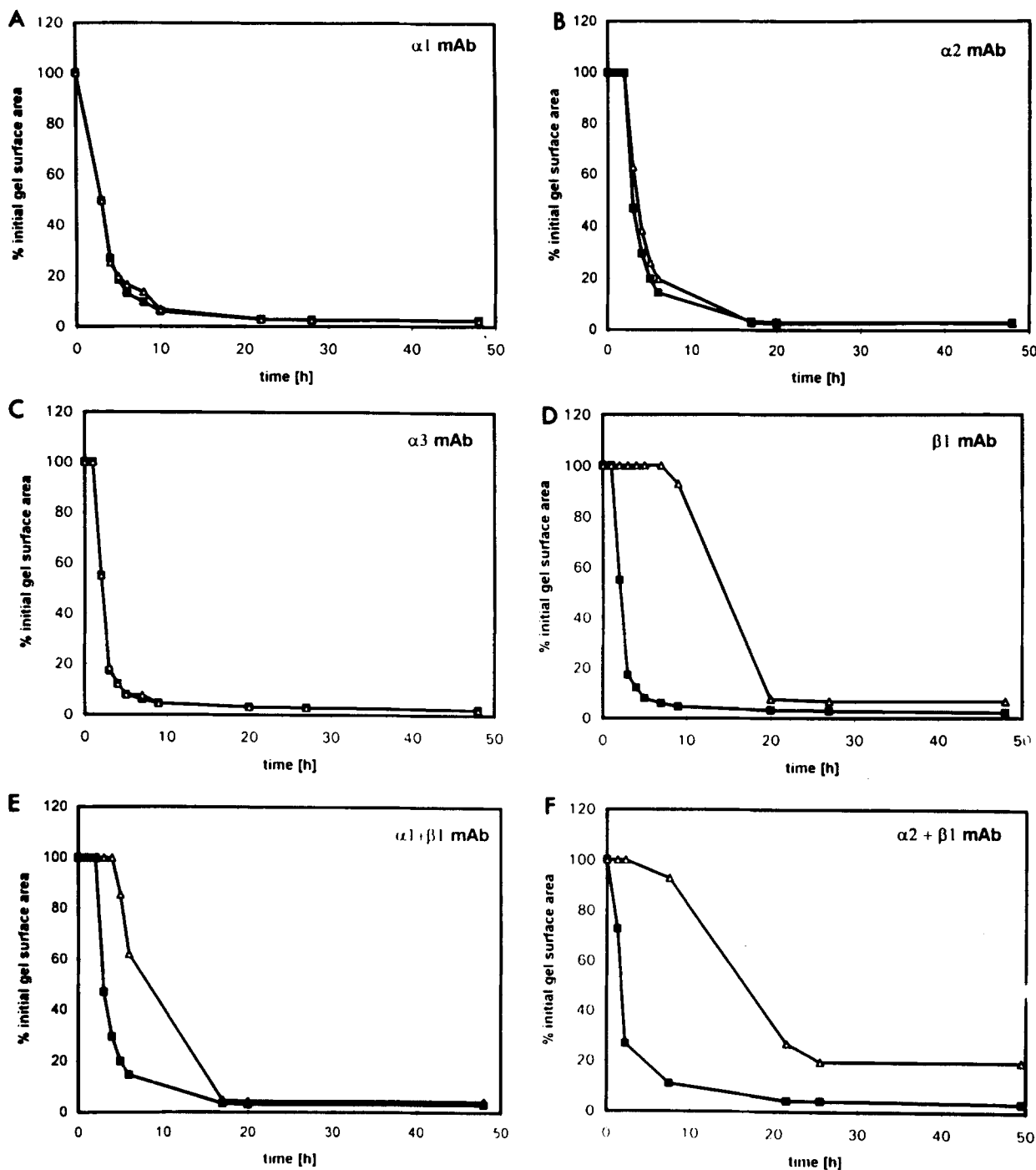


Figure 1. Contraction of type I collagen matrices by fibroblasts is inhibited by anti-integrin mAb. Plots of collagen matrix surface area are shown as percentage of initial gel surface area vs. time in hours. Fibroblasts were preincubated with mAbs at a concentration of 40 $\mu\text{g/ml}$ each in DME, 10% FCS for 30 min at 37°C, and then diluted 10-fold to final concentrations of 4 $\mu\text{g/ml}$ (triangles) or without antibody addition (squares). Gel contraction proceeded for the times indicated. In detail, the following mAbs were used: mAb 1B3.1 (A: anti- $\alpha 1$ integrin), mAb P1E6 (B: anti- $\alpha 2$ integrin), mAb P1B5 (C: anti- $\alpha 3$ integrin), 4B4 (D: anti- $\beta 1$ integrin), combination of mAb 1B3.1 and mAb 4B4 (E: anti- $\alpha 1$ and - $\beta 1$ integrin) or mAb P1E6 and mAb 4B4 (F: anti- $\alpha 2$ and - $\beta 1$ integrin).

Chemie GmbH), an inhibitor of protein tyrosine phosphatases, was used at final concentrations of 1, 10, and 100 μM and dissolved in H_2O . Different dilutions of these stocks in PBS were added to the collagen cell mix (see preparation of collagen gels) as the last component. Calphostin C incubations were conducted in a CO_2 incubator under ordinary fluorescent light (Bruns et al., 1991). After 24 h, cells were harvested for RNA isolation. Supernatants were tested for the presence of lactate dehydrogenase (LDH), indicating cytotoxic concentrations of the inhibitor. Effective concentrations showed no cytotoxic effects.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from monolayer and collagen lattice cultures using a modified guanidinium/CsCl method as described earlier (Sambrook et al., 1989; Eckes et al., 1993). Northern analysis was performed as described (Sambrook et al., 1989), using 5–10 μg of total RNA separated by electrophoresis in a 1% formaldehyde agarose gel and transferred to Gene Screen hybridization transfer membranes (New England Nuclear, Boston, MA). RNA was UV-cross-linked (Stratagene, La Jolla, CA) to membranes and hybridized with radioactive probes. Equal loading was confirmed by staining the membranes for 5 min in 0.04% methylene blue in 0.5 M Na acetate, followed by destaining in 25% ethanol. Probes were as follows: a 1.4-kb EcoRI fragment of the human collagen $\alpha 1(\text{I})$ cDNA from clone Hf677 (Chu et al., 1982), 2-kb PstI fragment of the human collagenase (MMP-1) cDNA clone X7 (Angel et al., 1987), 1.3-kb PstI fragment of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA (Fort et al., 1985). Insert sequences were radiolabeled by random priming with ^{32}P (Feinberg and Vogelstein, 1983). Hybridization was performed overnight at 42°C in a solution containing 50% formamide, $5 \times \text{SSC}$, 0.1% SDS, $5 \times$ Denhardt's buffer, and 0.1 mg/ml salmon sperm DNA. Filters were washed 2×15 min in $2 \times \text{SSC}$, 0.1% SDS at room temperature and 2×15 min in $0.1 \times \text{SSC}$, 0.1% SDS at 62°C (Hf677); 2×10 min in $2 \times \text{SSC}$, 0.1% SDS at room temperature and 1×10 min in $2 \times \text{SSC}$, 0.1% SDS at 62°C (X7); 2×15 min in $2 \times \text{SSC}$, 0.1% SDS at room temperature and 1×15 min in $1 \times \text{SSC}$, 0.1% SDS at 55°C (GAPDH), followed by autoradiography (X-Omat AR; Eastman Kodak, Rochester, NY) at -80°C . Autoradiographs were quantified densitometrically using a laser densitometer equipped with ImageQuant software (Molecular Dynamics GmbH, Krefeld, Germany). The data presented are representative of at least three independent experiments.

Results

Antibodies Directed against Subunits of the $\beta 1$ Integrin Family Inhibit Contraction of Collagen Matrices by Fibroblasts

When fibroblasts are provided with a three-dimensional matrix consisting mainly of collagen type I, the cells adhere to the collagen fibers and contract the initially loose network to a dense tissue-like structure. Under the conditions used in this study, contraction of the gels became visible 2–6 h after gel casting, being maximal between 12 and 48 h, and reaching a plateau at 72–80 h.

To analyze the functional role of different receptors of the $\beta 1$ integrin family on cellular functions during the contraction process, we applied monoclonal antibodies directed against the extracellular domains of integrin subunits to fibroblasts cultured in three-dimensional collagen lattices. Cultured human skin fibroblasts express all of the analyzed integrins (Schiro et al., 1991) as was confirmed by FACScan® analysis (Becton Dickinson & Co., Mountain View, CA; not shown). Cells, preincubated in the absence or presence of the indicated concentrations of monoclonal antibodies, were suspended in type I collagen lattices, and the effect of the added antibodies on fibroblast-mediated lattice contraction was studied.

Addition of an antibody (mAb 4B4), which recognizes the $\beta 1$ integrin subunit, resulted in a pronounced delay

and reduction of the contraction process, with contraction becoming visible after 8 h (Fig. 1 D). In contrast, incubation with an antibody directed against the $\alpha 1$ integrin chain (mAb 1B3.1) showed no detectable effect on the contraction process (Fig. 1 A). Treatment with an antibody directed against the $\alpha 2$ integrin chain (mAb P1E6) resulted only in a weak reduction of gel contraction in comparison to untreated cultures (Fig. 1 B). Incubation with an antibody that recognizes the $\alpha 3$ integrin subunit (mAb P1B5), the α -chain of the receptor that binds primarily epiligrin, showed no detectable effect on the contraction process (Fig. 1 C).

Addition of anti- $\beta 1$ antibodies (mAb 4B4) in combination with anti- $\alpha 1$ antibodies (mAb 1B3.1) to fibroblasts grown within collagen gels resulted in reduced contraction, which however did not exceed the effect of anti- $\beta 1$ antibody (mAb 4B4) alone, indicating that mAb 1B3.1 and mAb 4B4 do not function in a cooperative fashion (Fig. 1 E). Strongest inhibition of gel contraction by fibroblasts was obtained by a combination of anti- $\alpha 2$ antibody (mAb P1E6) and anti- $\beta 1$ mAb (mAb 4B4). This treatment inhibited the contraction process most significantly in comparison to untreated cultures (Fig. 1 F), indicating that antibodies directed to $\alpha 2$ and $\beta 1$ function cooperatively in inhibiting the contraction process. At 48 h, the extent of contraction inhibition displayed even higher levels than the addition of the values obtained with the two single mAb alone, suggesting a synergistic effect. In all cases the inhibition of gel contraction correlated directly with antibody concentration (shown for a combination of anti- $\alpha 2$ and anti- $\beta 1$ mAb in Fig. 2), suggesting that inhibition of gel contraction is a specific effect caused by these antibodies. Incubation of anti- $\beta 1$ mAb or a combination of anti- $\beta 1$

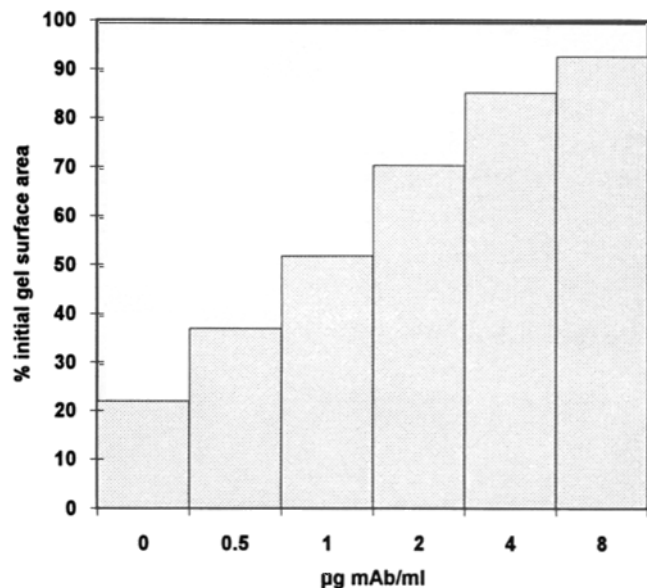


Figure 2. Dose-dependent inhibition of gel contraction by anti- $\alpha 2\beta 1$ integrin mAb. Human fibroblasts were incubated in the absence (0) or presence of a combination of mAb 4B4 (anti- $\beta 1$ integrin) and P1E6 (anti- $\alpha 2$ integrin) at final concentrations of 0.5, 1, 2, 4, and 8 $\mu\text{g}/\text{ml}$ of each mAb for 10 h as described in Fig. 1. Contraction is indicated as percentage of the initial gel surface area, shown for different concentrations of integrin mAb.

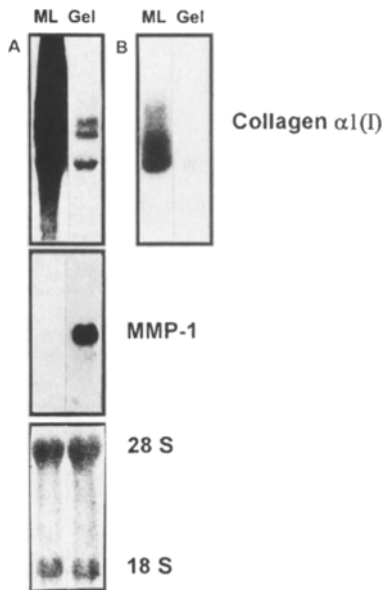


Figure 3. Regulation of collagen $\alpha 1(I)$ and interstitial collagenase (MMP-1) mRNA levels in three-dimensional collagen lattices and monolayer cultures. Total RNA was isolated from fibroblasts grown in collagen gels (*Gel*) and monolayer cultures (*ML*) for 48 h. Total RNA was separated in a denaturing agarose gel containing 0.66 M formaldehyde, blotted to nylon membranes, and hybridized with a radioactively labeled cDNA probe specific for collagen $\alpha 1(I)$ and collagenase (*MMP-1*). Transcript length is estimated relative to the migration of 18S and 28S rRNA. In the lower part of *A*, methylene blue staining of the blotted nylon membrane is shown as a control for loading equal amounts of total RNA. *B* displays a shorter exposure (5 h vs. 20 h in *A*) of the membrane hybridized with a collagen $\alpha 1(I)$ probe.

and anti- $\alpha 2$ followed by incubation with a secondary anti-IgG mAb was able to neutralize the inhibition completely. mAb directed to HLA class I antigens (mAb 1280) which were used as controls in all experiments had no influence on the contraction process (not shown).

Ligating $\alpha 1\beta 1$ Integrin with mAb Results in Enhanced Down-regulation of Type I Collagen mRNA Steady State Levels

The contraction process mediated by fibroblasts grown in three-dimensional collagen matrices is accompanied by dramatic down-regulation of collagen type I and an increase in collagenase (MMP-1) synthesis in comparison to monolayer cultures (Fig. 3). To address the question whether interfering with collagen binding to the integrins and resulting inhibition of gel contraction were accompanied by changes in transcription patterns, gene expression of collagen $\alpha 1(I)$ and collagenase (MMP-1) was studied in fibroblasts grown in three-dimensional gels in the presence of anti-integrin mAb.

Fibroblasts cultured in a three-dimensional collagen lattice and treated with a combination of mAb against $\alpha 1$ (mAb 1B3.1) and $\beta 1$ (mAb 4B4) integrin subunits showed an enhanced down-regulation of collagen type I mRNA in comparison to untreated controls (Fig. 4). Within 48 h, the signal for collagen $\alpha 1(I)$ disappeared nearly completely, whereas probing the identical RNA blot with a cDNA spe-

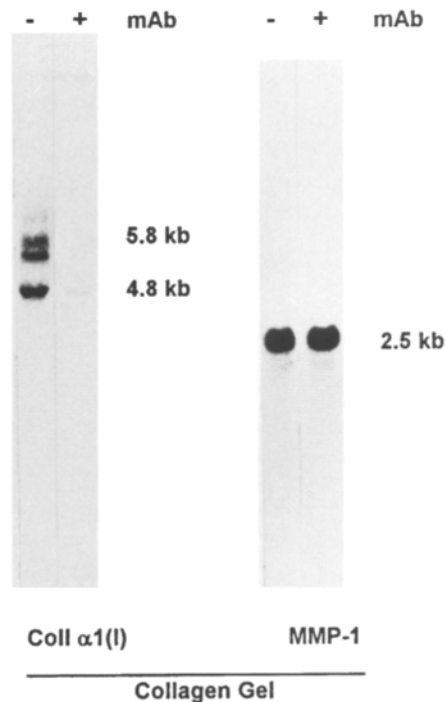


Figure 4. Regulation of collagen $\alpha 1(I)$ and interstitial collagenase (MMP-1) mRNA levels in three-dimensional collagen lattices by anti- $\alpha 1\beta 1$ integrin mAb. Total RNA was isolated from fibroblasts grown for 48 h in collagen gels incubated in the absence (-) or presence (+) of 4 $\mu\text{g/ml}$ each of monoclonal antibodies 1B3.1 and 4B4. Total RNA was separated in a denaturing agarose gel containing 0.66 M formaldehyde, blotted to nylon membranes, and hybridized with a radioactively labeled cDNA probe specific for collagen $\alpha 1(I)$ (*left*) and collagenase (*MMP-1*; *right*). Transcript length is indicated, estimated relative to the migration of 18S and 28S rRNA.

cific for interstitial collagenase (MMP-1) showed unaltered collagenase (MMP-1) transcript levels (Fig. 4). Using a different anti- $\alpha 1$ integrin-specific mAb (Ts2/7), that recognizes an epitope not overlapping with the one recognized by mAb 1B3.1, alone or in combination with mAb 4B4 (anti- $\beta 1$ integrin), had no effect on collagen $\alpha 1(I)$ or MMP-1 mRNA levels (not shown). Treating fibroblasts in three-dimensional collagen gels with either mAb against $\alpha 1$ integrin (mAb 1B3.1) or $\beta 1$ integrin (mAb 4B4) alone failed to induce any changes in MMP-1 or collagen $\alpha 1(I)$ mRNA expression, indicating that ligation of both $\alpha 1\beta 1$ integrin heterodimer subunits is a prerequisite for receptor activation. To demonstrate that the selective down-regulation of collagen $\alpha 1$ type (I) transcripts is directly related to ligating VLA-1 by $\alpha 1$ and $\beta 1$ integrin antibodies (mAb 1B3.1+4B4), we investigated the dose-dependence of this effect (Fig. 5). Collagen type I transcripts were reduced as a function of increasing amounts of the anti-integrin antibodies, when compared to the treatment with an unrelated control antibody (mAb 1280) directed to HLA class I molecules. Rehybridizing this blot to a collagenase (MMP-1) probe again showed no alteration in collagenase (MMP-1) expression, emphasizing the specificity for regulation of collagen. We further investigated the time course of the mAb-induced enhanced down-regulation of collagen $\alpha 1(I)$ by analyzing mRNA levels after 6, 10, and 24 h. The

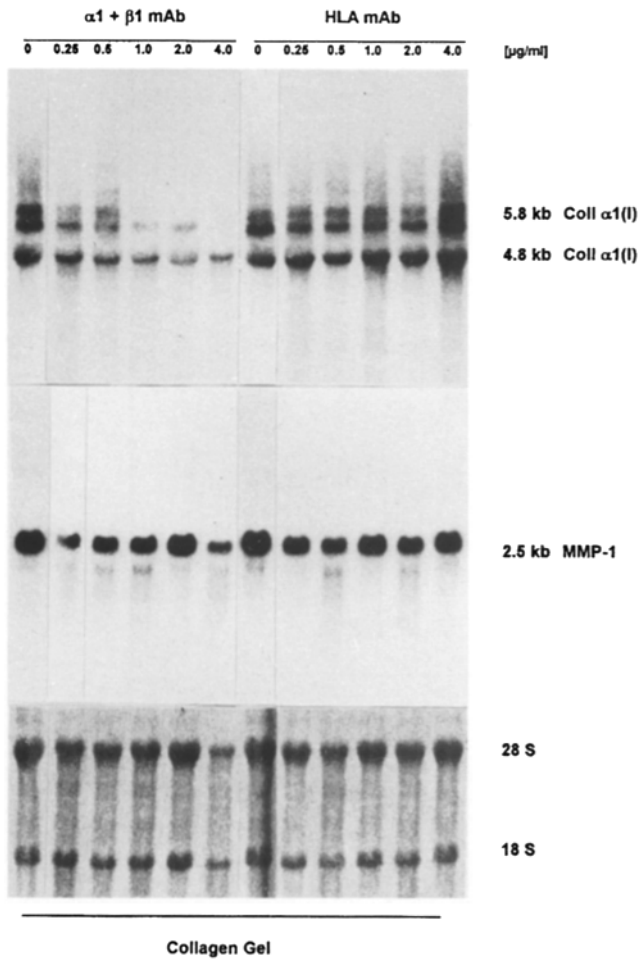


Figure 5. Dose-dependent down-regulation of collagen $\alpha 1(I)$ mRNA by anti- $\alpha 1\beta 1$ integrin mAb. Total RNA was isolated from fibroblasts cultured in three-dimensional collagen lattices incubated for 48 h with the indicated concentrations (0, 0.25, 0.5, 1.0, 2.0, and 4.0 $\mu\text{g/ml}$) each of mAb 4B4 and mAb 1B3.1 (anti- $\alpha 1 + \beta 1$ integrin) or mAb 1280 (anti-HLA-ABC). Total RNA was separated in a denaturing gel, blotted, and probed with cDNA specific for collagen $\alpha 1(I)$ or collagenase (MMP-1). Transcript length is indicated, estimated relative to the migration of 18S and 28S rRNA. In the lower parts of the panels, methylene blue staining of the blotted nylon membranes is shown as a control for loading equal amounts of total RNA.

effect appears after 10 h and still persists after 24 h (Fig. 6). These observations indicate that collagen $\alpha 1(I)$ regulation is specifically correlated to the binding of the $\alpha 1\beta 1$ integrin heterodimer by anti- $\alpha 1$ (mAb 1B3.1) and anti- $\beta 1$ antibodies (mAb 4B4).

Ligating $\alpha 2\beta 1$ Integrin Receptors with mAb Results in Superinduction of Collagenase (MMP-1) Transcription

We were further interested in finding out whether induction of MMP-1 during ECM remodeling in contracting collagen gels was correlated with a different collagen-binding integrin receptor of the $\beta 1$ family. We therefore investigated the influence of ligating $\alpha 2\beta 1$ integrin heterodimer with mAb on MMP-1 and type I collagen expression, using an analogous experimental approach.

Incubation of fibroblasts grown in a contracting collagen

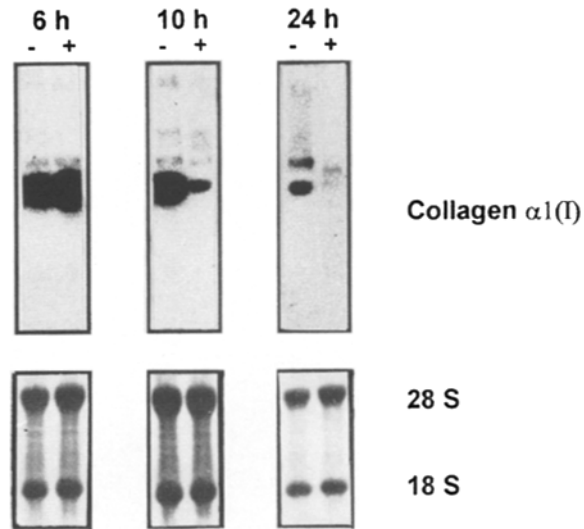


Figure 6. Time course of down-regulation of collagen $\alpha 1(I)$ mRNA by anti- $\alpha 1\beta 1$ integrin mAb. Total RNA was isolated from fibroblasts grown for different times in collagen gels (6, 10, and 24 h) in the absence (-) or presence (+) of 4 $\mu\text{g/ml}$ each of mAb 4B4 and 1B3.1 (anti- $\alpha 1 + \beta 1$ integrin) as indicated in Fig. 1. Total RNA was separated in a denaturing gel, blotted, and probed with cDNA specific for collagen $\alpha 1(I)$. In the lower parts of the panels, methylene blue staining of the blotted nylon membranes is shown as a control for loading equal amounts of total RNA.

gel in the presence of mAb against $\alpha 2$ (mAb P1E6) and $\beta 1$ (mAb 4B4) integrin exhibited enhanced induction (superinduction) of collagenase (MMP-1) transcript levels in comparison to fibroblasts grown in three-dimensional cultures in the presence of control antibodies (Fig. 7). In contrast, collagen $\alpha 1(I)$ expression was unaltered by treatment of the cells with mAb against $\alpha 2$ (mAb P1E6) and $\beta 1$ (mAb 4B4) integrins in comparison to that of cells incubated with a control antibody (mAb 1280). Applying $\alpha 2$ mAb or $\beta 1$ mAb alone to three-dimensional gels had no effect on either MMP-1 or type I collagen expression. Superinduction of MMP-1 gene expression was specifically achieved by the mAb directed against the $\alpha 2\beta 1$ receptor, as it positively correlated with antibody concentration (Fig. 8). Applying increasing amounts of anti- $\beta 1$ antibody (mAb 4B4) in combination with anti- $\alpha 2$ antibody (P1E6) resulted in increased induction of collagenase (MMP-1) transcript levels in fibroblasts cultured in collagen type I matrices (Fig. 8): the same phenomenon can be achieved by application of mAb 5E8, a further mAb directed against the $\alpha 2$ subunit, when combined with mAb 4B4. The time-course of collagenase (MMP-1) superinduction showed that the effect was evident as early as 5 h after lattice culture initiation, being most prominent at 10 h, and still persisted at 48 h (Fig. 9).

Triggering of Transmembrane Signal Transduction by Ligating Integrins with mAb Is Potentiated by the Presence of a Three-dimensional Environment

The preceding results indicate that ligating the two integrin receptors for type I collagen by mAb directed against $\alpha 1\beta 1$ and $\alpha 2\beta 1$ enhanced the normal physiological response of fibroblasts to the three-dimensional environment of a

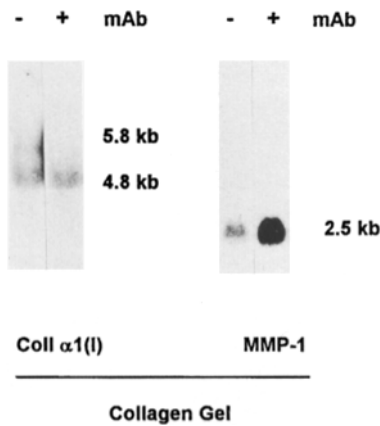


Figure 7. Expression of collagen $\alpha 1(I)$ and collagenase (MMP-1) in three-dimensional collagen lattices treated with anti- $\alpha 2\beta 1$ integrin mAb. Total RNA was isolated from fibroblasts grown in collagen gels incubated for 48 h with an unrelated control mAb (mAb 1280) (-) or with 2.5 $\mu\text{g}/\text{ml}$ each of mAb P1E6 and 4B4 (+). Total RNA was separated in a denaturing agarose gel, blotted, and hybridized with a radioactively labeled cDNA probe specific for collagen $\alpha 1(I)$ (left) and collagenase (MMP-1, right). Transcript length is indicated.

contracting collagen gel. The following experiments were undertaken to determine whether the ligation of integrins by mAb was sufficient to mimic the effects of physiological ligand in a three-dimensional independent manner. We therefore compared fibroblasts cultured in collagen gels with those maintained under analogous conditions in a conventional monolayer culture system.

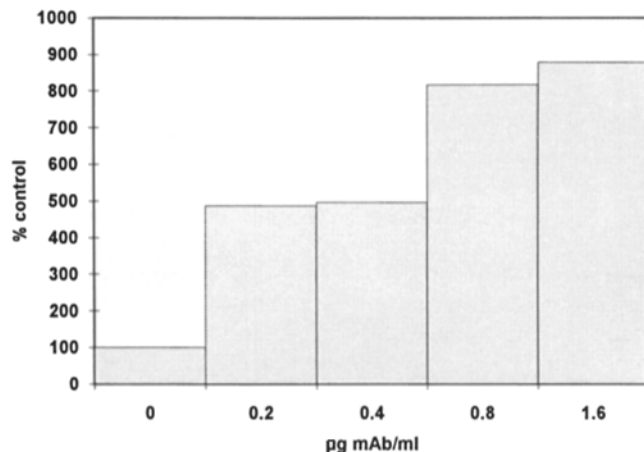


Figure 8. Dose-dependent superinduction of collagenase (MMP-1) expression by anti- $\alpha 2\beta 1$ integrin mAb. Total RNA was isolated from fibroblasts cultured in three-dimensional collagen lattices incubated for 48 h in the absence (0) or presence of increasing concentrations (0.2, 0.4, 0.8, and 1.6 $\mu\text{g}/\text{ml}$ each) of mAb 4B4 and P1E6 (anti- $\alpha 2 + -\beta 1$ integrin). Total RNA was separated in a denaturing gel, blotted, and probed with cDNA specific for collagenase (MMP-1) and GAPDH. Autoradiographs were scanned with a laser densitometer, and volume integration of signals was accomplished using ImageQuant software. Values obtained were normalized to GAPDH signal intensity and are expressed as percentage of untreated control (100%).

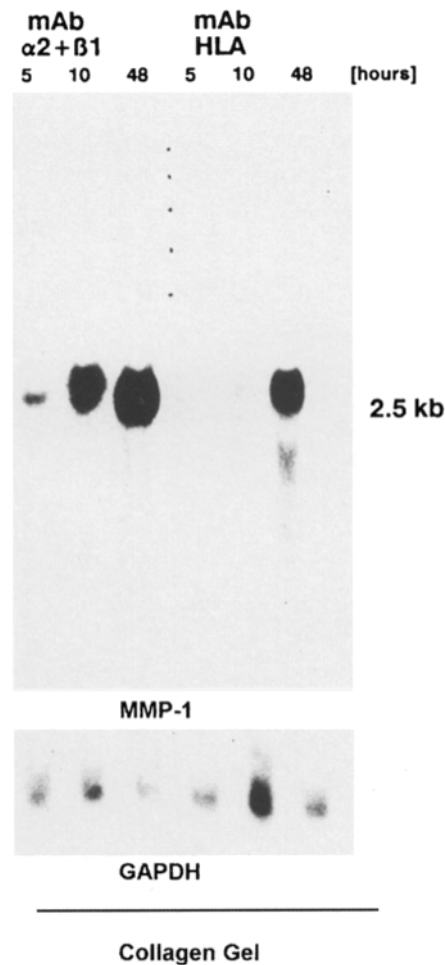


Figure 9. Time course of collagenase (MMP-1) superinduction by anti- $\alpha 2\beta 1$ integrin mAb. Total RNA was isolated from fibroblasts grown for different times in collagen gels (5, 10, and 48 h) in the presence of 2.5 $\mu\text{g}/\text{ml}$ each of mAb 4B4 and P1E6 (anti- $\alpha 2 + -\beta 1$ integrin) or mAb 1280 (anti-HLA-ABC). Total RNA was separated in a denaturing gel, blotted, and probed with cDNA specific for collagen $\alpha 1(I)$ and GAPDH as an internal standard. Transcript length is indicated.

As shown in Fig. 4, fibroblasts cultured in collagen lattices and treated with a combination of mAb against $\alpha 1$ and $\beta 1$ integrin subunits showed enhanced downregulation of collagen $\alpha 1(I)$ mRNA in comparison to untreated controls. Addition of the same mAb to fibroblast monolayer cultures seeded on uncoated dishes did induce changes in $\alpha 1(I)$ collagen expression that are similar but less prominent than the effects observed in a three-dimensional context (Fig. 10). In particular, laser-scanning densitometry revealed that the combination of mAb against $\alpha 1$ and $\beta 1$ integrin reduced type I collagen transcript levels in monolayers to 53% of the untreated control, in contrast to a reduction to 13% observed in three-dimensional systems. In analogy, combined mAb against $\alpha 2$ and $\beta 1$ integrin induced an increase in MMP-1 expression (Fig. 10) which was 331% in comparison with untreated controls, whereas in collagen gels an up to 10-fold induction was observed.

In addition, fibroblasts were seeded as monolayer culture on dishes coated with planar collagen type I substrates.

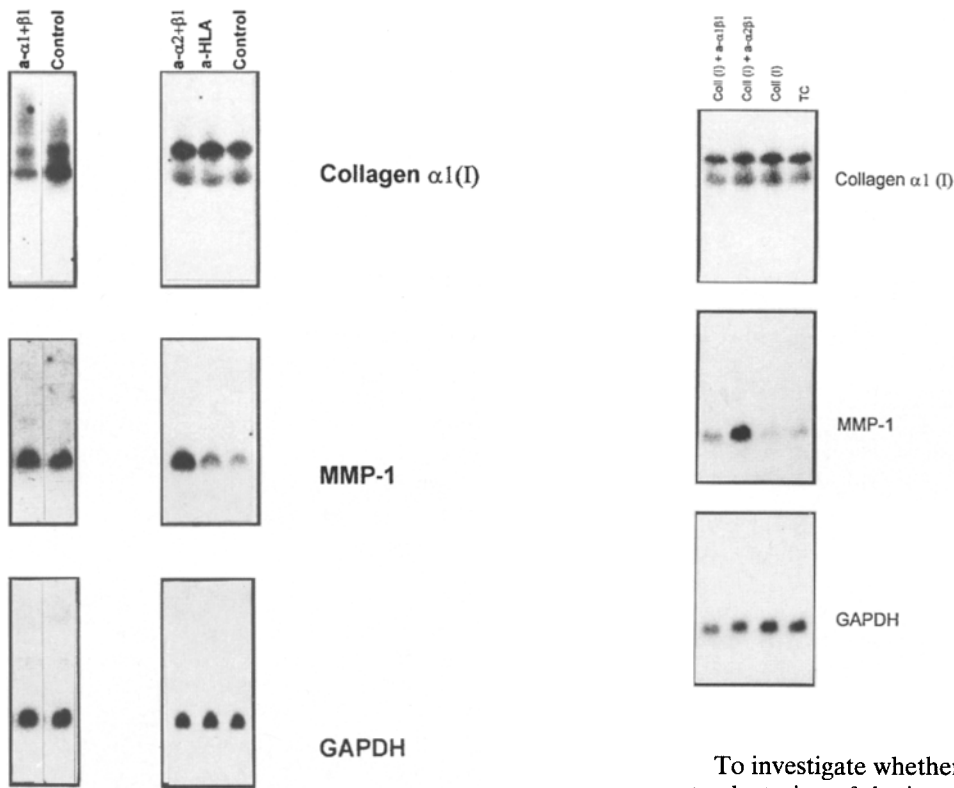


Figure 10. Regulation of collagen $\alpha 1(I)$ and interstitial collagenase (MMP-1) mRNA levels in fibroblasts grown in monolayer cultures by mAb directed against integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. Total RNA was isolated from fibroblasts grown for 24 h on tissue culture dishes as monolayer cultures incubated in the presence of a combination of monoclonal antibodies mAb P1E6 and mAb 4B4 (anti- $\alpha 2$ and $-\beta 1$ integrin), or mAb 1B3.1 and mAb 4B4 (anti- $\alpha 1$ and $-\beta 1$ integrin) or mAb 1280 (anti-HLA-ABC) or in the absence of any mAb (control). Fibroblasts were preincubated with mAb at a concentration of 40 $\mu\text{g/ml}$ each in DME, 10% FCS for 30 min at 37°C, and then diluted 10-fold to final concentrations of 4 $\mu\text{g/ml}$ each, or without antibody addition for 48 h. Total RNA was separated electrophoretically, blotted, and hybridized to radioactively labeled cDNA probe specific for collagen $\alpha 1(I)$, collagenase (MMP-1), and GAPDH as internal standard.

These revealed nearly identical effects as those observed on uncoated tissue culture dishes. As shown in Fig. 11, planar collagen type I is neither sufficient to induce MMP-1 expression nor to down-regulate collagen type I mRNA steady state levels, as indicated by the comparison of signals in Northern blot analysis of fibroblasts cultured on uncoated (Fig. 11, lane *Coll I*) and on collagen type I-coated dishes (Fig. 11, lane *TC*). In contrast, when fibroblasts were incubated with anti- $\alpha 1\beta 1$ or anti- $\alpha 2\beta 1$ mAb and plated on planar collagen, only a slight decrease in collagen type I and an increase in MMP-1 expression, respectively, was noted, similar to the results observed on uncoated dishes. These results indicate that ligation of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ by antibodies principally produces the same response in fibroblasts grown as monolayers that is achieved physiologically by culture within a three-dimensional lattice, but that this response is strongly potentiated by the presence of a three-dimensional environment.

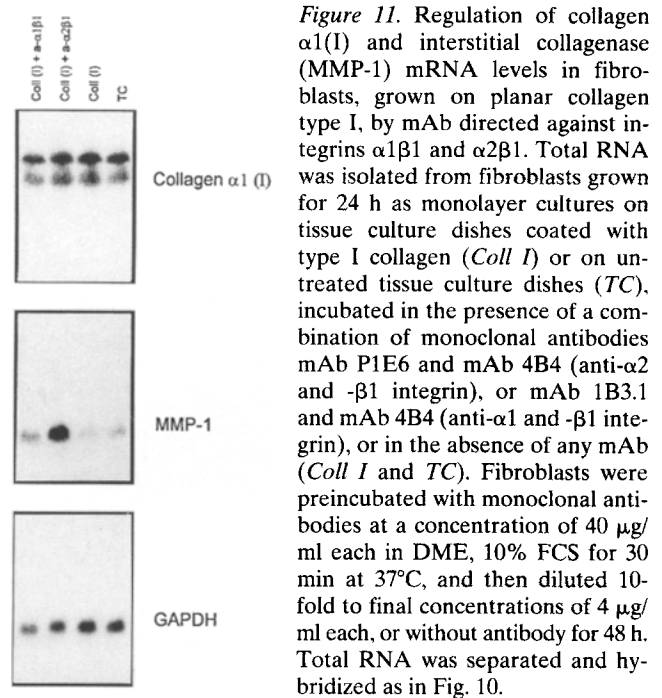


Figure 11. Regulation of collagen $\alpha 1(I)$ and interstitial collagenase (MMP-1) mRNA levels in fibroblasts, grown on planar collagen type I, by mAb directed against integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. Total RNA was isolated from fibroblasts grown for 24 h as monolayer cultures on tissue culture dishes coated with type I collagen (*Coll I*) or on untreated tissue culture dishes (*TC*), incubated in the presence of a combination of monoclonal antibodies mAb P1E6 and mAb 4B4 (anti- $\alpha 2$ and $-\beta 1$ integrin), or mAb 1B3.1 and mAb 4B4 (anti- $\alpha 1$ and $-\beta 1$ integrin), or in the absence of any mAb (*Coll I* and *TC*). Fibroblasts were preincubated with monoclonal antibodies at a concentration of 40 $\mu\text{g/ml}$ each in DME, 10% FCS for 30 min at 37°C, and then diluted 10-fold to final concentrations of 4 $\mu\text{g/ml}$ each, or without antibody for 48 h. Total RNA was separated and hybridized as in Fig. 10.

To investigate whether the observed effects could be due to clustering of the integrins via cross-linking by mAb, we investigated whether adhesion of fibroblasts to integrin mAb was able to induce similar effects. Therefore, fibroblasts were seeded on integrin mAb-coated dishes to induce clustering of integrins at focal plaques. Both collagen and MMP-1 expression were unaltered in fibroblasts cultured for 6 h on tissue culture dishes that had been coated with mAb directed against the $\alpha 1$, $\alpha 2$, $\beta 1$, $\alpha 1 + \beta 1$, $\alpha 2 + \beta 1$ (not shown), indicating that regulation of these two transcript species does not primarily depend upon integrin clustering.

To further exclude the possibility that the observed effects on collagen I and MMP-1 expression in three-dimensional collagen gels could be due to clustering of integrins, by, e.g., the $\beta 1$ mAb, we investigated the effect of clustering integrins by secondary antibodies. Fibroblasts were incubated with mAb 1B3.1 (anti- $\alpha 1$ integrin) or mAb P1E6 (anti- $\alpha 2$ integrin) or mAb 4B4 (anti- $\beta 1$ integrin), followed by incubation with an anti-IgG or anti-IgA, respectively, before being seeded within the three-dimensional environment of a collagen gel. The experimentally induced clustering of $\beta 1$ -integrins did not result in significant alterations in MMP-1 or collagen I expression in comparison to control cultures and did not display the decrease in collagen mRNA achieved by anti- $\alpha 1$ and $\beta 1$, or the MMP-1 superinduction seen with mAb 4B4 and P1E6 (anti- $\alpha 2$ and $-\beta 1$) (Fig. 12).

Remodeling of Collagen Lattices by Fibroblasts Depends upon Protein Kinase Activity and Involves Different Signal Transduction Pathways

To elucidate the role of various signal transducing pathways on gel contraction and expression of $\alpha 1(I)$ collagen and MMP-1, the following inhibitors were investigated: genistein, herbimycin A, vanadate, and calphostin C.

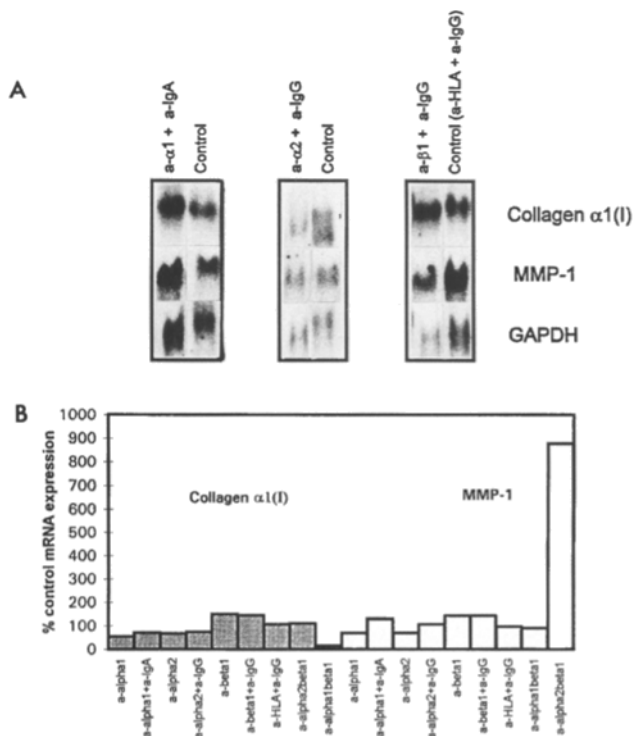


Figure 12. Effect of clustering integrin subunit chains $\alpha 1$, $\alpha 2$, and $\beta 1$ on collagen $\alpha 1(I)$ and collagenase (MMP-1) mRNA expression in three-dimensional collagen lattices. Clustering of integrins was experimentally induced by incubating fibroblasts with a secondary anti-immunoglobulin antibody after preincubation with a primary anti-integrin antibody. Total RNA was isolated from fibroblasts grown for 20 h in three-dimensional collagen lattices, incubated in the absence (*control*) or presence of monoclonal antibodies mAb 1B3.1 (anti- $\alpha 1$ integrin), mAb P1E6 (anti- $\alpha 2$ integrin), mAb 4B4 (anti- $\beta 1$ integrin), or mAb 1280 (anti-HLA-ABC) alone, or followed by incubation with a secondary antibody (anti-IgG and anti-IgA, respectively). Fibroblasts were preincubated with monoclonal antibodies at a concentration of 40 $\mu\text{g/ml}$ each in DME, 10% FCS for 15 min at 37°C, followed by a further 15 min incubation with a 10-fold excess of secondary antibody after a wash with PBS if indicated, and then diluted 10-fold to final concentrations of 4 $\mu\text{g/ml}$ of primary mAb or without antibody addition for 48 h. Total RNA was separated electrophoretically, blotted, and hybridized to radioactively labeled cDNA probe specific for collagen $\alpha 1(I)$, collagenase (MMP-1), and GAPDH as internal standard (A). Autoradiographs were scanned with a laser densitometer, and volume integration of signals was accomplished using ImageQuant software. Values obtained were normalized to GAPDH signal intensity, and untreated control cultures were set at 100% (B).

Genistein at 300 μM significantly inhibited the contraction process when compared with untreated cultures (Fig. 13). Quantification of mRNA levels of genistein-treated fibroblasts cultured in collagen gels revealed that in comparison with untreated controls, down-regulation of type I collagen message was not significantly altered, whereas induction of collagenase (MMP-1) expression was nearly completely inhibited (Fig. 13 A). Addition of mAb against $\alpha 2$ (mAb P1E6) and $\beta 1$ (mAb 4B4) integrin which mediates superinduction (see Fig. 7) failed to rescue the genistein effect (not shown). These observations suggest

that lack of collagenase (MMP-1) induction might be related to signaling events downstream of integrin ligand binding.

In contrast to genistein, the tyrosine kinase inhibitor, herbimycin A, displayed no effect on any of the three processes (Fig. 13), indicating the lack of involvement of *src* kinases and other protooncogene products. Similarly, vanadate, a potent tyrosine-specific protein phosphatase inhibitor, showed no effect (Fig. 13). In contrast, PKC appears to play a role in gel retraction since this was inhibited by the highly specific inhibitor of PKC, calphostin C, but had no effect on collagen $\alpha 1(I)$ or MMP-1 mRNA levels (Fig. 13).

In all cases, the observed effects were a function of inhibitor concentration (dose-dependent). Cytotoxic effects could be excluded since lactate dehydrogenase activity was not elevated in supernatants of the collagen gel cultures at the effective concentrations in comparison with untreated controls (data not shown).

Discussion

Reorganization of extracellular matrix occurs in any multicellular system. It is characterized by degradation of old and deposition of new collagenous and non-collagenous proteins. Collagen represents a family of many types, and collagen I is probably the most abundant component in vertebrate connective tissue. The homeostasis of fibroblast collagen metabolism is regulated in a complex manner by an interplay of various different mechanisms which include hormones and cytokines, but also cell-matrix interactions (for review see Grinnell, 1994). In this study, the role of different integrin receptors in mediating such cell-ECM interactions during ECM remodeling by human dermal fibroblasts was analyzed.

Using three-dimensional contracting collagen lattices as an *in vitro* model, binding of integrin receptors to type I collagen was perturbed by dispersing fibroblasts into the matrix in the presence of antibodies directed to $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\beta 1$ integrin subunits. In particular, effects on lattice contraction as well as on gene expression of $\alpha 1(I)$ collagen and interstitial collagenase (MMP-1) resulting from ligating single individual subunits or both chains of a receptor were studied. Distinct and different functions could be assigned to the two collagen receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$, suggesting that the two integrins mediate the transmembrane transduction of signals that ultimately leads to changes in gene expression patterns of fibroblasts during ECM remodeling in contracting collagen gels.

Using this approach, $\alpha 1$ and $\alpha 3$ integrins were found to play a rather negligible role in lattice contraction, whereas $\alpha 2$ and $\beta 1$ integrin mAb inhibited the contraction process significantly, indicating that the $\alpha 2\beta 1$ integrin heterodimer is involved in lattice contraction. This observation is consistent with elevated protein levels of $\alpha 2$ and $\beta 1$, but not $\alpha 1$ or $\alpha 3$ integrin (Klein et al., 1991) found in fibroblasts in contracting lattices. Interestingly, no antibody applied individually or in combination resulted in a complete inhibition of gel contraction, indicating that within the fibroblast receptor repertoire some redundancy for binding of type I collagen may exist, and moreover, that besides integrins other collagen receptors are likely to be involved in this process.

The observation that contraction is inhibited most strongly

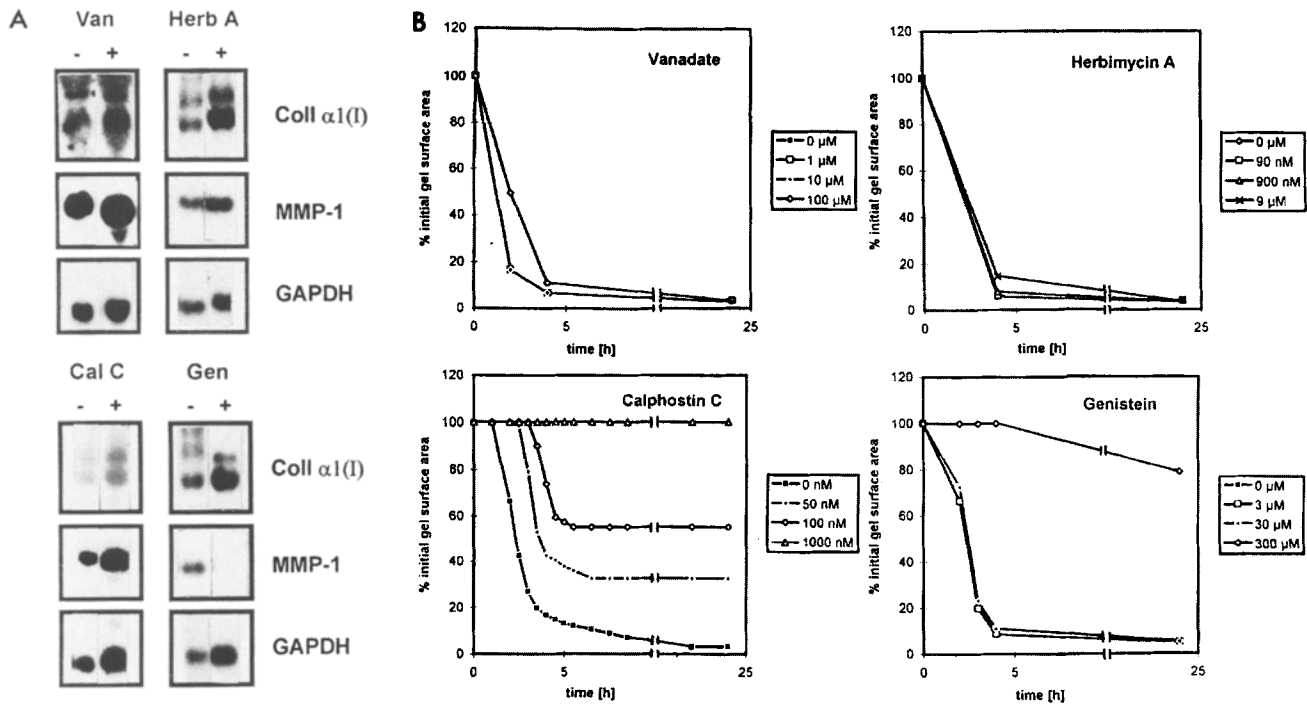


Figure 13. Effect of signal transduction inhibitors on collagen gel retraction, and expression of collagen $\alpha 1(I)$ and MMP-1. Inhibitors were applied to fibroblasts cultured in contracting collagen gels for 24 h. Influence on contraction was assessed by comparison of gel surface areas. (A) Influence on gene expression was analyzed by Northern blot analysis. Total RNA was separated electrophoretically, blotted, and hybridized to radioactively labeled cDNA probes specific for collagen $\alpha 1(I)$, collagenase (MMP-1), and GAPDH as internal standard. Final concentrations were as follows: vanadate (*Van*) 100 μM , herbimycin A (*Herb A*) 9 μM , calphostin C (*Cal C*) 50 nM, genistein (*Gen*) of 300 μM . (B) Dose-dependent influence of signal transduction inhibitors on lattice contraction.

in the presence of combined anti- $\alpha 2$ and - $\beta 1$ mAb rather than by either one alone suggests a cooperative effect of the two subunits. This is consistent with the finding that both integrin chains possess sites of ligand interaction (for review see Haas and Plow, 1994).

The mechanisms underlying inhibition of lattice contraction by antibodies ligating integrin subunits are as yet not fully understood. Steric hindrance of ligand binding by the added mAb appears unlikely because this effect could be neutralized by incubation with secondary antibodies. Binding of collagen I to $\alpha 2\beta 1$ integrin therefore seems to be crucial but not sufficient for contraction to proceed. The interaction of anti-integrin mAb could disturb the contraction process in a more complex way, e.g., modulating affinity by changes in spatial conformation. Our results are in good agreement with data obtained from transfecting $\alpha 2$ constructs which carried a mutation in the cytoplasmic domain, rendering the receptor capable of ligand binding, but incapable of transmitting the signal for lattice contraction (Schiro et al., 1991; Chan et al., 1992). Their data and ours strongly suggest $\alpha 2\beta 1$ integrin to be the only integrin receptor responsible for contraction of collagen gels by fibroblasts. However, the involvement of other nonintegrin receptors cannot be excluded.

In addition to mediating lattice contraction, $\alpha 2\beta 1$ integrin could be shown to trigger the pathway resulting in elevated transcript levels of interstitial collagenase (MMP-1). Based on previous observations (Mauch et al., 1989), increased mRNA levels are assumed to correlate with an in-

crease in MMP-1 activity. Interfering with $\alpha 1\beta 1$ function, in contrast, had no effect on collagenase (MMP-1) gene expression, which indicates that $\alpha 2\beta 1$ is the receptor mediating cell-matrix interactions that result in the intracellular events of MMP-1 induction.

Analyzing receptor function and signaling by antibody perturbation has become a widely used tool in cell biology. In many instances, antibodies physically interfere with cell adhesion. However, they do not necessarily inhibit all other functions performed by the receptor. In fact, antibody binding can even trigger signal transduction involving adhesion molecules, resulting in a mixed cellular response (for review see Gumbiner and Yamada, 1993). Certain mAb have been shown, among other external factors, to up-regulate the function of $\beta 1$ integrins (for review see Hynes, 1992). These mAb most likely induce a conformational change of the receptor that increases the affinity/avidity for its ligands, or act as ligands by themselves. There is increasing evidence that integrins undergo a transition between different activation states that can be induced or conserved by mAb (Hynes, 1992). Such antibody-induced changes in receptor conformation have become an accepted concept explaining variable affinity states of integrin receptors (Arroyo et al., 1993; O'Toole et al., 1991; Gailit and Ruoslahti, 1988).

Since the effects that occur in untreated control lattices are enhanced by antibody ligation (i.e., even greater reduction of collagen synthesis after blocking $\alpha 1\beta 1$ by mAb 1B3.1 and 4B4, and increased induction of collagenase (MMP-1)

synthesis by blocking $\alpha 2\beta 1$ with mAb P1E6 and 4B4), we postulate that regarding synthesis of collagen and collagenase (MMP-1), these antibodies mimic the effects of physiologic ligands, in addition to their property of inhibiting cell adhesion to collagen in monolayer cultures.

A similar example has recently been described by Symington et al. (1993) who found that mAb P1B5 directed against $\alpha 3$ in VLA-3 inhibits keratinocyte adhesion to epiligrin, yet induces intercellular adhesion by activating $\alpha 3\beta 1$ and accelerating an ongoing process by mimicking the physiologic trigger. In our hands, ligating $\alpha 3\beta 1$ receptor had no effect on contraction or collagen and collagenase (MMP-1) synthesis.

Induction of interstitial collagenase (MMP-1) and stromelysin had previously been achieved by ligation of the fibronectin receptor in rabbit synovial fibroblasts (Werb et al., 1989). In contrast to our results, addition of adhesion inhibiting antibodies directed to the $\beta 1$ subunit was sufficient to mediate this effect in an RGD-dependent fashion. Unlike $\alpha 5\beta 1$, the collagen receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$ recognize sequence motifs in collagen other than RGD (Gullberg et al., 1992). In particular, the induction of collagenase (MMP-1) by anti-fibronectin receptor mAb differed from results presented here, in that receptor ligation did not reproduce the effects of the natural ligand, as native fibronectin does not induce collagenase (MMP-1). In this context, a recent report by Pacifici and co-workers (1994) is of particular interest to those who mention that binding of soluble fibronectin to $\alpha 5\beta 1$ integrin activates the $\alpha 2\beta 1$ integrin receptor and potentiates the normal response of $\alpha 2\beta 1$ integrin ligand binding.

The gene regulatory effects that are induced by the presence of specific mAb can also be detected to a certain extent in a two-dimensional environment (i.e., fibroblasts seeded as monolayer cultures on plastic or planar collagen). However, significant down-regulation of type I collagen and induction of collagenase (MMP-1) synthesis by mAb is only seen in the three-dimensional lattice system, indicating that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin signaling during ECM remodeling is strongly influenced by three-dimensional matrix structure. In vivo, integrins are assembled into rather immobile structures triggering persistent signals with subtle modulation around a baseline value. Such "on-off" effects, like induction of collagenase (MMP-1) and down-regulation of type I collagen, occur naturally during ECM remodeling, reflected by the dynamic character of contracting collagen gels. This observation makes it appear likely that transducing the information three-dimensional structure depends on a three-dimensional conformation of the relevant receptors and/or its ligand. This is consistent with the common concept that activation of integrin receptors is mediated by conformational changes (Adams and Watt, 1990; Hotchin and Watt, 1992; Takada and Puzon, 1993).

Obviously, a combination of mAb directed against both subunits of a particular receptor is essential for maintaining an active receptor state for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in contracting collagen gels. We could exclude that receptor clustering (by simply cross-linking with the anti- $\beta 1$ mAb or the anti- $\alpha 1$ or - $\alpha 2$ mAb) is the reason for that finding, since experimentally induced clustering with a secondary antibody did not result in alterations of MMP-1 or collagen type I mRNA levels. Interestingly, Miyamoto and co-work-

ers (1995) were recently able to demonstrate synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. In the case of the fibronectin receptor $\alpha 5\beta 1$, they were able, using different functional mAb for integrin chains, to distinguish between receptor occupancy, which induced receptor redistribution but only minimal phosphorylation signaling, and aggregation, which resulted in phosphorylation signaling of, e.g., focal adhesion kinase and tensin. Only the combination of both clustering and ligand occupancy was able to mimic the physiologic multivalent interaction with fibronectin. They concluded that most integrin transmembrane effects require both ligand occupancy and clustering of integrin receptors. This could also provide an explanation for our observations, namely the requirement for a combination of $\beta 1$ - and $\alpha 1$ or $\alpha 2$ -integrin subunit ligation, respectively, to mimic the physiologic response to the natural ligand collagen. According to this concept, the anti- $\beta 1$ mAb would induce aggregation, but this signal alone would not be sufficient for transmembrane signaling inducing regulation of collagen I and MMP-1, whereas the anti- α integrin mAb would only induce receptor occupancy. Only the combination of both could then trigger the physiologic response. This would be consistent with the concept that the $\beta 1$ subunit is responsible for the localization of integrins into sites of focal adhesion, while the α -subunit seems to suppress this function until ligand occupancy releases constraints on receptor localization (Ylänne et al., 1993).

Further experiments were designed to investigate the signal transduction pathways triggered by the collagen-integrin binding. Therefore, different signaling inhibitors were applied to fibroblasts in contracting collagen lattices. The data indicated that different, independent intracellular pathways for signaling by $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin must exist, controlling ECM remodeling with induction of MMP-1 and down-regulation of type I collagen (Fig. 13). The inhibition of lattice contraction by genistein and calphostin C suggests the involvement of tyrosine-specific protein kinases and PKC in this process. The dependence of the contraction process on PKC is consistent with reports by Guidry and co-workers (1993). Besides its effect on contraction, genistein inhibits the induction of MMP-1, but not the down-regulation of type I collagen. The differences in genistein action (i.e., blockage of $\alpha 2\beta 1$ mediated collagenase (MMP-1) induction, but lack of effect on regulation of type I collagen expression) strongly suggest that these two processes involve distinctly differing signal cascades. This would provide a sensitive mechanism to guarantee the subtle temporal and spatial control required for separating ECM synthesis and degradation (for review see Gailit and Clark, 1994). Interestingly, herbimycin A, another tyrosine-specific protein kinase inhibitor, did not display the effect on collagenase regulation, nor did it affect collagen regulation or lattice contraction. Furthermore, tyrosine-specific phosphatases are not involved in any of these three processes. This is consistent with emerging evidence that intracellular integrin signaling in general is connected with kinase cascades (for review see Juliano and Haskill, 1993). The differential influence of the different inhibitors on the three processes analyzed suggests that different pathways for all three events exist, and that one distinct integrin can control several distinct downstream

pathways. Moreover, these observations indicate that the three processes are not per se dependent upon each other.

This work supports the concept that integrins are used by cells as cell surface receptors to interact with components of the ECM by transducing specific signals from the matrix into the cell, thereby being responsible for modifying the three-dimensional structure of the surrounding ECM.

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