Extracellular Matrix Alters PDGF Regulation of Fibroblast Integrins

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Abstract. Extracellular matrix (ECM) and growth factors are potent regulators of cell phenotype. These biological mediators of cellular responses are potentially interactive and as such could drive cells through progressive phenotypes to create new tissue as in morphogenesis and wound repair. In fact, ECM composition changes during tissue formation accompanied by alterations in cell growth and migration. How alterations in the ECM regulate cell activities is poorly defined. To address this question in wound repair, we cultured normal human dermal skin fibroblasts in relaxed collagen gels, fibronectin-rich cultures or stressed fibrin gels, and stressed collagen gels to model normal dermis, early wound provisional matrix, and late granulation tissue, respectively. Integrin subunits, α_2, α_3, and α_5, that define receptor specificity for collagen and provisional matrix, respectively, were measured at mRNA steady-state level before and after stimulation with platelet-derived growth factor-BB (PDGF-BB), a potent mitogen and chemoattractant for fibroblasts. Fibronectin-rich cultures and fibrin gels supported PDGF-BB induction of α_3 and α_5 mRNA. In contrast, both stressed and relaxed collagen attenuated these responses while promoting maximal α_2 mRNA expression. Posttranscriptional regulation was an important mechanism in this differential response. Together PDGF-BB and collagen gels promoted α_2, but not α_3 and α_5, mRNA stability. Conversely, when fibroblasts were in fibronectin-rich cultures, PDGF-BB promoted α_3 and α_5, but not α_2, mRNA stability. We suggest that ECM alterations during wound healing or any new tissue formation cause cells to respond differently to repeated growth factor stimuli. An ordered progression of cell phenotypes results, ultimately consummating tissue repair or morphogenesis.

DURING cutaneous wound repair, cells must attach to new matrix molecules and migrate (Welch et al., 1990). Soon after wounding, a provisional matrix of fibronectin, fibrinogen, fibrin, and vitronectin forms in the wound area (Gailit and Clark, 1994*a*). In the fibrin network, platelets release growth factors such as PDGF that stimulate proliferation and chemoattraction of fibroblasts (Deuel et al., 1991). Activated fibroblasts must move from surrounding collagenous connective tissues into a fibrin/fibronectin-filled wound and subsequently synthesize new collagenous matrix. Clearly, fibroblast responses to these extracellular matrix (ECM)[1] molecules and growth factors are essential for the healing process to progress.

To address the mechanisms by which ECM molecules regulate cell activities, three-dimensional ECM-based culture systems are increasingly used. Since type I collagen is a ubiquitous component of dermal ECM much attention has been devoted to cells cultured in stressed and/or relaxed collagen gels. The relaxed collagen gel is considered a reconstituted dermal model whereas the stressed gel is similar to late granulation tissue (Grinnell, 1994). Compared to fibroblast cultures on plastic, cells in a relaxed collagen gel exhibit little proliferative response to PDGF (Lin and Grinnell, 1993) and little collagen production (Eckes et al., 1993). Nevertheless, cells harvested from stressed collagen gels proliferate and synthesize collagen robustly. Collagen matrices can affect fibroblasts function by altering both gene expression and second messenger pathways. Fibroblasts in collagen gels that are stimulated with PDGF decrease α_1(I) collagen gene expression (Berthod et al., 1994; Eckes et al., 1993), increase collagen receptor gene expression (Klein et al., 1991), and decrease autophosphorylation of PDGF receptors (Lin and Grinnell, 1993). In contrast, fibroblasts in fibrin gels or in fibronectin-rich tissue culture conditions, proliferate robustly and produce exuberant ECM (Clark et al., 1995*a*; Clark, R. A. F., G. A. McCoy, J. M. Folkvord, and J. McPherson, manuscript submitted for publication; Grinnell et al., 1989; Nakagawa et al., 1989).

The molecules primarily responsible for cell adherence to ECM are integrins, a family of cell surface receptors. Integrins are heterodimers composed of an α chain and a β chain. β_1 integrin can form a complex with at least ten different α subunits, namely α_1-α_9 and α_v (Hynes, 1992; Haas

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; RGD, arginine-glycine-aspartic acid; TGF-β1, transforming growth factor-β1.

and Plow, 1994). Among them, $\alpha_2\beta_1$ is a receptor for various collagen types and laminin. $\alpha_3\beta_1$ binds to the arginine-glycine-aspartic acid (RGD) sequence in fibronectin and laminin. $\alpha_3\beta_1$ interaction with native collagen is controversial and at best a weak interaction (Pfaff et al., 1993; Yamamoto and Yamamoto, 1994). $\alpha_5\beta_1$ is specific for the fibronectin RGD sequence.

The expression of integrins is regulated by both growth factors and ECM proteins. For example, transforming growth factor-β1 (TGF-β1), released from platelets after wounding, upregulates β1 and β3 integrin receptors (Enenstein et al., 1992; Heino et al., 1989; Heino and Massague, 1989). PDGF-BB, also released from platelets, stimulates gene expression of integrin β_1 in Swiss 3T3 cells (Bellas et al., 1991), α_5 and β_3 in aortic smooth muscle cells (Janat et al., 1992) and α_2 in human foreskin fibroblasts (Ahlen and Kristofer, 1994). Type I collagen upregulates α_2 integrin mRNA level in human fibroblast and melanoma cell lines (Klein et al., 1991). Thus, the level of specific integrins is probably controlled by interactive signals from both ECM molecules and growth factors in the pericellular environment.

The global goal of our laboratory is to investigate how variations in ECM composition during wound healing correlates with changes in cell growth and migration. We hypothesized that the alteration in ECM during wound healing differentially regulates cell response to cytokines such as PDGF, resulting in a progression of cell phenotypes necessary for the ordered healing of wounds. Specifically, we hypothesize that fibrin and collagen differentially regulate integrin gene expression, which in turn affects how fibroblasts physically and biochemically relate to ECM. Multiple cycles of these events would lead to a progression of cell-ECM interaction.

To test our hypothesis, in vitro systems to model different stages of wound repair were established. Relaxed collagen gels, stressed fibrin gels or fibronectin-rich cultures, and stressed collagen gels were used to model normal dermis, early wound provisional matrix, and late granulation tissue, respectively. Steady-state levels of specific integrin mRNAs, collagen integrin subunit, α_2, and provisional matrix integrin subunits, α_3 and α_5, were analyzed in these different ECM environments. By adding PDGF-BB to these culture systems, we found that fibrin gels and fibronectin-rich cultures supported induction of provisional matrix receptor α_3 and α_5 mRNAs while collagen gels facilitated induction of collagen receptor α_2 mRNAs and attenuated provisional matrix receptor responses. Thus fibroblasts surrounded by fibrin respond to PDGF by expressing receptors necessary for interaction with the wound provisional matrix. In contrast fibroblasts in collagen increase their collagen receptors and fail to support PDGF-induction of provisional matrix receptors. Finally, PDGF-BB was able to prolong half-lives of α_3 and α_5 mRNAs in stressed fibrin gel. Collagen gels failed to support this response. In contrast, α_2 mRNA stability was greatly increased by PDGF-BB when the cells were cultured in collagen but not when cultured on plastic. These results suggest that coordinate signals from PDGF-BB and fibrin increase the epigenetic pressure in fibroblasts to express provisional matrix receptors which may be one of the inductive processes of granulation tissue formation. In addition, one new mechanism, modulation of integrin mRNA decay rate, is found to greatly alter integrin mRNA steady state.

Materials and Methods

Cell Culture

Human fibroblasts cultures were established by outgrowth from healthy human skin biopsies. The cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD), supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, 100 U/ml streptomycin (GIBCO BRL), and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells between population doubling levels (PDL) 15 and 20 (the 6th and 10th passage) were used for the experiments.

Preparation of Collagen and Fibrin Gels

Collagen gels were prepared with pepsin-solubilized bovine dermal collagen dissolved in 0.012 M HCl (Vitrogen 100). The bovine skin collagen is 99.9% pure containing 95–98% type I collagen and type III as the remainder (Cetrix Laboratories, Palo Alto, CA). Collagen for cultures was prepared by mixing 2.0 mg/ml of type I collagen, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% FCS in DMEM at pH 7.0–7.4. Human dermal fibroblasts from subconfluent cultures were mixed with 10 ml of collagen solution for a final concentration of 5×10^5 cells/ml. The collagen cell suspension (4 ml) was immediately placed onto 60-mm tissue culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) and incubated for 2 h at 37°C before the addition of 5 ml of 10% FCS/DMEM to each dish. Relaxed collagen gels were obtained by detaching the gels from the plate after polymerization and they were allowed to float in the medium. Stressed gels remained anchored to the culture dishes.

Fibrin for cultures was prepared by mixing 3.0 mg/ml human plasma fibrinogen (>98% clottable proteins and homogenous by SDS-PAGE, Calbiotech, La Jolla, CA), 100 U/ml penicillin, 100 U/ml streptomycin, and 10% FCS in DMEM. 0.1 N HCl was added to adjust pH at 7.0–7.4. Human dermal fibroblasts from subconfluent cultures were mixed with the fibrinogen solution for a final concentration of 5×10^5 cells/ml. Immediately thereafter human thrombin (GIBCO BRL) was added at 0.2 U/ml. The fibrin(ogen) cell suspensions (4 ml) were placed onto 60-mm petri dishes (Falcon). After gentle stirring, gels formed in less than 5 min. After 2 h of incubation at 37°C, 5 ml of 10% FCS/DMEM was added.

After incubation at 37°C in 95% air, 5% CO_2 and 100% humidity for 24 h, the cultures were carefully washed two times in DMEM, switched to 1.0% FCS/DMEM and remained in the same media for 4 d with medium change every 2 d. In experiments with PDGF-BB (ZymoGenetics, Seattle, WA), the growth factor was added on the fourth day of culturing. All subsequent experiments were performed according to specific conditions.

PDGF-BB and Platelet Releasate

Sterile, endotoxin-free, recombinant BB isoform of PDGF was kindly provided by Charles Hart (Zymogenetics, Seattle, WA) (Hart et al., 1988).

Platelets were isolated from pooled human blood according to previously described methods (Knighton et al., 1986) and kindly provided by Curative Technology Inc. (East Setauket, NY). The platelet-containing pellet was resuspended in platelet buffer which contained 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes), 100 mM NaCl, 6 mM KCl and 3 mM glucose to a final platelet concentration of 5×10^9 platelets/ml. Bovine thrombin (Thrombinar, Armour Pharmaceutical, Kankakee, IL) was then added at a concentration of 1 U/10^9 platelets and the thrombin platelet mixture incubated at room temperature for 10 min. After granule release, the aggregated platelets were resuspended by aspiration and centrifuged for 10 min at 2,000 *g* at room temperature. The supernatant containing the released platelet factors was retained and diluted 1:10 in DMEM. PDGF concentrations in the platelet releasate were determined using a sandwich EIA with anti-PDGF from Collaborative Research Inc. (Bedford, MA) which recognizes all PDGF isoforms, and PDGF-AB (Boehringer Mannheim, Indianapolis, IN) for the standard curve.

RNA Isolation and Northern Blot Hybridization

Total RNA was isolated from monolayers and gel cultures using a modification of guanidinium thiocyanate method (Chromczynski and Sacchi, 1987). After stressed gel cultures were spun in a microcentrifuge at 14,000 *g* to remove H_2O, gel cultures of both types were dissolved in 4 M guanidinium isothiocyanate and repeatedly passed through a 20 1/2-gauge needle. For Northern blot hybridization, 5–7.5 μg of total RNA was treated with glyoxal/DMSO, separated by electrophoresis on a 1% agarose gel in 10 mM phosphate buffer, pH 7.0, and transferred to Hybond$^+$ nylon membranes (Amersham, Arlington Heights, IL). Ethidium bromide (0.5 μg/ml) was included in the gel to monitor equal loading by the quantity of 18S and 28S ribosomal RNA present. cDNA probes were labeled with [α-^{32}P] dCTP by the random primer procedure (Du Pont New England Nuclear, Boston, MA). Oligonucleotide probes were end-labeled with [γ-^{32}P]ATP (Du Pont NEN) and polynucleotide kinase (Boehringer Mannheim). The filters were hybridized to the labeled probes in QuickHyb solution (Stratagene, La Jolla, CA) for 3 h at 68°C and washed according to manufacturer's protocol. After autoradiography (Kodak X-Omat AR) at −80°C for optimal exposure, signal intensity was determined by densitometry. Values shown are representative of at least two independent experiments. Human cDNAs were generous gifts from Dr. Yoshikazu Takada, Scripps Institute for α_2 (Takada and Hemler, 1989) and α_3 (Takada et al., 1991). α_5 cDNAs were purchased from GIBCO BRL. An oligonucleotide complementary to 28S ribosomal RNA was purchased from Clontech (Palo Alto, CA).

Measurement of mRNA Stability

The procedure was a modification of the method by Penttinen et al. (1988). After 1 d in 10% FCS/DMEM, cells on plastic and in collagen gels were cultured in 1% FCS/DMEM for an additional 72 h. Unless otherwise specified, PDGF-BB was added at 30 ng/ml 15 min before the addition of 60 μM 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB, Sigma Chem. Co., St. Louis, MO), an inhibitor of transcription initiation (Zandomeni et al., 1983). Total RNA isolation and Northern analysis were performed as described above.

Porcine Cutaneous Wounds and Immunofluorescence Staining

Full-thickness cutaneous wounds were made with an 8-mm punch on the backs of White Yorkskire pigs and harvested at the times indicated (Welch et al., 1990). Specimens were bisected; one half was fixed in formalin and stained with Masson trichrome; the other half was frozen in liquid nitrogen for immunofluorescence studies. Frozen sections were prepared for immunofluorescence as previously described (Folkvord et al., 1989).

The following antibodies were used to identify integrins of interest. The monoclonal antibodies against α_2 and α_3 (Clones P1E6 and P1B5, respectively) were purchased from GIBCO BRL. A rabbit polyclonal antibody against the carboxy-terminal sequence AQLKPPATSDA, from the cytoplasmic tail of α_5, was the gift of John McDonald (Mayo Clinic).

All antibodies were used at dilutions that gave maximal specific fluorescence and minimal background fluorescence on frozen tissue specimens. Immunofluorescence controls included sections stained with an irrelevant monoclonal antibody instead of the primary antibody, as well as sections in which either the primary and/or secondary antibody(ies) was omitted from the staining procedure. Bound antibody was detected by the avidin–biotin-complex (ABC) technique as previously described (Folkvord et al., 1989). Nonspecific staining was blocked by incubating the tissue sections with 50 μg/ml horse IgG, 5% human serum and 200 μg/ml Avidin D for 60 min. The slides were incubated with primary antibody solution in

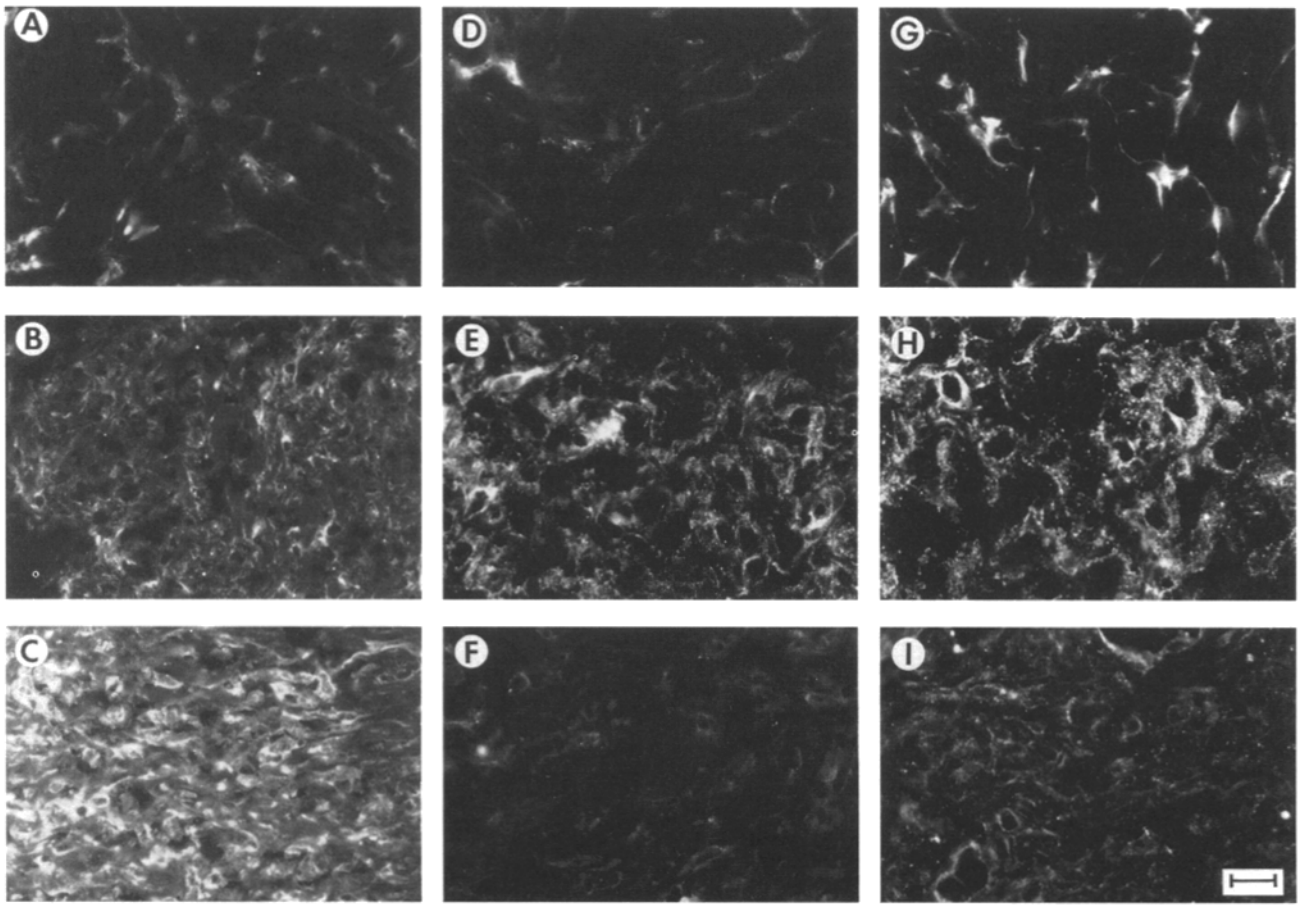

Figure 1. Expression of integrin subunits in porcine dermis and wound fibroblasts. Normal porcine skin and wounds were stained with antibodies against α_2, α_3, and α_5, respectively. (*A–C*) α_2; (*D–F*) α_3; (*G–I*) α_5; (*A*, *D*, and *G*) normal pig dermis; (*B*, *E*, and *H*) granulation tissue in day 5 wounds; (*C*, *F*, and *I*) granulation tissue in day 7 wounds. Bar, 10 μm.

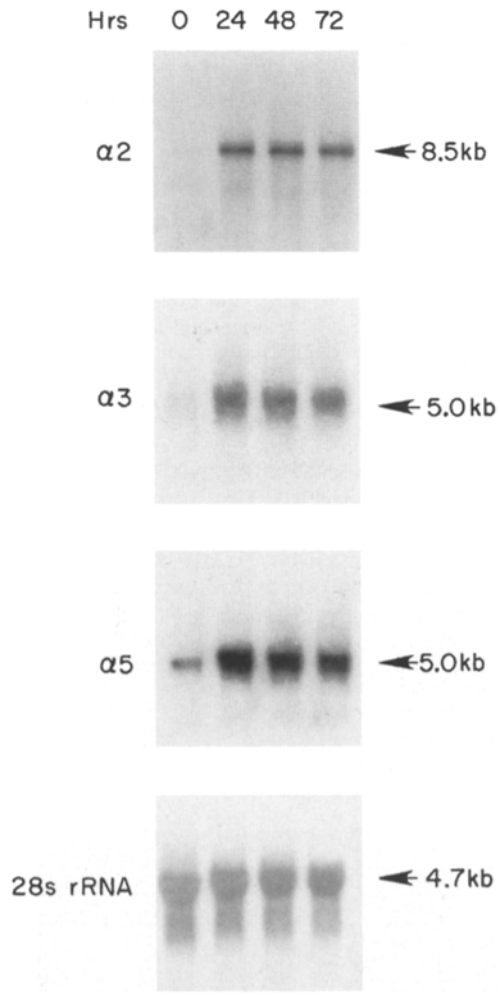

Figure 2. Time course of PDGF-BB effects on integrin mRNA levels in fibroblasts cultured on plastic. Fibroblasts were cultured 1 d in 10% FCS/DMEM followed by 3 d in 1% FCS/DMEM. Such cultures were then stimulated by 30 ng/ml PDGF-BB and incubated for the time indicated. Total RNA was probed with human integrin cDNAs, α_2, α_3, and α_5, respectively. Equal loading was monitored by UV light examination of ethidium bromide stained gel and confirmed by hybridization of the same blot with ^{32}P-labeled probe for 28S ribosomal RNA.

PBS containing 0.02% sodium azide and 0.1% BSA (Miles, Kankakee, IL) overnight at 4°C, washed three times, and incubated with 2.5 μg/ml biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) followed by 15 μg/ml streptavidin-FITC (Vector Laboratories) for 30 min at room temperature.

Vectashield mounting medium (Vector Laboratories) was used to retard quenching. Slides were photographed on 35-mm Tmax 400 film (Eastman Kodak, Rochester, NY) using a Nikon Microphot FXA epifluorescence microscope equipped with a halogen light source, a 470–490-nm excitation filter and a 515-nm barrier filter for fluorescein emission. The filters selected excluded cross excitation resulting in pure yellow/green fluorescein and red rhodamine fluorescence.

Results

Progressive Expression of α_2, α_3, and α_5 Integrin Subunit during Wound Healing Process

To understand the relation between integrin expression and different ECM components during granulation tissue formation, we prepared tissue specimens from normal porcine skin and 4, 5, and 7 d porcine granulation tissue (GT). Previous investigations have showed that 4- and 5-d wounds mainly have a network of fibrin or fibronectin, respectively, whereas 7-d wounds have a substantial organized collagen fiber network (Welch et al., 1990; Clark et al., 1995*a*). Furthermore, PDGF is abundantly present in wounds during these early time periods but not in normal dermis (Ansel et al., 1993). Fig. 1 shows that α_2 expression was greatest in collagen-rich day 7 wounds (panel *C*) compared to either normal dermis (panel *A*) or fibronectin-rich day 5 wounds (panel *B*), while α_3 and α_5 expression

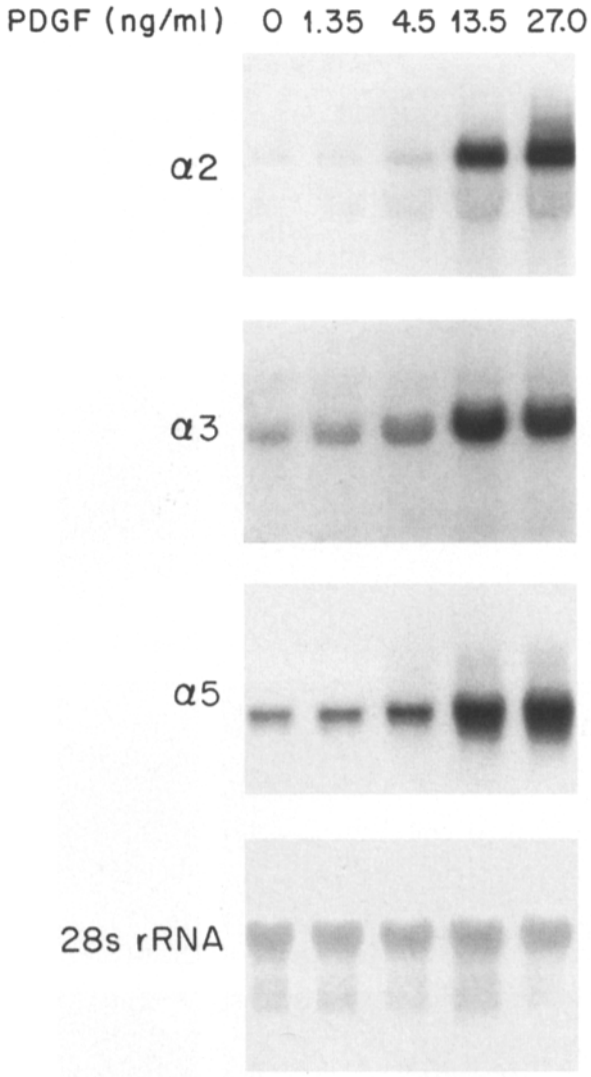

Figure 3. Dose dependence of PDGF-BB effects on integrin mRNA levels in fibroblasts cultured on plastic. Fibroblasts were cultured 1 d in 10% FCS/DMEM followed by 3 d in 1% FCS/DMEM. Subsequently, cultures were stimulated with PDGF at the concentrations indicated for 24 h. Total RNA transblot was probed with the same integrin cDNAs as indicated in Fig. 1. Molecular size for α_2, α_3, and α_5 mRNAs is 8.5, 5.0, and 5.0 kb, respectively. Equal loading was monitored by UV light examination of ethidium bromide stained gel and confirmed by hybridization of the same blot with ^{32}P-labeled probe for 28S ribosomal RNA.

was greatest in fibronectin-rich 5-d wounds (panels *E* and *H*, respectively) compared to either normal dermis (*D* and *G*, respectively) or collagen-rich 7-d wounds (panels *F* and *I*, respectively). Thus, the maximal expression of these integrins in the cytokine-rich wound environment correlated with the presence of abundant ECM ligand.

PDGF-BB Regulation of α_2, α_3, and α_5 Integrin Subunit mRNA

To assess whether the integrins of interest were PDGF responsive, we compared mRNA steady-state levels from normal human dermal fibroblasts grown on plastic dishes treated with PDGF-BB for different time periods (Fig. 2) and at different dosages (Fig. 3). PDGF-BB upregulated α_2, α_3, and α_5 mRNA (Fig. 2). The mRNA molecules were ~8.5 kb for α_2 and 5.0 kb for α_3 and α_5 as reported earlier (Argraves et al., 1987; Takada and Hemler, 1989; Takada et al., 1991; Tsuji et al., 1991). PDGF-induced changes in mRNA steady state were first observed 4 h after addition (data not shown) but reached a maximum at 24 h and persisted for at least 2 d (Fig. 2).

PDGF regulated integrin mRNA in a dose-dependent manner (Fig. 3). In general, steady-state mRNA reached a maximum change at 13.5 ng/ml PDGF-BB, a dose response similar to that of PDGF-stimulated cell proliferation (data not shown). One exception was α_2 mRNA levels which increased further with 27.0 ng/ml PDGF-BB. To insure optimal conditions, we chose PDGF-BB at 30.0 ng/ml and 24 h of incubation as our standard condition for the following studies.

ECM Regulation of Integrin Subunits α_2, α_3, and α_5 mRNA Levels

To determine whether ECM found during the different phases of wound repair differentially regulates integrin mRNA levels in the absence or presence of PDGF-BB, we measured mRNA steady-state levels from cells grown in stressed fibrin gel, stressed and relaxed collagen gels as in vitro models of early wound clot, late granulation tissues, and normal dermis, respectively. Cells grown on tissue plastic dishes were included as a general control and for comparison with cells in fibrin gels since fibroblasts in these conditions produce an abundant fibronectin pericellular matrix (Hynes, 1973; Ruoslahti and Vaheri, 1974; Yamada and Weston, 1974). Compared to fibronectin-rich tissue culture conditions, basal mRNA levels of α_3 and α_5 were altered slightly by collagen gels, while α_2 was increased (Fig. 4). There was little if any difference in inte-

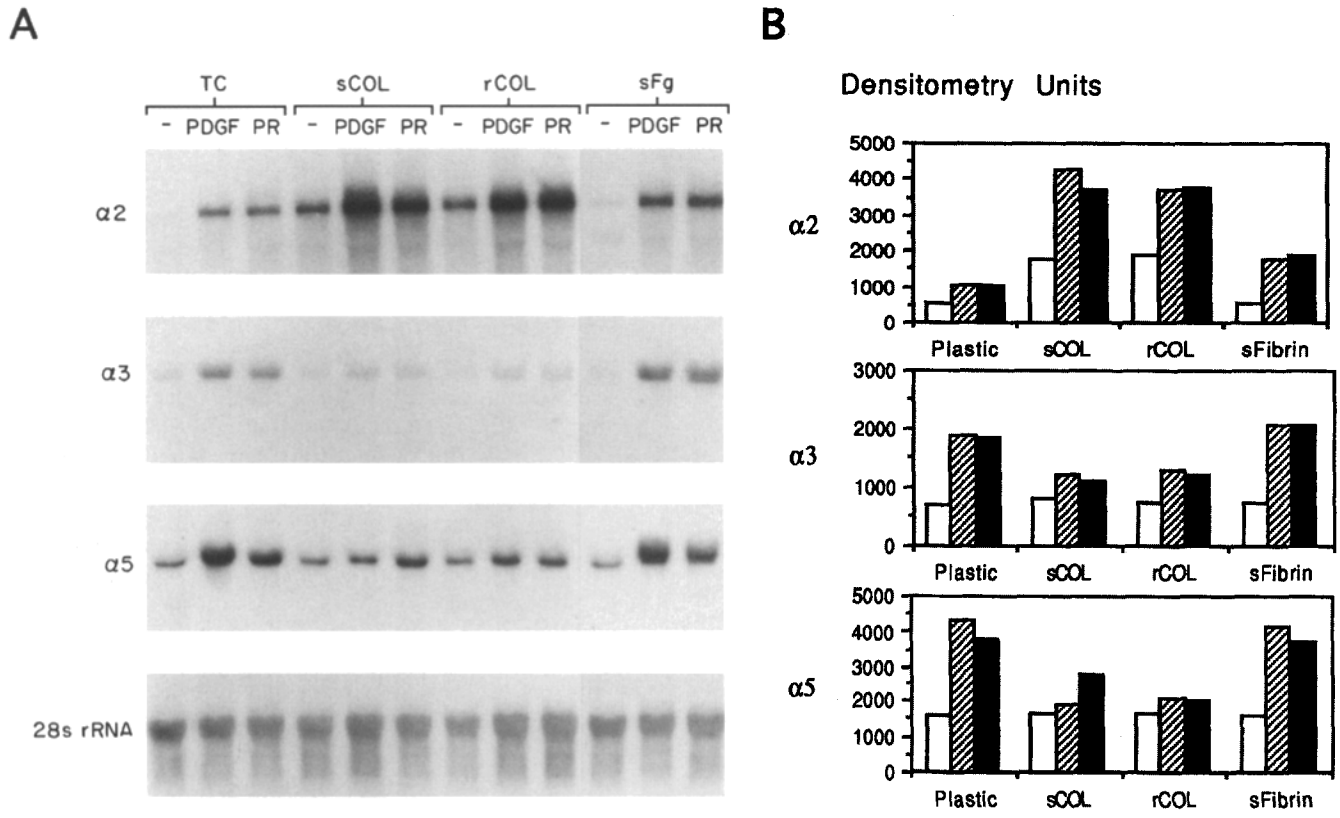

Figure 4. Regulation of integrin mRNAs by PDGF-BB in fibroblasts cultured on plastic, stressed fibrin gel, stressed and relaxed collagen gels. Fibroblasts were cultured on plastic, in stressed fibrin, stressed, and relaxed collagen gels for 1 d in 10% FCS/DMEM followed by 3 d in 1% FCS/DMEM. Subsequently, cultures were incubated with fresh DMEM in the absence (−) or presence of 30 ng/ml PDGF-BB or platelet releasates (PR) for 24 h. Total RNA was probed with the same integrin cDNAs as indicated in Fig. 1. (*A*) Northern blots. (*B*) Northern blots analyzed by densitometric scanning. Open, hatched, and dark bars represent treatment without and with PDGF-BB and platelet releasates, respectively. *TC*, tissue culture plastic; *sCOL*, stressed collagen gel; *rCOL*, relaxed collagen gel; *sFg*, stressed fibrin gel; *PR*, platelet releasates. Equal loading was monitored by UV light examination of ethidium bromide stained gel and confirmed by hybridization of the same blot with ^{32}P-labeled probe for 28S ribosomal RNA.

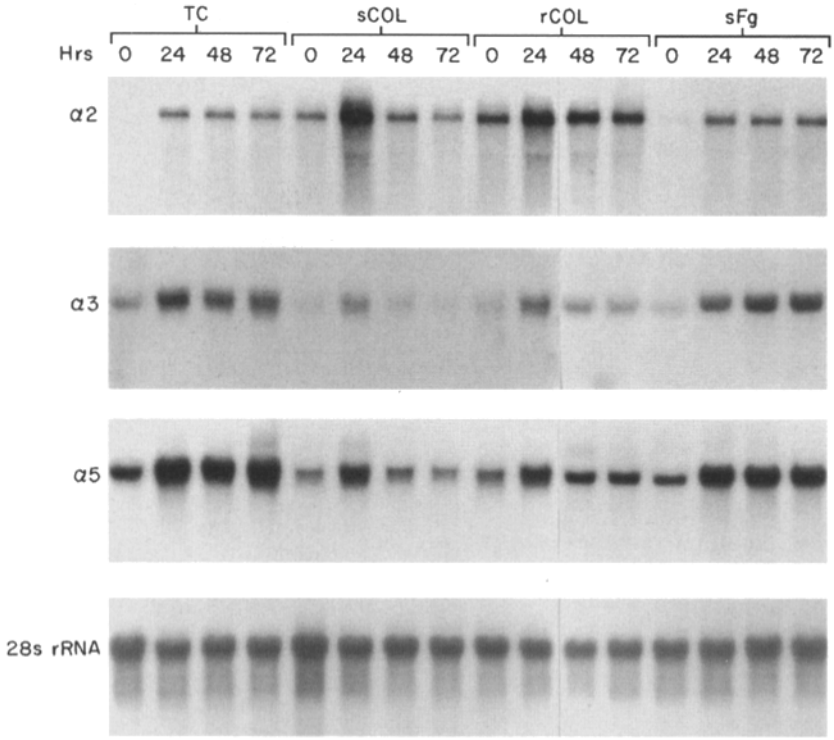

Figure 5. Time course of PDGF-BB effects on α_2, α_3, and α_5 mRNA levels in fibroblasts cultured on plastic, stressed fibrin gel, stressed and relaxed collagen gels. Fibroblasts were cultured on plastic, in stressed fibrin, stressed and relaxed collagen gels for 1 d in 10% FCS/DMEM followed by 3 d in 1% FCS/DMEM. Subsequently, cultures were stimulated with 30 ng/ml PDGF-BB for the time indicated. Total RNA was isolated and probed with α_2, α_3, and α_5 cDNAs. Equal loading was monitored by UV light examination of ethidium bromide stained gel and confirmed by hybridization of the same blot with ^{32}P-labeled probe for 28S ribosomal RNA.

grin mRNA levels when cells were cultured in stressed vs relaxed collagen gels. Fibroblasts grown in stressed fibrin gels had similar integrin mRNA levels as cells grown in fibronectin-rich tissue culture conditions. Since cells exhibited quite distinct morphology in stressed collagen vs relaxed collagen gels and similar morphology in stressed collagen and fibrin gels (data not shown), we concluded that although cell shape may play a role in regulating basal levels of some genes (Grinnell, 1994; Ingber, 1993; Singhvi et al., 1994), ECM regulates integrin expression mainly by its chemical nature.

Influence of ECM on PDGF-BB Regulation of Integrin Subunits α_2, α_3, and α_5 mRNA Levels

Since all integrins examined were regulated by recombinant PDGF-BB when fibroblasts were cultured on plastic dishes (Figs. 2 and 3), we examined whether collagen and fibrin gels would influence these PDGF-BB effects. Diluted human platelet releasate, containing 13.5 ng/ml PDGF, was included in the experiment in parallel to recombinant PDGF-BB. Integrin α_2, α_3, and α_5 mRNA levels were all increased by PDGF-BB and platelet releasate whether cells were grown in fibronectin-rich culture conditions or in stressed fibrin gels (Fig. 4). In comparison, collagen gels greatly attenuated the α_3 and α_5 mRNA response to PDGF-BB and platelet releasate and enhanced the stimulated increase of α_2 mRNA.

Since other laboratories have reported that fibroblasts cultured in collagen gels incrementally decrease c-*myc* mRNA (Shimbara et al., 1992) and collagen α1(I) mRNA (Eckes et al., 1993) during prolonged incubation, we examined the effects of collagen and fibrin gel on PDGF-stimulated α_2, α_3, and α_5 mRNAs beyond 24 h of incubation. Cells grown in fibronectin-rich culture conditions and stressed fibrin gel maintained the same stimulated levels of α_2, α_3, and α_5 mRNA levels for up to 72 h (Fig. 5). In contrast, when cultured in collagen gels, α_3 and α_5 mRNA returned to basal levels by 48 h (Fig. 5). The synergistic stimulation of α_2 mRNA by PDGF and collagen was maximal at 24 h but still present at 48 h.

Collagen Gels Impair the Ability of PDGF-BB to Increase α_3 and α_5 mRNA Stability

Since collagen gels appeared to shorten the response time as well as blunt the maximal response of α_3 and α_5 mRNA to PDGF-BB, the effect of collagen gels on α_3 and α_5 mRNA stability was examined. An RNA transcription initiation inhibitor, DRB, at 60 μM was used to treat cells grown in fibronectin-rich tissue culture conditions and in relaxed collagen gel with or without PDGF-BB. Quantitative Northern analysis of α_3 and α_5 mRNAs were examined as a function of time. In cells grown in tissue culture conditions and treated with PDGF-BB, a clear increase in stability of α_3 and α_5 mRNAs was detected compared to cells not treated with PDGF-BB (Fig. 6). In contrast, cells grown in collagen gel demonstrated no significant increase in α_3 and α_5 mRNA stability with PDGF-BB treatment. It thus appears that fibroblasts in collagen gels are unable to increase α_3 and α_5 mRNA levels in response to PDGF partially due to their inability to stabilize α_3 and α_5 mRNAs.

PDGF Increases α_2 mRNA Stability When Fibroblasts Are Cultured in Collagen, but Not on Plastic

To address whether collagen abrogated PDGF regulation of mRNA stability of all integrins, α_2 mRNA stability of

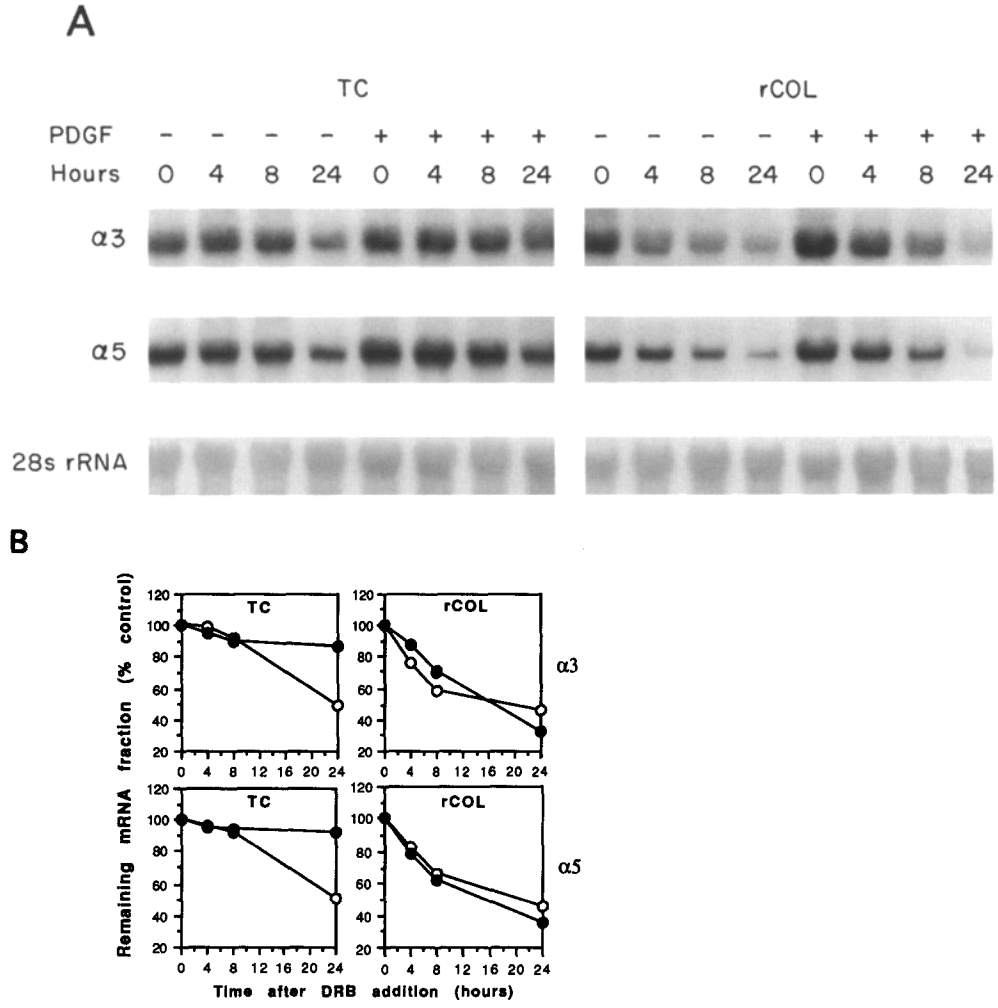

Figure 6. Effects of PDGF-BB on stability of integrin α_3 and α_5 mRNAs from cells cultured on plastic and relaxed collagen gel. Fibroblasts were cultured on plastic and relaxed collagen gel for 1 d in 10% FCS/DMEM followed by 3 d in 1% FCS/DMEM. Day 4 cultures were treated with an RNA synthesis inhibitor, 5,6-dichloro-1β-D-ribofuranosylbenzimidazole (DRB), 15 min before experimental conditions. Total RNA was isolated at 0 h; 4 h; 8 h and 24 h, and probed with α_3 and α_5 cDNAs. (*A*) Northern blots. (*B*) Densitometric scans of Northern blots. Open and dark circles represent without and with PDGF-BB treatment, respectively. Equal loading was monitored by UV light examination of ethidium bromide stained gel and confirmed by hybridization of the same blot with ^{32}P-labeled probe for 28S ribosomal RNA.

cells grown in fibronectin-rich tissue culture conditions and in collagen gels was compared. Because the basal level of α_2 mRNA from cells grown on tissue culture dishes is almost nondetectable, α_2 mRNA was first induced with PDGF and then its decay rate determined. Surprisingly, cells grown in fibronectin-rich tissue culture conditions failed to increase α_2 mRNA stability in response to PDGF-BB (Fig. 7). In contrast, PDGF significantly increased α_2 mRNA stability in cells grown in collagen gels (Fig. 7). Therefore, the synergistic effects of collagen gels and PDGF on α_2 mRNA clearly involve mRNA stability. Thus, posttranscriptional regulation appears to play an important role in both PDGF and ECM control of integrin expression.

Discussion

The early wound environment is composed of a provisional matrix consisting largely of fibrin and fibronectin and a fluid-phase milieu rich in factors arising mostly from degranulated platelets (Clark, 1993). Among the components of this platelet cocktail, PDGF stands out as a potent mesenchymal cell mitogen and chemoattractant (Ross et al., 1990). To move into and through the provisional matrix, however, the cells must express the integrin receptors that can bind fibrin, fibronectin, or vitronectin (Hynes, 1992; Ruoslahti, 1991). In this report we demonstrate that fibroblasts moving into the provisional matrix of a wound dramatically upregulate the fibronectin receptors, $\alpha_3\beta_1$ and $\alpha_5\beta_1$, but not the collagen receptor $\alpha_2\beta_1$ (Fig. 1). $\alpha_2\beta_1$ receptors increase later at 7 d when great quantities of collagen matrix accumulate in the wound (Welch et al., 1990; Clark et al., 1995*a*). Human dermal fibroblasts cultured with PDGF-BB either in fibronectin-rich tissue culture conditions or in fibrin gels, conditions that approximate an early wound environment, increase mRNA steady-state levels of provisional matrix integrin receptors compared to collagen receptors (Fig. 4). Fibroblasts cultured in collagen gels, conditions that approximate normal dermis or later wound environments, fail to give this response even in the presence of PDGF. Clearly these results demonstrate that ECM and PDGF act synergistically to generate the proper cellular signal(s) to elicit integrins needed for the situation at hand.

PDGF-BB is a known positive regulator of integrin α_5 and β_3 mRNA in aortic smooth muscle cells (Janat et al., 1992), α_2 in human foreskin fibroblasts (Ahlen and Kristofer, 1994) and β_1 gene transcription in Swiss 3T3 mouse fibroblasts (Bellas et al., 1991). Here we studied its influence in normal human dermal skin fibroblasts on α_2 collagen receptor subunit mRNAs, and α_3, α_5 provisional matrix receptor subunit mRNAs (Figs. 2 and 3), and the modula-

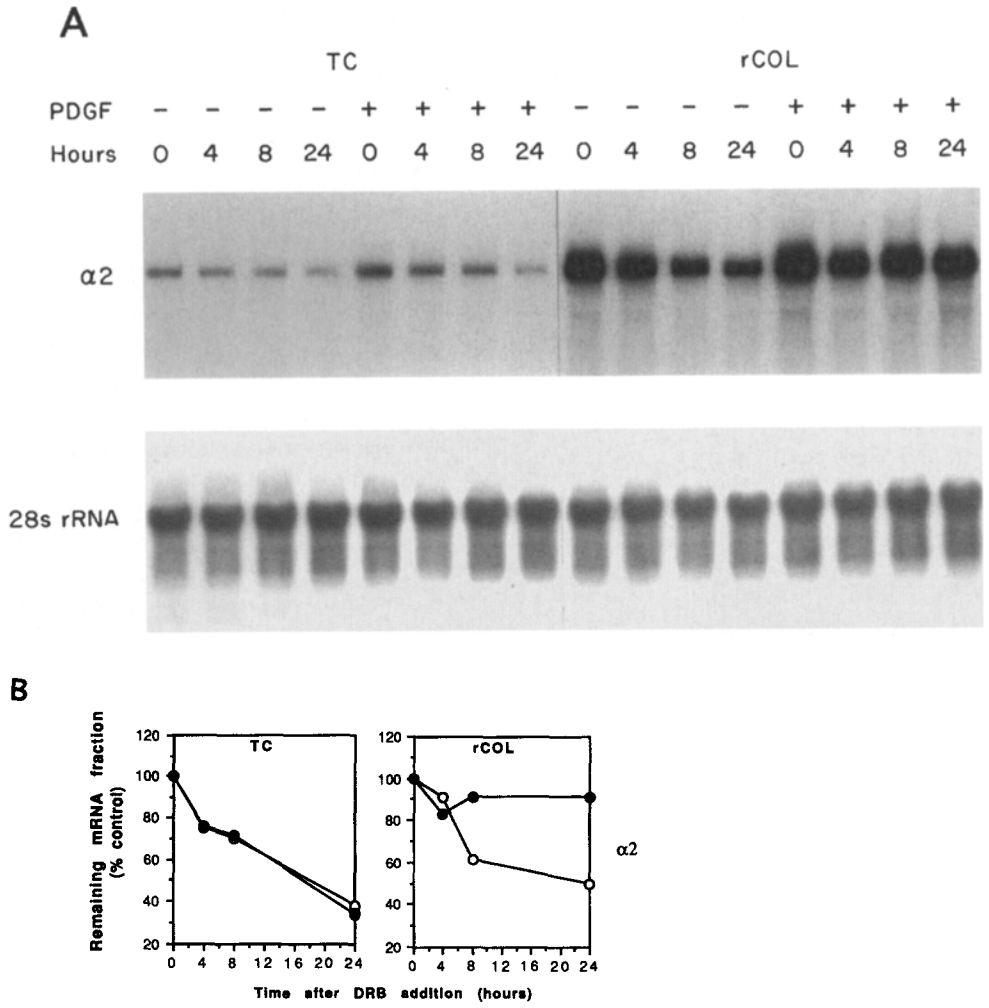

Figure 7. Effects of PDGF-BB on stability of integrin α_2 mRNAs from cells cultured on plastic and in relaxed collagen gels. Fibroblasts were cultured on plastic and in relaxed collagen gels for 1 d in 10% FCS/DMEM followed by 3 d in 1% FCS/DMEM. Day 4 cultures were treated with 30 ng/ml PDGF-BB for 24 h. Extensively washed cells were then treated with DRB with or without PDGF-BB. Total RNA was isolated at 0 h; 4 h; 8 h and 24 h, and probed with α_2 cDNA. (*A*) Northern blots. (*B*) Densitometric scans of Northern blots. Open and dark circles represent without and with PDGF-BB treatment, respectively. Equal loading was monitored by UV light examination of ethidium bromide stained gel and confirmed by hybridization of the same blot with ^{32}P-labeled probe for 28S ribosomal RNA.

tion of its effects by two distinct types of ECM, collagen, and fibrin gels (Fig. 4).

When fibroblasts were cultured in fibrin gels they responded to PDGF in a fashion similar to fibroblast on plastic (Fig. 4). This finding is perhaps not so surprising when one considers that fibroblasts cultured on plastic form a fibronectin-rich pericellular matrix (Hynes, 1973; Ruoslahti and Vaheri, 1974; Yamada and Weston, 1974). Thus fibroblasts in an environment of either fibrin or fibronectin responded to PDGF by increasing α_3 and α_5 mRNAs for the integrin receptors that interact with these proteins. The PDGF-induced increases in α_3 and α_5 mRNA observed in fibrin/fibronectin-cultured fibroblasts were attenuated when fibroblasts were cultured in collagen while the increase in α_2 mRNA was accentuated. The overall effect of collagen-cultured fibroblasts in the presence or absence of PDGF was to increase their collagen receptor mRNAs and decrease their provisional matrix receptor mRNAs. The integrin responses of fibroblasts in these various culture conditions to human platelet releasate containing 13.5 μg/ml PDGF were parallel to the responses to recombinant PDGF-BB (Fig. 4).

Collagen gel attenuation of PDGF-stimulated integrin α_3 and α_5 mRNA levels evokes a familiar theme of suppression of cell responsiveness to growth factors, such as PDGF-stimulated DNA synthesis (Nishiyama et al., 1991) and TGF-β–induced type I collagen synthesis (Clark et al., 1995*a*). However, the similar regulatory pattern of α_3 and α_5 by collagen and PDGF-BB was initially disconcerting since we expected that α_3 would respond to these agents similar to $\alpha_2\beta_1$ since the $\alpha_3\beta_1$ integrin was known as a receptor for collagen by affinity chromatography and antibody blocking of cell adhesion to collagen-coated substrata (Elices et al., 1991; Takada et al., 1988; Wayner and Carter, 1987). However, $\alpha_3\beta_1$ has several features different from collagen receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$: (*a*) Gullberg et al. (1992) found that $\alpha_3\beta_1$ is not involved in the initial attachment of rat hepatocytes and cardiac fibroblasts to type I collagen as judged by affinity chromatography and antibody blocking of the cell adhesion; (*b*) unlike $\alpha_1\beta_1$ and $\alpha_2\beta_1$, $\alpha_3\beta_1$ has no collagen binding I-domain but rather is a RGD-dependent receptor for fibronectin (Elices et al., 1991); (*c*) $\alpha_3\beta_1$ recognizes denatured type I collagen (Yamamoto and Yamamoto, 1994) possibly through an RGD-dependent mechanism (Aumailley et al., 1989; Pfaff et al., 1993; Vandenberg et al., 1991) in contrast to $\alpha_1\beta_1$ and $\alpha_2\beta_1$ binding to collagen via non-RGD sites (Kupper and Ferguson, 1993; Staatz et al., 1991). Thus the similar modulation of α_3 and α_5 mRNAs is concordant with the possibility that $\alpha_3\beta_1$ in human fibroblasts is a provisional

matrix (fibronectin and denatured collagen) receptor rather than a receptor for native collagen.

Although there are several reports that relaxed collagen gels attenuate the PDGF-responsiveness of fibroblasts more than stressed collagen gels (for review see Grinnell, 1994), we failed to observe such differences among the integrin subunit mRNAs. We did confirm, however, that fibroblasts are elongated in stressed collagen gels and stellate in relaxed collagen gels (data not shown) and that PDGF-stimulated fibroblast proliferation rate is more attenuated by relaxed collagen gels than stressed collagen gels (data not shown). For the most part, however, it appears that collagen matrix modulation of both basal and PDGF-regulated integrin mRNA levels are achieved by the biochemical nature of the collagen and not by isotropic or anisotropic forces established within a three-dimensional collagen gel. Although cell shape has often been implicated in cell function (Folkman and Moscona, 1978; Ingber, 1993), more and more evidence showed that integrin-mediated changes in intracellular milieu can occur in the absence of changes in cell morphology or overt reorganization of actin microfilaments (Werb et al., 1989). For example, interaction of $\alpha_5\beta_1$ with fibronectin induced cytoplasmic alkalization by activating the Na^+/H^+ antiporter without involving cell shape (Ingber et al., 1990; Schwartz et al., 1991). Therefore it is possible that in our systems, interaction of collagen with its receptors, rather than changes in cell shape, sends signals distinct from those sent by fibrin or fibronectin receptor interaction. The chemical nature of the ECM would be more important than physical nature of the ECM in this type of regulation. Indeed, ECM and growth factor stimuli may cooperate at the level of second messengers or genes to achieve control of gene expression.

Collagen gel enhancement of PDGF-stimulated α_2 mRNA level provided new insight into collagen's role on growth factor regulation of cellular function. Grinnell and coworkers have observed that PDGF receptor autophosphorylation was impaired when human foreskin fibroblasts were cultured in relaxed collagen gel (Lin and Grinnell, 1993) and Marx et al. (1993) have reported that collagen gels reduce PDGF receptor β subunits (Lin and Grinnell, 1994; Marx et al., 1993). Thus collagen modulation of PDGF pathways might occur at a very early stage of the stimulation. On the other hand, since we detected both positive and negative impact of collagen gels on PDGF-stimulation of selective integrins and since increasing evidence showed that ECM-induced signal pathways share several components identified in growth factor or cytokine pathways, e.g., G-protein (Symons and Mitchison, 1992), src family kinase (Huang et al., 1991), PIP_2 (McNamee et al., 1993), and phospholipase D (Yeo et al., 1995), it is possible that more distal point(s) along PDGF regulatory pathways may be affected by ECM. This would allow a diversified pattern of the combinatorial effects of ECM and PDGF on cell activities. We confirmed the general view that collagen gel possesses a suppressive nature by showing that the PDGF-stimulation of integrin α_3 and α_5 mRNA levels was attenuated by collagen gels. However, the collagen gel can also synergize with PDGF on increasing integrin α_2 mRNA levels.

The mechanisms by which ECM and growth factors cooperate with one another largely remain unclear. In this study, we describe a novel mechanism by which collagen gels modulate fibroblast responsiveness to PDGF. PDGF-stimulated integrin α_3 and α_5 mRNA steady-state levels were attenuated by collagen gels through alteration in message half-life. Our experiments clearly showed that α_3 and α_5 integrin mRNAs from fibroblasts cultured in collagen gels had shorter half-lives than the mRNAs from cells cultured on plastic in the presence or absence of PDGF (Fig. 6). On the other hand, PDGF greatly prolonged α_2 mRNA half-life when fibroblasts were cultured in collagen gels, but did not have any effect on α_2 mRNA stability from cells cultured on plastic (Fig. 7). Thus mRNA stability clearly played a role in the ECM and PDGF regulation of integrins.

Collagen posttranscriptional regulation of gene expression has been reported for albumin in mouse hepatocytes (Zaret et al., 1988) and collagen α1(I) in adult dermal human fibroblasts (Berthod et al., 1994; Eckes et al., 1993). A comprehensive review by Juliano and Haskill of integrin-mediated adhesion-induced immediate early genes in monocytes, revealed that all responsive genes contained repetitive AU-rich sequences which regulate mRNA stability and translational efficiency (Juliano and Haskill, 1993). Therefore, the ECM may alter integrin expression, in large part, through regulation of mRNA stability.

Based on our results reported here and ongoing in vivo studies, we propose a working hypothesis for the initiation of wound repair. After injury, fibrinogen/fibronectin and PDGF leak from blood vessels around the wound and envelop biosynthetically inactive fibroblasts that were previously embedded in a collagen-rich matrix. The combination of fibrin/fibronectin provisional matrix environment and PDGF stimulates fibroblasts to proliferate and to express relatively high levels of provisional matrix receptor mRNAs compared to collagen receptor mRNAs. This alteration in integrin mRNA steady state ultimately leads to surface changes in integrin receptors (Gailit and Clark, 1995). Once accumulation of provisional matrix integrins on the cell surface reaches threshold (3 or 4 d after the initial signals), cells migrate into the clot-filled wound space under the direction of chemotactic signals and stay there via ECM-integrin recognition forces (Clark et al., 1995*b*). In a provisional matrix bed, cells respond to growth factors and cytokines by additional proliferation and new ECM synthesis. Once a new matrix of collagen prevails, collagen attenuates cell proliferation and ECM synthesis and stimulates α_2 integrin synthesis synergistically with PDGF to facilitate collagen contraction and ECM reorganization.

In summary ECM and growth factors have the ability to coordinately alter cell phenotypes. The pretranslational events reported by this paper clearly support this concept. The posttranslational events are on-going studies in our lab. Specifically we show here a mechanism by which cells can respond differently to repeated stimulation by the same growth factor. Since cells have the ability to secrete ECM, the matrix environment continuously changes leading to a progression of cell phenotypes even in the face of a constant growth factor milieu. Ultimately the changes in cell phenotype and ECM results in new tissue formation. Coordinate ECM and growth factor regulation of cell phenotype probably is partially responsible for tissue develop-

ment during embryogenesis and morphogenesis (Schmidt, 1994) as well as wound repair (Clark et al., 1995*a*; Clark, 1995).

We thank Dr. Charles Hart (ZymoGenetics, Seattle, Washington) for PDGF-BB; Dr. Yoshikazu Takada of The Scripps Institute (San Diego, California) for cDNA probes; Dr. John McDonald of Mayo Clinic (Scottsdale, AZ) for anti-α_5 antibodies; and Curative Technology Inc. (East Setauket, NY), for platelet releasate.

Funding for this work was provided by National Institutes of Health grant AG10114309 to R. A. F. Clark. J. Xu is a postdoctoral fellow supported by funding from the School of Medicine, SUNY at Stony Brook and the Dermatology Foundation.

Received for publication 20 April 1995 and in revised form 29 August 1995.

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