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, \( \alpha_2 \), \( \alpha_3 \), and \( \alpha_5 \), 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 \( \alpha_3 \) and \( \alpha_5 \) mRNA. In contrast, both stressed and relaxed collagen attenuated these responses while promoting maximal \( \alpha_2 \) mRNA expression. Posttranscriptional regulation was an important mechanism in this differential response. Together PDGF-BB and collagen gels promoted \( \alpha_2 \), but not \( \alpha_3 \) and \( \alpha_5 \), mRNA stability. Conversely, when fibroblasts were in fibronectin-rich cultures, PDGF-BB promoted \( \alpha_3 \) and \( \alpha_5 \), but not \( \alpha_2 \), 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.
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broblasts surrounded by fibrin respond to PDGF by
bronectin-rich cultures supported induction of provisional
finally, greatly increased by PDGF-BB when the cells were cul-
tured in collagen but not when cultured on plastic. These
fibrin increase the epigenetic pressure in fibroblasts to ex-
tenuated provisional matrix receptor responses. Thus fi-
gel. Collagen gels failed to sup-
that fibrin and collagen differentially regul-
expression, which in turn affects how fi-
physically and biochemically relate to ECM.
which in turn affects how fi-
blood fibroblasts from subconfluent cultures were mixed with 10 ml of col-
lagen solution for a final concentration of 5 × 10^6 cells/ml. The collagen
cell suspension (4 ml) was immediately placed onto 60-mm tissue culture
Bovine thrombin (Thrombinar, Armour Pharmaceutical,
mM KCl and 3 mM glucose to a final platelet concentration of 5 x 10^9
After granule release, the aggregated platelets were resuspended by aspi-
tioned using a sandwich EIA with anti-PDGF from Collaborative Re-
search Inc. (Bedford, MA) which recognizes all PDGF isoforms, and
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% CO₂ 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 col-
lagen 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 der-
mal fibroblasts from subconfluent cultures were mixed with 10 ml of col-
lagen solution for a final concentration of 5 × 10^6 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 fi-
brinogen (>98% clottable proteins and homogenous by SDS-PAGE, Cal-
biochem, 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 fibrin-
gen solution for a final concentration of 5 × 10^6 cells/ml. Immediately
thereafter human thrombin (GIBCO BRL) was added at 0.2 U/ml. The fi-
brinogen 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% CO₂ 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 subse-
quent experiments were performed according to specific conditions.

PDGF-BB and Platelet Releasate

Sterile, endotoxin-free, recombinant BI 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 previ-
ously 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
hydroxyethylpiperazin-N-2-ethanesulfonic acid (Hepes), 100 mM NaCl, 6
mM KCl and 3 mM glucose to a final platelet concentration of 5 × 10^6
platelets/ml. Bovine thrombin (Thrombinar, Armour Pharmaceutical,
Kankakee, IL) was then added at a concentration of 1 U/10^6 platelets and
the thrombin platelet mixture incubated at room temperature for 10 min.
After granule release, the aggregated platelets were resuspended by aspi-
ration and centrifuged for 10 min at 2,000 g at room temperature. The su-
pernatant containing the released platelet factor was retained and diluted
1:10 in DMEM. PDGF concentrations in the platelet releasate were deter-
mined using a sandwich EIA with anti-PDGF from Collaborative Re-
search 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 H2O, 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-32P]dCTP by the random primer procedure (Du Pont New England Nuclear, Boston, MA). Oligonucleotide probes were end-labeled with [γ-32P]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 ~2 (Takada and Hernler, 1989) and ~5 (Takada et al., 1991). α5 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 Mason 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 α2 and α3 (Clones PIE6 and P1B5, respectively) were purchased from GIBCO BRL. A rabbit polyclonal antibody against the carboxy-terminal sequence AQLKPPATSDA, from the cytoplasmic tail of α5, 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). Non-specific 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 α5, α3, and α5, respectively. (A-C) α2; (D-F) α3; (G-I) α5; (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.
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. 1995a). 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 $\alpha_2$ 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 $\alpha_3$ and $\alpha_5$ expression

**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, $\alpha_2$, $\alpha_3$, and $\alpha_5$, 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 $\mu$g/ml biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) followed by 15 $\mu$g/ml streptavidin-FITC (Vector Laboratories) for 30 min at room temperature.

Vectorshield 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 $\alpha_2$, $\alpha_3$, and $\alpha_5$ Integrin Subunit during Wound Healing Process**

To understand the relation between integrin 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 $\alpha_2$, $\alpha_3$, and $\alpha_5$ 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 L, 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 α2, α3, and α5 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 α2, α3, and α5 mRNA (Fig. 2). The mRNA molecules were ~8.5 kb for α2 and 5.0 kb for α3 and α5, 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 α2 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 α2, α3, and α5 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 α3 and α5 were altered slightly by collagen gels, while α2 was increased (Fig. 4). There was little if any difference in inte-

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**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 32P-labeled probe for 28S ribosomal RNA.
Figure 5. Time course of PDGF-BB effects on α2, α3, and α5 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 α2, α3, and α5 cDNAs. Equal loading was monitored by UV light examination of ethidium bromide stained gel and confirmed by hybridization of the same blot with 32P-labeled probe for 28S ribosomal RNA.

Influence of ECM on PDGF-BB Regulation of Integrin Subunits α2, α3, and α5 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 α2, α3, and α5 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 α3 and α5 mRNA response to PDGF-BB and platelet releasate and enhanced the stimulated increase of α2 mRNA.

Since other laboratories have reported that fibroblasts cultured in collagen gels incrementally decrease c-myc mRNA (Shimbara et al., 1992) and collagen α1(1) mRNA (Eckes et al., 1993) during prolonged incubation, we examined the effects of collagen and fibrin gel on PDGF-stimulated α2, α3, and α5 mRNAs beyond 24 h of incubation. Cells grown in fibronectin-rich culture conditions and stressed fibrin gel maintained the same stimulated levels of α2, α3, and α5 mRNA levels for up to 72 h (Fig. 5). In contrast, when cultured in collagen gels, α3 and α5 mRNA returned to basal levels by 48 h (Fig. 5). The synergistic stimulation of α3 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 α3 and α5 mRNA Stability

Since collagen gels appeared to shorten the response time as well as blunt the maximal response of α3 and α5 mRNA to PDGF-BB, the effect of collagen gels on α3 and α5 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 α3 and α5 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 α3 and α5 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 α3 and α5 mRNA stability with PDGF-BB treatment. It thus appears that fibroblasts in collagen gels are unable to increase α3 and α5 mRNA levels in response to PDGF partially due to their inability to stabilize α3 and α5 mRNAs.

PDGF Increases α2 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, α2 mRNA stability of...
cells grown in fibronectin-rich tissue culture conditions and in collagen gels was compared. Because the basal level of \( \alpha_2 \) mRNA from cells grown on tissue culture dishes is almost nondetectable, \( \alpha_2 \) mRNA was first induced with PDGF and then its decay rate determined. Surprisingly, cells grown in fibronectin-rich tissue culture conditions failed to increase \( \alpha_2 \) mRNA stability in response to PDGF-BB (Fig. 7). In contrast, PDGF significantly increased \( \alpha_2 \) mRNA stability in cells grown in collagen gels (Fig. 7). Therefore, the synergistic effects of collagen gels and PDGF on \( \alpha_2 \) 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 chemotacttractant (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, \( \alpha_5 \beta_1 \) and \( \alpha_5 \beta_3 \), but not collagen receptor \( \alpha_2 \beta_1 \) (Fig. 1).\( \alpha_5 \beta_1 \) receptors increase later at 7 d when great quantities of collagen matrix accumulate in the wound (Weibel et al., 1990; Clark et al., 1995a). 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 \( \alpha_5 \) and \( \beta_1 \) mRNA in aortic smooth muscle cells (Janat et al., 1992), \( \alpha_5 \) in human foreskin fibroblasts (Ahlen and Kristofer, 1994) and \( \beta_1 \) gene transcription in Swiss 3T3 mouse fibroblasts (Bellas et al., 1991). Here we studied its influence in normal human dermal skin fibroblasts on \( \alpha_2 \) collagen receptor subunit mRNAs, and \( \alpha_5 \alpha_5 \) provisional matrix receptor subunit mRNAs (Figs. 2 and 3), and the modula-
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 \( \alpha_3 \) and \( \alpha_5 \) mRNAs for the integrin receptors that interact with these proteins. The PDGF-induced increases in \( \alpha_3 \) and \( \alpha_5 \) mRNA observed in fibrin/fibronectin-cultured fibroblasts were attenuated when fibroblasts were cultured in collagen while the increase in \( \alpha_3 \) 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 \( \mu \)g/ml PDGF were parallel to the responses to recombinant PDGF-BB (Fig. 4).

Collagen gel attenuation of PDGF-stimulated integrin \( \alpha_3 \) and \( \alpha_5 \) 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-\( \beta \)-induced type I collagen synthesis (Clark et al., 1995a). However, the similar regulatory pattern of \( \alpha_3 \) and \( \alpha_5 \) by collagen and PDGF-BB was initially disconcernting since we expected that \( \alpha_3 \) would respond to these agents similar to \( \alpha_1 \beta_1 \) since the \( \alpha_1 \beta_1 \) integrin was known as a receptor for collagen by affinity chromatography and antibody blocking of cell adhesion to collagen-coated substrata (Eliees 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 \( \alpha_5 \beta_1 \): (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 \( \alpha_1 \beta_1 \), the collagen-binding I-domain of \( \alpha_3 \beta_1 \) has no collagen binding I-domain but rather is a RGD-dependent receptor for fibronectin (Eliees et al., 1991); (c) \( \alpha_3 \beta_1 \) has several features different from collagen receptors \( \alpha_1 \beta_1 \) and \( \alpha_5 \beta_1 \); (d) unlike \( \alpha_5 \beta_1 \) and \( \alpha_3 \beta_1 \), has no collagen binding I-domain but rather is a RGD-dependent receptor for fibronectin (Eliees et al., 1991); (e) \( \alpha_3 \beta_1 \) 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 \( \alpha_5 \beta_1 \) binding to collagen via non-RGD sites (Kupper and Ferguson, 1993; Staatz et al., 1991). Thus the similar modulation of \( \alpha_3 \) and \( \alpha_5 \) mRNAs is concordant with the possibility that \( \alpha_3 \beta_1 \) in human fibroblasts is a provisional

Figure 7. Effects of PDGF-BB on stability of integrin \( \alpha_2 \) 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 \( \alpha_2 \) 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.
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 stimulate 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 over reorganization of actin microfilaments (Werb et al., 1989). For example, interaction of α5β1 with fibronectin induced cytoplasmic alkalinization by activating the Na"/H"+ antipporter 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 α2 mRNA level provided new insight into collagen's role on growth factor regulation of cellular function. Grinnell and co-workers 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), PIP2 (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 α2 and α5 mRNA levels was attenuated by collagen gels. However, the collagen gel can also synergize with PDGF on increasing integrin α2 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 α2 and α5 mRNA steady-state levels were attenuated by collagen gels through alteration in message half-life. Our experiments clearly showed that α2 and α5 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 α2 mRNA half-life when fibroblasts were cultured in collagen gels, but did not have any effect on α2 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 (Galili and Clark, 1995). Once accumulation of provisional matrix integrins on the cell surface reaches threshold (3 or 4 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., 1995b).

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 α2 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., 1995a; Clark, 1995).

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