

A Three-dimensional Collagen Lattice Induces Protein Kinase C- ζ Activity: Role in α_2 Integrin and Collagenase mRNA Expression

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Abstract. A three-dimensional collagen lattice can provide skin fibroblasts with a cell culture environment that simulates normal dermis. Such a collagen matrix environment regulates interstitial collagenase (type I metalloproteinase [MMP-1], collagenase-1) and collagen receptor α_2 subunit mRNA expression in both unstimulated or platelet-derived growth factor-stimulated dermal fibroblasts (Xu, J., and R.A.F. Clark. 1996. *J. Cell Biol.* 132:239–249). Here we report that the collagen gel can signal protein kinase C (PKC)- ζ activation in human dermal fibroblasts. An in vitro kinase assay demonstrated that autophosphorylation of PKC- ζ immunoprecipitates was markedly increased by a collagen matrix. In contrast, no alteration in PKC- ζ protein levels or intracellular location was observed. DNA binding activity of nuclear factor κ B (NF- κ B), a downstream regulatory target of PKC- ζ , was also increased by fibroblasts grown in collagen gel. The composition of the NF- κ B/Rel complexes that contained p50, was

not changed. The potential role of PKC- ζ in collagen gel-induced mRNA expression of collagen receptor α_2 subunit and human fibroblast MMP-1 was assessed by the following evidence. Increased levels of α_2 and MMP-1 mRNA in collagen gel-stimulated fibroblasts were abrogated by bisindolylmaleimide GF 109203X and calphostin C, chemical inhibitors for PKC, but retained when cells were depleted of 12-myristate 13-acetate (PMA)-inducible PKC isoforms by 24 h of pretreatment with phorbol PMA. Antisense oligonucleotides complementary to the 5' end of PKC- ζ mRNA sequences significantly reduced the collagen lattice-stimulated α_2 and MMP-1 mRNA levels. Taken together, these data indicate that PKC- ζ , a PKC isoform not inducible by PMA or diacylglycerol, is a component of collagen matrix stimulatory pathway for α_2 and MMP-1 mRNA expression. Thus, a three-dimensional collagen lattice maintains the dermal fibroblast phenotype, in part, through the activation of PKC- ζ .

THE interactions of cells with extracellular matrix (ECM)¹ are essential for cell behavior such as morphology, growth, motility, differentiation, and gene expression. In many biological and pathophysiological processes such as embryonic development, wound healing, tumor invasion and metastasis, and fibrosis, ECM plays this important role not only by its different components but also by its tightly regulated spatial and temporal organizations (Hay, 1991; Lin and Bissell, 1993; Grinnell, 1994; Clark, 1996). Three-dimensional ECM culture systems have been developed to simulate natural interactions be-

tween cells and ECM more closely than the traditional in vitro monolayer culture (Grinnell, 1994; Clark et al., 1995; Ronnov-Jessen et al., 1995; Streuli et al., 1995; Sankar et al., 1996). Among those systems, a relaxed collagen lattice populated by fibroblasts is considered an in vitro system representative of a normal fibrous stroma in vivo such as the dermis (Grinnell, 1994). When fibroblasts are embedded in the lattice consisting mainly of type I collagen, they contract the initially loose network to a dense tissue-like structure. This process is accompanied by a fundamental reprogramming of fibroblast morphology and metabolism. This results in down-regulation of type I collagen synthesis (Eckes et al., 1993), attenuation of cellular response to growth factors (Lin and Grinnell, 1993; Clark et al., 1995), induction of collagenase (Unemori and Werb, 1986) and the collagen receptor α_2 integrin subunit (Klein et al., 1991), and modulation of platelet-derived growth factor (PDGF) effects on integrin receptor expression (Xu and Clark, 1996).

Much attention has been paid to the role of protein kinase C (PKC) in ECM-regulated cellular activities. A family of serine/threonine-specific protein kinases, PKC has been linked to cell proliferation, differentiation, and regu-

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1. *Abbreviations used in this paper:* AA, arachidonic acid; BIM, bisindolylmaleimide GF 109203X; CalC, calphostin C; CAM, cell adhesion molecule; CHX, cycloheximide; COL, collagen gel; DAG, diacylglycerol; DOTMA, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride; ECM, extracellular matrix; MMP-1, type I metalloproteinase; NF- κ B, nuclear factor κ B; PC-PLC, phosphatidylcholine-hydrolyzing phospholipase C; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TC, tissue culture plates; TNF, tumor necrosis factor.

lation of gene expression. This enzyme family can be divided into three groups (for review see Nishizuka, 1995). The classic group containing isoforms α , β I, β II, and γ depends on Ca^{2+} and phorbol ester/diacylglycerol (DAG) for activity. The novel group containing isoforms δ , ϵ , η , θ , and μ is phorbol ester/DAG-dependent but does not require Ca^{2+} . An atypical group containing PKC- λ , τ , and ζ is not activated by phorbol ester/DAG. Cell adhesion has been reported to signal PKC activation. For example, adhesion of HeLa cells to a collagen substratum induces PKC activity (Chun and Jacobson, 1992, 1993). During HeLa cell adhesion to a gelatin substratum, PKC- ϵ is translocated from cytosolic to membrane fractions (Chun and Jacobson, 1996). Integrin cell adhesion molecules could be direct substrates for activated PKC since PKC phosphorylates the cytoplasmic domain of α 6A integrin subunit in vitro (Gimond et al., 1995). The PKC activity has been shown to be required for the spreading of several cell types, such as macrophage on immunoglobulin-coated surfaces (Li et al., 1996), HeLa cells on a collagen substratum (Chun and Jacobson, 1992, 1993), and CHO cells on fibronectin (Vuori and Ruoslahti, 1993). PKC is also involved in the formation of focal contacts by human embryo fibroblasts on substrata composed of fibronectin since its inhibitors reduced focal adhesion and stress fiber formation (Woods and Couchman, 1992).

The physiological activators of atypical PKC isoforms are not known. Evidence in the past few years has suggested that products of phosphoinositol 3-kinase, phosphoinositol 3,4-bisphosphate, and phosphoinositol 3,4,5-trisphosphate can induce PKC- ζ autophosphorylation activity in a cell-free assay (Nakanishi et al., 1993). Also, transducers of tumor necrosis factor (TNF)- α , such as ceramide, the product of sphingomyelin hydrolysis by acidic sphingomyelinase, and arachidonic acid (AA), a product of cellular phospholipase A2, can activate PKC- ζ (Nakanishi and Exton, 1992; Muller et al., 1995). The increased PKC- ζ activity by sphingomyelinase has also been observed (Lozano et al., 1994). In addition, PKC- ζ is directly associated with Ras (Diaz-Meco et al., 1994b).

The regulatory role of PKC- ζ in collagen-mediated cellular process has not been addressed. Nevertheless, studies have shown that cell adhesion to collagen substratum increases AA release (Chun and Jacobson, 1992; Auer and Jacobson, 1995) and Ras activity (Kapron-Bras et al., 1993), suggesting potential correlations between collagen signaling processes and PKC- ζ activation. The aim of this study is to understand how a three-dimensional collagen gel sends biochemical signals to induce cellular expression of a collagen receptor integrin subunit, α_2 , and collagenase type I metalloproteinase (MMP-1). In this report, we demonstrate that the collagen lattices activate fibroblast PKC- ζ . The PKC- ζ activity appears to be a component of the nuclear signaling cascade that leads to integrin α_2 and MMP-1 expression.

Materials and Methods

Cell Culture

Human fibroblast cultures established by outgrowth from healthy human skin biopsies were kindly provided by Marcia Simon (Department of Der-

matology, SUNY at Stony Brook). The cells were maintained in DME (GIBCO BRL, Gaithersburg, MD), supplemented with 10% FCS (Hyclone Labs, Logan, UT), 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 15 and 20 (the 6th and 10th passage) were used for the experiments.

Antibodies and Inhibitors

Cycloheximide (CHX) was obtained from Sigma Chemical Co. (St. Louis, MO). Bisindolylmaleimide GF 109203X (BIM) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Calphostin C and chelerythrine were purchased from LC Laboratories (Woburn, MA). Polyclonal antibodies against human protein kinase C- ζ were purchased from GIBCO BRL. Monoclonal antibodies against human protein kinase C- τ , λ and α were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against PKC- μ , nuclear factor κ B (NF- κ B), p65, and p50 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against human PDGF AB and cyclin A were purchased from Upstate Biotechnology (Lake Placid, NY).

Preparation of Collagen Lattices

Collagen gels were prepared according to a procedure previously described (Xu and Clark, 1996). Pepsin-solubilized bovine dermal collagen dissolved in 0.012 M HCl was 99.9% pure containing 95–98% type I collagen and 2–5% type III collagen (Vitrogen 100; Cetrax 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 1% FCS in DME at pH 7.0–7.4. Human dermal fibroblasts from subconfluent cultures starved in 1% FBS for 24 h were mixed with 10 ml collagen solution for a final concentration of 5×10^5 cells/ml. The collagen cell suspension (4 ml) was immediately placed onto 2% BSA (Kankakee, IL)-coated 60-mm petri dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) and incubated at 37°C for 2 h (or for the time periods described in figure legends) before the addition of 5 ml of 1% FCS/DME to each dish.

After incubation at 37°C in 95% air, 5% CO_2 , and 100% humidity for the indicated time, cultures were carefully washed twice in DME and processed for various analyses. In experiments where inhibitors were used, the levels of lactate dehydrogenase activity released were measured (LD Diagnostic kit, Sigma Chemical Co.) and found to be similar to cells cultured in the absence of inhibitors.

Coating of Petri Dishes

For monolayer collagen coating of plastic dishes, the collagen used for lattices was diluted to a final concentration of 50 μ g/ml with PBS. This solution was added to plastic dishes at a final concentration of 6.4 μ g/cm² and incubated overnight at 4°C. Coated dishes were blocked with 2% BSA for 2 h at room temperature and rinsed with PBS twice before use.

Incubation with Antibodies

Collagen gels minus fibroblasts and 1% FCS/DME were preincubated with polyclonal antibodies against PDGF AB (100 μ g/ml) and, as a control, cyclin A (100 μ g/ml) for 1 h at 4°C. Fibroblasts starved in 1% FCS/DME for 1 d were detached by trypsinization and then seeded into collagen gels containing relevant antibodies. For antibody blocking of PDGF stimulation, the medium was replaced with fresh medium containing 30 ng/ml PDGF-BB preincubated with or without anti-PDGF for 1 h. (PDGF-BB was generously provided by Charles Hart of ZymoGenetics, Seattle, WA.) All the control media were replaced accordingly, minus PDGF-BB. Cells were further incubated for 18 h.

Northern Analysis of Total Cellular RNA

Total RNA was isolated from cell monolayers and collagen gel cultures using a modification of guanidinium thiocyanate method (Chromczynski and Sacchi, 1987). After centrifugation at 14,000 g to remove culture medium, collagen gels were dissolved in 4 M guanidinium isothiocyanate and repeatedly passed through a 20 1/2-gauge needle. For Northern blot hybridization, 3–5 μ g 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 Corp., Arlington Heights, IL). Ethidium bromide (0.5 μ g/ml) was included in the gel to monitor equal loading by the quantity of 18 S and 28 S

ribosomal RNA present. cDNA probes were labeled with [α - 32 P]dCTP by the random primer procedure (Du Pont/NEN, Boston, MA). Oligonucleotide probes were end-labeled with [γ - 32 P]ATP (Du Pont/NEN) and in the presence of polynucleotide kinase (Boehringer Mannheim Corp., Indianapolis, IN). 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. The signals were detected by autoradiography (model X-Omat AR; Kodak Eastman, Rochester, NY) at -80°C for optimal exposure. All results shown are representative of at least two independent experiments. Human α_2 cDNA was a generous gift from Dr. Yoshikazu Takada (Scripps Institute, La Jolla, CA) (Takada and Hemler, 1989). Human MMP-1 cDNA was purchased from American Type Culture Collection (Rockville, MD). Human α_5 cDNA was purchased from GIBCO BRL. An oligonucleotide complementary to 28 S ribosomal RNA was purchased from Clontech (Palo Alto, CA).

Preparation of Cell Extracts

Fibroblasts grown in collagen gels were released by digestion of gels with collagenase D (Boehringer Mannheim Corp.). Cells grown on plastic plates were scraped and subsequently processed as those released from collagen gel. Cells from all culture conditions were then washed with ice-cold PBS twice and resuspended in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, and proteinase inhibitors). After incubation on ice for 30 min, cells were repeatedly passed through a 26 1/2-gauge needle followed by centrifugation at 14,000 g for 20 min. Protein content was determined using a bicinchonic acid assay (Pierce, Rockford, IL).

Isolation of Nuclei

The nuclei were prepared according to a protocol previously described with some modifications (Greenberg and Ziff, 1984). Cells released from collagen gel or scraped from plastic plates were washed twice with ice-cold PBS. Cells were precipitated by centrifugation at 500 g for 5 min at 4°C. Cell pellet was washed in buffer A (10 mM Tris, pH 7.4, 3 mM CaCl₂, 2 mM MgCl₂), resuspended in 1 ml lysis buffer B (0.5% NP-40 in buffer A) and homogenized in a Dounce homogenizer with a "B" pestle. After examination under a microscope to monitor the release of nuclei, the homogenate was centrifuged at 500 g at 4°C. The pellet was designated as nuclei, whereas the supernatant was designated as cytoplasmic fraction. The nuclear pellet was stored in buffer C (50 mM Tris, pH 8.0, 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM PMSF, 1 μ M leupeptin). The protein content in cytoplasm and nuclei was determined using the bicinchonic acid assay (Pierce).

Western Immunoblotting

Proteins from cell and nuclear extracts were separated on 8% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Samples containing 4–7 μ g of total proteins were loaded onto the gel. The membranes were incubated with a blocking solution containing 2% BSA, 2% horse serum, 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for 1 h at room temperature and then incubated overnight at 4°C with various antibodies: monoclonal PKC- α , - τ , - λ and β -tubulin, and polyclonal PKC- ζ and - μ . After incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1,000 dilution; Amersham Corp.) in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for 1 h at room temperature, the blots were then visualized with enhanced chemiluminescence (Amersham Corp.).

Immunoprecipitation and In Vitro PKC- ζ Activity Assay

Cell extracts prepared from 10⁶ cells were incubated at 4°C overnight with a rabbit polyclonal antibody against human PKC- ζ used in Western analysis. The immune complexes were recovered by anti-rabbit IgG agarose beads (Sigma Chemical Co.). The resulting immunoprecipitates were washed three times with a cold buffer containing 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 25 μ g/ml leupeptin, and 25 μ g/ml aprotinin before they were mixed in a final volume of 50 μ l assay solution (35 mM Tris, pH 7.5, 15 mM MgCl₂, 1 mM MnCl₂, 0.5 mM EGTA, 0.1 mM CaCl₂, 1 mM sodium orthovanadate, and 100 μ M [γ - 32 P]ATP with or without 280 μ g/ml phosphatidyserine) and incubated at 30°C for 10 min. After reactions were stopped by the addition of the equal volume of 2 \times gel loading

buffer, the samples were boiled for 3 min and precipitated. The supernatant was analyzed by SDS-PAGE followed by autoradiography.

Gel Mobility Shift Assay

Nuclear extracts were prepared by a modified miniextraction protocol (Schreiber et al., 1989). Cells were washed with ice-cold PBS twice and hypotonic buffer A once (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM PMSF, 0.5 mM DTT). Cells were lysed by incubation in lysis buffer (0.2% NP-40 in buffer A) for 10 min on ice. After centrifugation at 500 g for 4 min at 4°C, the nuclear pellet was resuspended in extraction buffer C (20 mM Tris, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 20 mM KCl, 0.5 mM PMSF, 0.5 mM DTT) and D (20 mM Tris, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 1.2 M KCl, 0.5 mM PMSF, 0.5 mM DTT) at a 1:2 ratio followed by incubation on ice for 20 min. The supernatant was collected as nuclear extracts after centrifugation at 14,000 g for 8 min. The nuclear protein was determined using the bicinchonic acid assay (Pierce).

Gel mobility shift assay was performed with nuclear extracts prepared as described above. NF- κ B and Sp-1 enhancer element consensus sequences 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 5'-ATT CGA TCG GGG CGG GGC GAG C-3', respectively, were purchased (Promega Corp., Madison, WI). These oligonucleotides were labeled by [γ - 32 P]ATP. The nuclear extracts (3–5 μ g) were incubated with 1 μ g poly (dI/dC) (Boehringer Mannheim Corp.) and 2 μ g BSA (GIBCO BRL) in a binding buffer (10 mM Tris, pH 7.9, 5 mM MgCl₂, 50 mM KCl, 10% glycerol, and 1–5 \times 10⁴ cpm end-labeled oligonucleotides) for 20 min at room temperature. The samples were separated on a 5% native polyacrylamide gel in 0.5 \times TBE buffer (Tris-borate-EDTA). For supershift assays, the reaction mixture minus the probe was incubated with 32 P-labeled oligonucleotides for 20 min followed by incubation with 2 μ l antibodies for 30 min at room temperature. The samples were separated on a 4% native polyacrylamide gel.

Down-Regulation of PKC- ζ Protein by Antisense Inhibition of Translation

The procedure was essentially as previously described (Xu et al., 1996) with modifications to accommodate the collagen gel culture. Phosphorothioate DNA oligonucleotides with the sequences 5'-ATGCCAG-CAGGACC-3' (sense 1143), 5'-GGTCCTYGCTGGGCAT-3' (antisense 1142), and 5'-GGTCCTGCTGGGCATGCG AAAGC-3' (antisense 1144) were synthesized by Promega. Subconfluent adult human dermal fibroblasts were treated with oligonucleotides in DME containing 20 μ g/ml lipofectin (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride [DOTMA]; GIBCO BRL) for 6–8 h at 37°C in the presence of 5% CO₂. After this time, the medium containing lipofectin was replaced by fresh medium containing appropriate oligonucleotides. After 48 h, cells were subcultured into collagen gel containing freshly added oligonucleotides. After incubation for 18 h, total RNA or cell extracts were prepared and analyzed with Northern blotting, Western blotting, or immunoprecipitation and kinase assay.

Results

Inhibition of Collagen Gel-induced α_2 and MMP-1 mRNA Levels

Previous studies have shown that collagen gel increases α_2 integrin mRNA steady-state levels in human foreskin (Klein et al., 1991) and adult dermal (Xu and Clark, 1996) fibroblasts. Relaxed collagen gels also induce fibroblast collagenase (Unemori and Werb, 1986; Langholz et al., 1995). Here we demonstrate that the induction of α_2 and MMP-1 mRNA occurred only when cells were cultured in a three-dimensional collagen gel, not on collagen monolayer-coated surface (Fig. 1 A), in agreement with Langholz et al. (1995). A time course showed that the mRNAs appeared as early as 4 h after cells were placed in collagen lattices (Fig. 1 B). α_2 mRNA reached the maximum after 24 h, whereas MMP-1 continued to increase up to 72 h (Fig. 1 B).

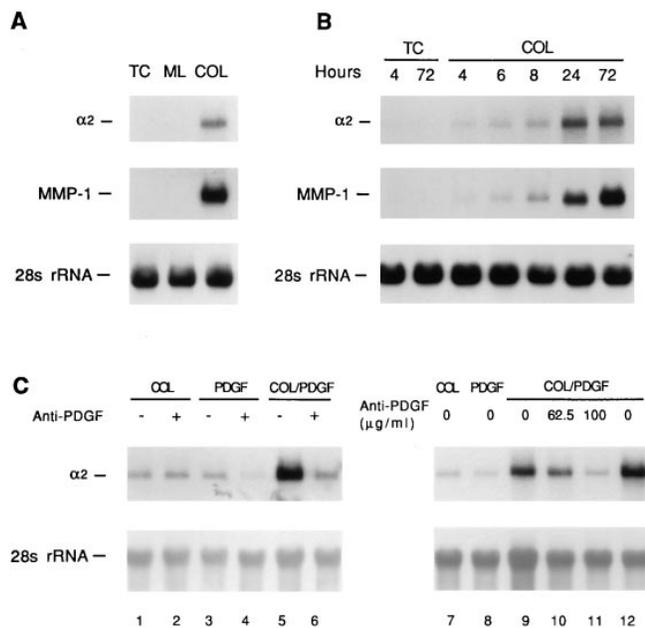


Figure 1. Northern analysis of integrin α_2 subunit and MMP-1 mRNA induced by three-dimensional collagen lattices. Normal human dermal fibroblasts were starved 1 d in 1% FCS/DME before subculture in test conditions. (A) Cells were subcultured on tissue culture plates (TC), collagen monolayer coated surface (ML), and in three-dimensional collagen lattices (COL). (B) Cells were subcultured on tissue culture plates (TC) and in three-dimensional lattices (COL) for the time indicated. (C) Collagen gel mix was preincubated with anti-PDGF at 100 ng/ml (lanes 2 and 6) and at indicated concentrations (lanes 10 and 11), and with anti-cyclin A at 100 ng/ml (lane 12). Cells in collagen gel (lanes 5, 6, and 10–12) or on TC (lanes 3, 4, and 8) were stimulated with 30 ng/ml PDGF-BB preincubated with (lanes 4, 6, 10, and 11) or without anti-PDGF (lanes 3, 5, 8, and 9) or with anti-cyclin A (lane 12) 4 h after subculture. Total RNA was probed with human α_2 integrin or MMP-1 cDNAs as indicated. 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 28 S ribosomal RNA. Results are representative of two experiments.

Since PDGF-BB also induces steady-state levels of α_2 mRNA (Ahlen and Rubin, 1994; Xu et al., 1996), we asked whether the induction by collagen gel was caused by PDGF present in collagen preparations. Antibody against PDGF has been shown to neutralize PDGF biological effects (Ferns, 1991). Polyclonal antibody against PDGF AB was therefore included in the collagen gel cultures and, as control, in fibroblasts grown on conventional tissue culture plates (TC) and in collagen gel (COL) before PDGF stimulation. As shown in Fig. 1 C, the antibodies did not affect the induction by COL (lanes 1 and 2) but drastically inhibited PDGF effects (lanes 3 and 4). The possibility that the failure of anti-PDGF to inhibit COL induction may result from PDGF-BB in collagen gel higher than 30 ng/ml, a concentration used for PDGF effects on TC, was examined. Since the saturating concentration of PDGF-BB to induce α_2 expression is between 10–15 ng/ml (Xu and Clark, 1996), we rationalized that if high concentration of PDGF in COL was responsible for α_2 induction, the addition of

PDGF cannot further increase α_2 expression. Fibroblasts grown in collagen gel were further stimulated by PDGF-BB at 30 ng/ml, and integrin α_2 mRNA level was greatly increased compared to COL or PDGF alone (Fig. 1 C, lanes 5 and 9), confirming our previous observation (Xu and Clark, 1996). This further induction was inhibited by anti-PDGF in a dose-dependent manner (Fig. 1 C, lanes 10 and 11). A control antibody, cyclin A, did not have any effect (Fig. 1 C, lane 12). Taken together, signals from three-dimensional collagen lattices responsible for induction of integrin α_2 and MMP-1 do not emanate from PDGF contamination.

The requirement of newly synthesized proteins and posttranslational modifications was investigated next. Protein synthesis was required since both types of mRNA diminished in the presence of CHX, a protein synthesis inhibitor (Fig. 2 A). To understand what role PKC might play, three specific PKC inhibitors, BIM, calphostin C (CalC), and chelerythrine, were used in the study. Both BIM and CalC blocked the induction of both mRNAs in a dose-dependent manner, indicating that protein phosphorylation by PKC is required for both α_2 and MMP-1 mRNA

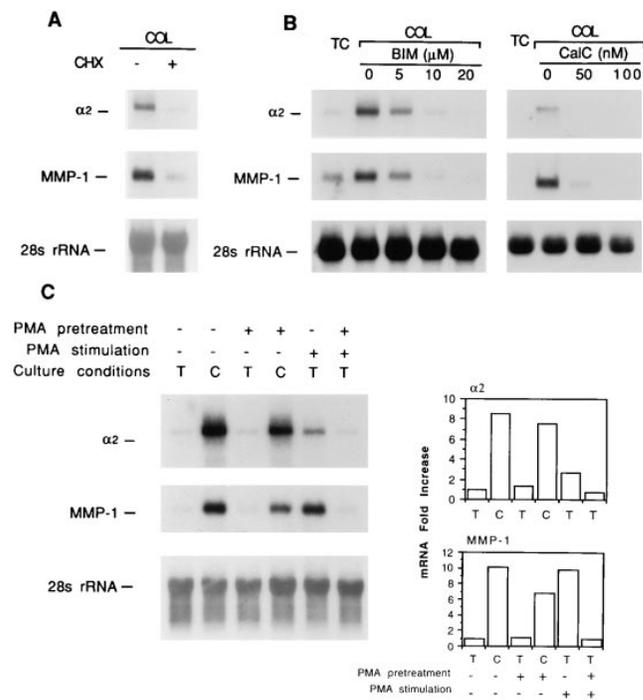


Figure 2. Inhibition of collagen gel induction of α_2 and MMP-1 mRNA levels. (A and B) Cells were preincubated with inhibitors before subculture. (A) 10 μ g/ml cycloheximide (CHX), a protein synthesis inhibitor, for 15 min. (B) PKC inhibitors bisindolylmaleimide GF 109203X (BIM) and calphostin C (CalC) at concentrations indicated for 1 h and 30 min, respectively. The incubation with inhibitors was continued for 18 h after subculture. (C) Quiescent cells either untreated or incubated for 24 h with PMA (300 ng/ml) were subcultured in collagen lattices or stimulated with PMA (50 ng/ml) for 16 h. Total RNA was probed with human α_2 integrin and MMP-1 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. Results are representative of two independent experiments. T, tissue culture plates; C, collagen lattices.

expression (Fig. 2 B). Chelerythrine, however, did not demonstrate the inhibitory effect on the induction (data not shown).

Since there are 12 PKC isoforms identified so far, nine of which are phorbol 12-myristate 13-acetate (PMA)/DAG-inducible, whereas three were not induced by PMA, the identity of the PKC isoform(s) that mediated collagen-induced α_2 and MMP-1 mRNA expression was sought. As a first step, we investigated whether this PKC was PMA-inducible or not. A widely used strategy is to deplete cellular PMA-inducible PKC levels by treating cell culture chronically with PMA (Larrodera et al., 1990). This approach depletes PKC isoforms sensitive to PMA/DAG activation. Thus, quiescent human fibroblast cultures were exposed to PMA (300 ng/ml) for 24 h before they were subcultured into collagen gel. PMA induction of cells cultured on tissue culture plates were performed in parallel as a control. α_2 and MMP-1 mRNA steady-state levels were then determined. As expected, PMA was unable to induce either α_2 or MMP-1 mRNA in cells chronically treated with PMA (Fig. 2 C, right lane). In contrast, collagen gel promoted a potent response in α_2 mRNA expression in PMA-pretreated cells, which was only slightly lower than that in untreated cells (Fig. 2 C, middle two lanes). This result indicates the involvement of atypical PKC(s). However, MMP-1 mRNA response to collagen gel was reduced from 10-fold to 6.3-fold increase in cells chronically treated with PMA (Fig. 2 C, middle two lanes). These results suggest that collagen lattices activate an atypical PKC isoform that, to a different extent, is required for α_2 and MMP-1 mRNA expression.

The Expression of Atypical PKC Isoforms

To determine which atypical PKC may be required for the collagen gel induction, protein levels of atypical PKC isoforms in human dermal fibroblasts were first examined. We performed Western analysis of total cell extracts after cells were grown in collagen gels for various time periods. In these experiments, HeLa cell extracts were used as a positive control (data not shown), as they contain all of the PKC isoforms examined (Chun and Jacobson, 1996). All three known members of the atypical PKC subfamily, ζ , τ , and λ , were detected in human dermal fibroblasts (Fig. 3). The antibody against the PKC- ζ isoform detected a high level of immunoreactive proteins from the lysates. Collagen gel did not change its amount. PKC- λ and τ were detected at relatively low levels in both monolayer and collagen gel cultures, and no obvious differences could be discerned in the levels from the two culture conditions (Fig. 3). When lysate amount, antibody concentration, or exposure time was increased, the results became questionable because of the high background. A novel PKC isoform, PKC- μ , was also examined. Western blot detected PKC- μ at a level comparable with PKC- ζ . In contrast to atypical PKC isoforms that were not synthetically affected by the collagen gel culturing, the cellular protein level of PKC- μ decreased as cells were incubated in collagen gel for 18 h (Fig. 3). These results demonstrate that collagen lattices, which were capable of altering some PKC isoform protein levels, did not change the protein amount of atypical PKCs in human fibroblasts.

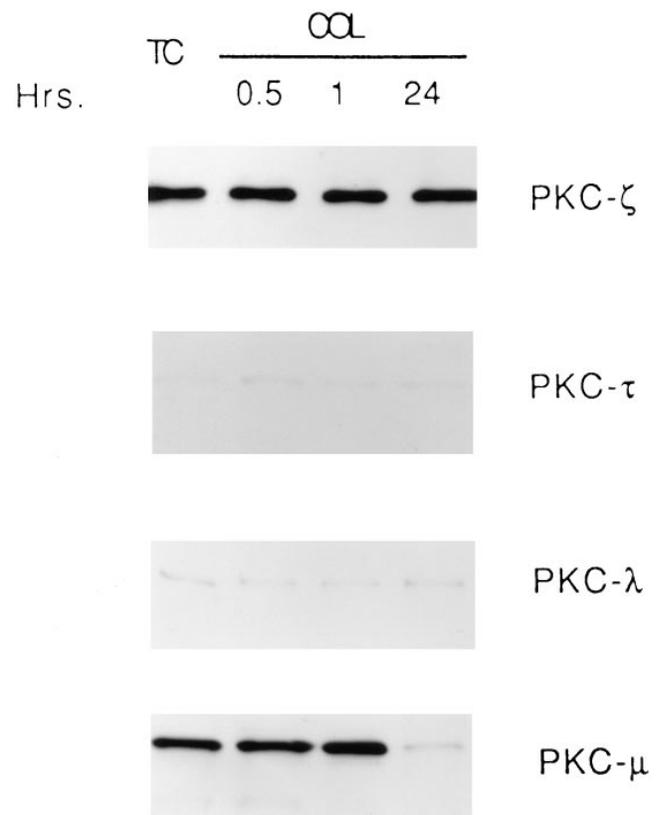


Figure 3. Western analysis of PKC isoforms in total cell lysates. Normal human dermal fibroblasts were cultured 1 d in 1% FCS/DME before subculture in test conditions. Cells were subcultured on tissue culture plates (TC) or in three-dimensional collagen lattices (COL) for the time indicated. Total cellular proteins were extracted, quantified with BCA assay, blotted, and detected with antibodies against PKC- ζ , τ , λ , and μ .

Collagen Gel Induction of PKC- ζ Kinase Activity

Since collagen gel induction of α_2 integrin and MMP-1 mRNA involved atypical PKC isoforms, collagen lattices may induce atypical PKC enzyme activity. The kinase activity of PKC- ζ was investigated for the following reasons. PKC- ζ is CalC (Larivee et al., 1994) and BIM sensitive (Xu et al., 1996), chelerythrine insensitive (Thompson and Fields, 1996), and not down-regulated by PMA (Wooten, 1994), which is consistent with the results observed thus far (Fig. 2, B and C). Furthermore, PKC- ζ was the only member of atypical PKC subfamily detected in human fibroblasts in significant amounts (Fig. 3). To assess whether collagen gel can induce PKC- ζ kinase activity, fibroblasts grown in collagen gel for various time periods were assayed for PKC- ζ kinase activity. PKC- ζ present in the cell extracts was immunoprecipitated with a polyclonal antibody. PKC- ζ was autophosphorylated by kinase activities associated with the immunoprecipitates. The kinase activity was induced 30 min after fibroblasts cultured in collagen gel (4.6-fold), reached the maximum at 4 h (9.2-fold), and decreased after 4 h (Fig. 4, A and C). Cells grown on tissue culture plates, however, remained unstimulated during the entire time course (data not shown). The presence of phosphatidylserine, a PKC activator, increased

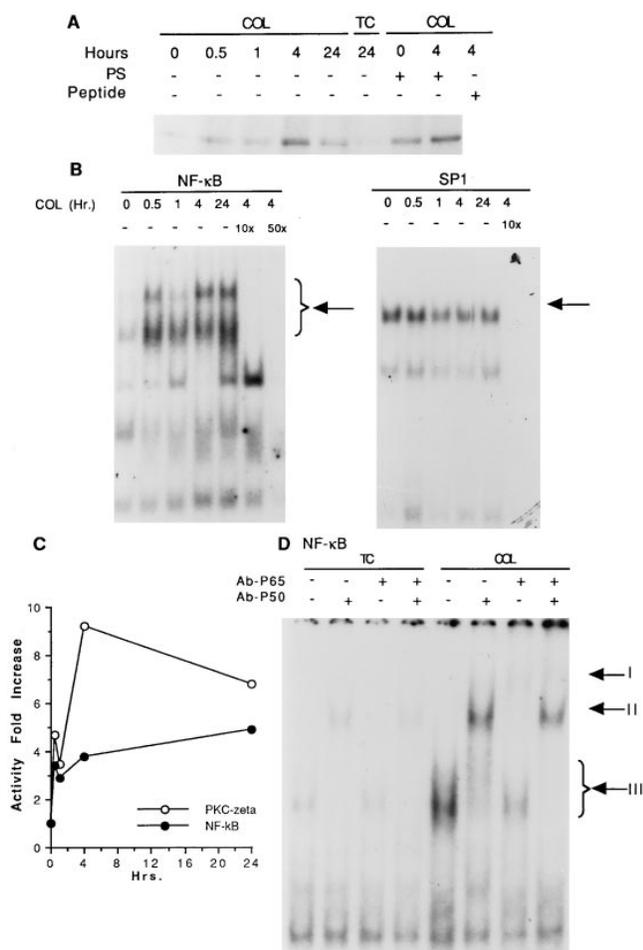


Figure 4. Stimulation of PKC- ζ and NF- κ B DNA binding activity by three-dimensional collagen lattices. Normal human dermal fibroblasts were cultured 1 d in 1% FCS/DME before subculture in test conditions. Cells were subcultured on tissue culture plates (TC) or in three-dimensional collagen lattices (COL) for the times indicated. (A) PKC- ζ kinase activity assay. Total cellular proteins were extracted, quantified with BCA assay, and immunoprecipitated with a polyclonal antibody against PKC- ζ in the presence or absence of a synthetic peptide to which the antibody was raised (Peptide). The immunoprecipitates were incubated in a kinase assay buffer for 10 min at 30°C. Unless specified, the reactions were carried out in the absence of phosphatidylserine. The kinase activity was determined by autophosphorylation as described in Materials and Methods. The results are representative of three independent experiments. (B) Gel mobility shift assay. Nuclear extracts were prepared and assayed for DNA binding activity of NF- κ B and SP1 by gel mobility shift as described in Materials and Methods. Arrows indicate specific bindings. The results are representative of three independent experiments. (C) Quantification of A and B. (D) Supershift assay. Nuclear extracts prepared from cells stimulated with collagen gel (COL) for 24 h and on tissue culture plates (TC) were incubated with labeled NF- κ B consensus sequences before further incubation with antibodies against p65 and p50. I, the supershift caused by p65; II, the supershift caused by p50; III, the specific NF- κ B binding.

the PKC- ζ activity impressively from unstimulated (sevenfold) and slightly from stimulated cells (11.5-fold, a 20% increase). Therefore, collagen gel appears to be an activator for fibroblast PKC- ζ , as determined by kinase activity associated with PKC- ζ immunoprecipitates.

Collagen Gel Induction of DNA Binding Activity of Transcription Factor NF- κ B

PKC- ζ has been reported as a positive regulator of the activity of a transcription factor, NF- κ B (Diaz-Meco et al., 1993). Cells that display enhanced PKC- ζ phosphorylation activity also increase NF- κ B DNA binding activity in response to sphingomyelinase (Lozano et al., 1994), *ras* p21 (Diaz-Meco et al., 1993), and TNF- α (Muller et al., 1995). The induction of PKC- ζ activity by cells incubated in collagen gels prompted us to ask whether NF- κ B DNA binding activity was also induced. Nuclear extracts were prepared from human fibroblasts grown in collagen gel from 30 min to 24 h. The NF- κ B DNA binding activity present in the nuclear extracts was detected by gel mobility shift assay using a DNA probe encompassing the κ B motif (see Materials and Methods). As seen in Fig. 4 B, a 30-min incubation of human fibroblasts in collagen gel induced the formation of specific κ B DNA-protein complexes. The NF- κ B DNA binding activity remained similar from 30 min to 4 h (Fig. 4 C, 2.9–3.8-fold increases) but increased modestly after 24 h of incubation in collagen gel (Fig. 4 C, 4.9-fold increases). The quantification of the NF- κ B DNA binding activity revealed a kinetic pattern in concord with, if not identical to, that of PKC- ζ kinase activity (Fig. 4 C). Cells grown on tissue culture plates, on the contrary, did not demonstrate any change in the NF- κ B DNA binding activity (data not shown). Competition experiments with an unlabeled κ B consensus sequence confirmed the specificity of the binding complexes. As a control, the binding activity to the Sp1, a transcription factor, consensus sequence was also examined (Fig. 4 B). The Sp1 DNA binding activity present in nuclear extracts was not altered by cells cultured in collagen gel. Therefore, we conclude that this three-dimensional cell culture system increased PKC- ζ activity in association with the activation of NF- κ B DNA binding complexes. The composition of the NF- κ B DNA binding complexes was examined next. The supershift assay showed that p50 of NF- κ B/Rel family proteins was one component of the binding complex (Fig. 4 D, II) regardless of culture condition. Antibody to p65, however, only shifted a very low amount of the binding complex (Fig. 4 D, I), suggesting that the binding complex may be composed of other member(s) of NF- κ B/Rel family. Therefore, collagen gel induced the p50-containing complex binding to NF- κ B site without changing protein composition.

Antisense Translational Inhibition of PKC- ζ

The results presented so far have suggested that collagen gel was an activator of PKC- ζ activity and that collagen gel-induced α_2 and MMP-1 mRNA expression required atypical PKC isoform activity. The potential role of PKC- ζ in collagen gel-induced α_2 and MMP-1 mRNA expression was then investigated. To establish a direct connection between these two events, we used a previously described strategy to remove PKC- ζ protein through translation inhibition by antisense oligonucleotides (Xu et al., 1996). Antisense phosphorothioate oligonucleotides 1142 and 1144 have sequences targeted at the beginning of the open reading frame of PKC- ζ cDNA (Barbee et al., 1993). This GC-rich site is in a nonconserved variable region (V1) of PKC family (Nishizuka, 1992), which differs significantly among

PKC isoforms. Antisense oligonucleotides 1142 and 1144 are essentially the same except that 1144 was designed to have an additional looping secondary structure at its 3' end to reduce the possible degradation caused by 3' exonuclease (Tang et al., 1993). Previously we have found that the antisense oligonucleotides at concentrations higher than 2.5 μ M can inhibit human fibroblast PKC- ζ protein level by at least 70% (Xu et al., 1996). Therefore, fibroblasts were lipid-transfected with antisense oligonucleotides 1142 or 1144 at 2.5 μ M. As a control, some cells were transfected with "sense" oligonucleotide 1143. The transfected cells were then subcultured into collagen gels and further treated with oligonucleotides for 18 h. The depletion of PKC- ζ protein levels by antisense inhibition of translation was monitored by Western blotting (Fig. 5 A) and kinase activity of PKC- ζ immunoprecipitates (Fig. 5 B). The results from Fig. 5 demonstrated a significant reduction of PKC- ζ protein and its associated kinase activity after treatment with both oligonucleotides, 1142 and 1144. The specificity of antisense inhibition was confirmed by probing the membrane with an mAb against PKC- α . The equal loading was confirmed by detection with an mAb against β -tubulin (Fig. 5 A).

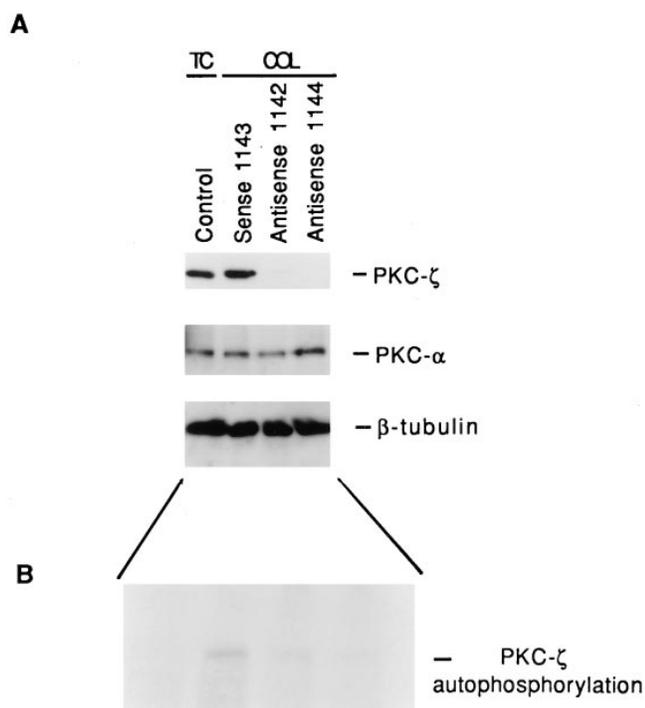


Figure 5. Specific down-regulation of protein levels and PKC- ζ activity by antisense inhibition of translation. Subconfluent normal dermal human fibroblasts were either untreated or treated with sense (1143) or antisense oligonucleotides (1142 and 1144) at 2.5 μ M in DME containing 20 μ g/ml DOTMA for 6–8 h. After this time, the medium containing DOTMA was replaced by fresh medium containing appropriate oligonucleotides. Cells were subcultured 48 h later on tissue culture plates (TC) or in three-dimensional collagen lattices (COL) and continuously incubated in appropriate oligonucleotides for 18 h. Total cellular proteins were extracted, quantified with BCA assay, and assayed for PKC- ζ in vitro kinase activity or protein level. (A) Western blot detection of PKC- ζ , PKC- α , and β -tubulin. (B) In vitro kinase activity in immunoprecipitates of PKC- ζ .

Such cells were harvested for analysis of α_2 and MMP-1 mRNA expression. Antisense treatment inhibited collagen gel induction of α_2 expression up to 60% (Fig. 6). Both oligonucleotides 1142 and 1144 had similar effects. These results are consistent with observations of cells treated with the PKC inhibitors, BIM and CalC (Fig. 2 B), and chronically with PMA (Fig. 2 C). MMP-1 expression was also inhibited by antisense treatment, although less effectively with 1142 and more with 1144. The expression of a fibronectin receptor integrin subunit, α_5 , was not affected by either a collagen gel environment or antisense treat-

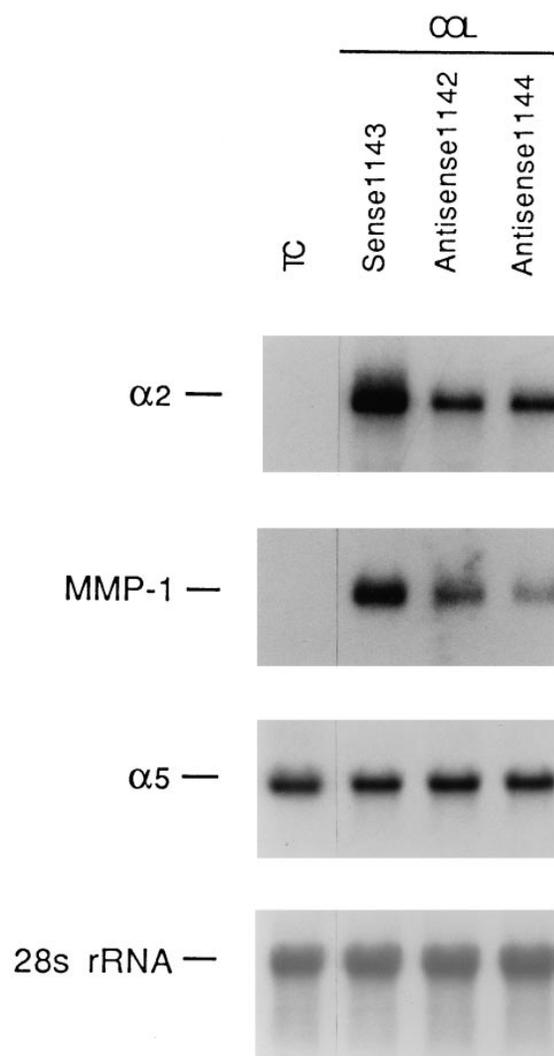


Figure 6. Antisense-mediated down-regulation of PKC- ζ protein inhibits collagen gel induction of integrin α_2 and MMP-1 mRNA expression. Total cellular RNA was extracted from normal human dermal fibroblasts after incubation in the presence or absence of sense (1143) or antisense (1142 and 1144) oligonucleotides complementary to the 5'-end of the PKC- ζ transcript at 2.5 μ M. Before RNA harvest, cultures were incubated in collagen lattices for 18–24 h. Total RNA was probed with human α_2 integrin, MMP-1 and α_5 integrin 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. Results are representative of two independent experiments.

ment, confirming the specificity of the antisense inhibition. Therefore, PKC- ζ is involved in collagen gel induction of the α_2 integrin subunit and MMP-1 mRNA expression.

The Cytoplasmic and Nuclear Localization of PKC- ζ

Several PKC isoforms have been reported to be present in the nucleus, including PKC- δ and PKC- ϵ (Ventura et al., 1995). The nuclear localization of those PKC isoforms seems directly correlated to its functional role in regulating cellular biosynthetic activities. For example, the phorbol ester-regulated expression of opioid peptide gene in rat myocardial cells was reported as a possible target of nuclear PKC- δ and - ϵ through autocrine or paracrine mechanisms (Ventura et al., 1995). The involvement of PKC- ζ in mRNA expression of α_2 and MMP-1 prompted us to ask whether PKC- ζ is a cytosolic or nuclear protein in human dermal fibroblasts. Previously, PKC- ζ has been reported present in cytoplasm and nucleus in species such as rat and rabbit (Masmoudi et al., 1989; Hagiwara et al., 1990; Disatnik et al., 1994; Rosenberger et al., 1995). To assess this possibility of human PKC- ζ , the distribution of PKC- ζ as well as - μ in cytoplasm and nucleus was examined. Intact nuclei were isolated from fibroblasts grown as monolayer or in three-dimensional collagen gel. Western analysis was performed with cytoplasmic and nuclear fractions. While PKC- μ was predominantly present in the cytoplasm, PKC- ζ was detected in both cytoplasm and nucleus (Fig. 7). Cells grown in collagen gel did not change the distribution between the two subcellular compartments. Therefore, PKC- ζ , the kinase required for collagen gel regulatory pathway leading to α_2 and MMP-1 mRNA expression in human dermal fibroblasts, is constitutively a nuclear as well as a cytoplasmic protein.

Discussion

We have previously shown that a three-dimensional collagen lattice, either stressed or relaxed, can induce integrin α_2 mRNA expression in human dermal fibroblasts (Xu and Clark, 1996). Interestingly, no induction of α_2 or MMP-1 mRNA is observed when fibroblasts are plated on a collagen monolayer (Fig. 1 A) as previously reported by Langholz et al. (1995) for MMP-1. The evidence presented in this report indicates that PKC- ζ is required for the full response of integrin α_2 subunit and MMP-1 mRNA to collagen lattice (Fig. 6).

Although the nature of precise primary signals triggered by a three-dimensional type I collagen construct is undefined, we report here that these collagen gels activate PKC- ζ (Fig. 4 A) as a component of secondary transduction signals (second messenger pathway). How might collagen gel activate PKC- ζ ? Previously, we showed that PDGF can induce PKC- ζ activity (Xu et al., 1996). Could collagen gel use the same pathway as PDGF to activate PKC- ζ ? In fact, adhesion molecules such as CAMs (cell adhesion molecules) and basic fibroblast growth factor both activate FGF receptor to induce cell contact-dependent neurite outgrowth (Williams et al., 1994). However, several lines of evidence argue against the similar scheme with collagen gel and PDGF. First, Lin and Grinnell (1993) have presented evidence that PDGF, but not collagen gel,

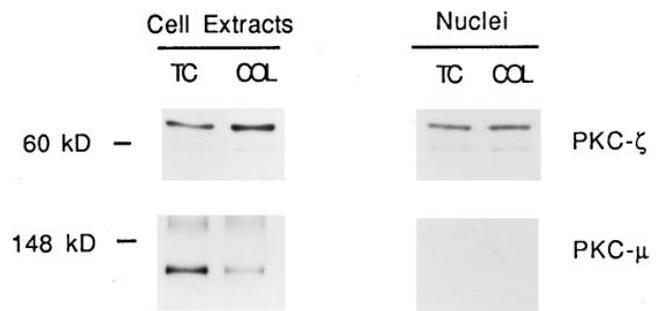


Figure 7. Western analysis of PKC isoforms in cytoplasm and nuclei. Normal human dermal fibroblasts were starved 1 d in 1% FCS/DME before subculture in test conditions. Cells were subcultured on tissue culture plates (TC) or in three-dimensional collagen lattices (COL) for 18–24 h. Nuclear (Nuclei) and cytoplasmic (Cell Extracts) fractions were prepared, quantified with BCA assay, blotted, and detected with antibodies against PKC- ζ and - μ .

induces tyrosine phosphorylation of PDGF receptor in fibroblasts. Second, the same report also showed that collagen gel, especially relaxed collagen gel, actually reduces PDGF-stimulated receptor autophosphorylation. Third, we previously reported that collagen gel does not stimulate expression of integrin α_3 and α_5 subunits, two PDGF-inducible genes (Xu and Clark, 1996). Fourth, collagen gel possesses both positive and negative impact on PDGF stimulation of integrin subunit mRNA expression: positive on α_2 , negative on α_3 and α_5 (Xu and Clark, 1996). The data taken together, in fact, indicate that collagen gel interferes with some PDGF pathways. Thus, collagen gel and PDGF signaling pathways appear not to converge at PDGF receptor site.

Among known activators of PKC- ζ are AA generated by phospholipase A2 and ceramide generated by phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC)/sphingomyelinase. Adhesion of HeLa cells to collagen (Chun and Jacobson, 1993) or β_1 integrin clustering (Auer and Jacobson, 1995) increased the release of AA. AA could activate PKC- ζ either directly (Nakanishi and Exton, 1992) or indirectly by triggering DAG release (Auer and Jacobson, 1995). DAG released from AA metabolism may activate PKC- ζ by sphingomyelinase-ceramide pathway since it was able to increase sphingomyelin hydrolysis in some cell types (Kolesnick, 1987). Alternatively, collagen gel may sequentially activate PC-PLC and PKC- ζ . In our system, disruption of PC-PLC activity with its inhibitor D609 (Schutze et al., 1992) blocked collagen gel induction of α_2 mRNA expression (data not shown), implicating the importance of this pathway. Another possibility for PKC- ζ activation by collagen gel may be its phosphorylation by a second serine/threonine kinase. Several reports have suggested that the phosphorylation may precede the PKC activation (Pears et al., 1992; Cazaubon and Parker, 1993). This phosphorylation requirement has been shown with PKC- α and - β (Cazaubon et al., 1994; Orr and Newton, 1994). Potential phosphorylation sites are thought to be present in several members of PKC family, including ζ (Tsutakawa et al., 1995). In agreement with this, the clustering of α_2 and β_1 , the subunits of collagen receptor integrin $\alpha_2\beta_1$, which was reported to mediate collagen gel-induced

MMP-1 expression (Langholz et al., 1995), can induce p21ras activation (Kapron-Bras et al., 1993). Ras has been shown to be coprecipitated with PKC- ζ and to induce PKC- ζ activity (Diaz-Meco et al., 1994b). Indeed, our data showed that collagen gel did not alter the cellular level of PKC- ζ (Fig. 3). Thus, posttranslational modification is a possible mechanism by which PKC- ζ is regulated.

The downstream targets of PKC- ζ activity are yet to be discovered. One consequence of PKC- ζ activation by extracellular stimuli such as TNF- α is the induction of NF- κ B activity (Diaz-Meco et al., 1994a). Here we present evidence from gel mobility shift assays that collagen lattices induced NF- κ B DNA binding activity in dermal fibroblasts rapidly (≤ 30 min) and persistently (≥ 24 h) (Fig. 4 B). This is in concordance with the observation that cells stimulated with TNF- α and p21Ras demonstrate both increased PKC- ζ and NF- κ B activities (Diaz-Meco et al., 1994a; Muller et al., 1995). In fact, TNF- α was also shown to induce NF- κ B DNA binding rapidly and persistently (1/3–20 h) (Johnson et al., 1996; Roff et al., 1996), similar to collagen gel induction. One puzzling observation was that during a 24-h incubation period, PKC- ζ kinase activity peaked at 4 h, whereas NF- κ B DNA binding activity reached the maximum at 24 h with modest increase over the binding at 30 min (Fig. 4 C). While rapid increase of NF- κ B activity is predominantly caused by posttranslational modification (for review see Siebenlist et al., 1994), sustained nuclear NF- κ B activity over longer periods of stimulation may be caused by either sustained reduction of NF- κ B inhibitor B, as demonstrated in vascular endothelial cells (Johnson et al., 1996), or induced expression of NF- κ B subunits other than RelA (p65), as reported for HL60 cells (Hohmann et al., 1991). Both c-Rel and RelB can be transcriptionally regulated through the κ B element in their promoters (Hannink and Temin, 1990; Ryseck et al., 1992), which p65 lacks (Ueberla et al., 1993). Stimulation of Jurkat cells over several hours results in increasing amounts of c-Rel relative to p65 in the nucleus, possibly because of preferentially induced levels of c-Rel (Molitor et al., 1990; Doerre et al., 1993). In support of this, results from supershift assay showed very low levels of p65 in the κ B-binding complex (Fig. 4 B). We speculate that the rapid binding observed may be caused by phosphorylation of existing NF- κ B/Rel in cellular pool, whereas the persistent binding may result from the newly synthesized NF- κ B/Rel. Therefore, although PKC- ζ reached the maximum at 4 h, NF- κ B binding activity may not show identical kinetics and fold-increases because of regulatory transition from posttranscriptional modification of existing protein factors to synthesis and modification of new proteins. In agreement with this, we obtained evidence that collagen gel induction of α_2 and MMP-1 mRNA expression, a PKC- ζ -mediated event, requires both NF- κ B (unpublished data) and protein synthesis (Fig. 2 A).

It is of great interest that collagen gel and PDGF (Xu et al., 1996) both require PKC- ζ activity to induce integrin α_2 mRNA expression. How is the induction further enhanced when both signals are present (Fig. 1 C)? Although the study is at a stage too early to provide answers, there are several possibilities. First, PKC- ζ activity has been shown to be regulated by protein-protein interactions with apoptosis gene *par-4* product (Diaz-Meco et al., 1996b), λ -inter-

acting protein (LIP) (Diaz-Meco et al., 1996a), and Ras (Diaz-Meco et al., 1994b). Thus, collagen gel and PDGF might induce different protein factors that in turn activate PKC- ζ by protein-protein interaction. Copresence of both stimuli could synergistically induce the kinase activity by the interaction among multifactors. Second, there are multiple potential phosphorylation sites in PKC- ζ molecules (Tsutakawa et al., 1995). The fact that PKC- ζ is physically interacting with and activated by Ras (Diaz-Meco et al., 1994b) suggests that phosphorylation is an important means of regulating its activity. Thus, collagen gel and PDGF may activate PKC- ζ by inducing phosphorylation at different amino acid residue(s). A similar scheme was suggested in the synergistic activation of NF- κ B by calcium and Ras/Raf (for review see Baeuerle and Baltimore, 1996). Third, PKC- ζ can induce transactivating activities of different transcription factors such as NF- κ B and AP-1 (Bjorkoy et al., 1995). A differentially activated PKC- ζ could stimulate distinct downstream pathways. We have observed that collagen gel stimulated DNA binding activity of NF- κ B (Fig. 4 B) but not AP-1 (unpublished data). In contrast, PDGF induced DNA binding of substantial AP-1 but little NF- κ B (unpublished data). It is tempting to suggest that collagen gel/PKC- ζ and PDGF/PKC- ζ activate different transcription factors to induce integrin α_2 and MMP-1 mRNA expression. The synergistic action then would occur at the level of gene transcription. Investigations of these possibilities are ongoing in our laboratory.

It is well established that three-dimensional collagen matrix sends integrated physical and chemical signals to the interior of the cell. Nevertheless, detailed knowledge of collagen matrix signal transduction is very limited. Results reported here connect a signal transduction protein, PKC- ζ , to the upstream initiator, the collagen lattice, and to downstream outputs, α_2 and MMP-1 mRNA expression.

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