Type I Collagen Structure Regulates Cell Morphology and EGF Signaling in Primary Rat Hepatocytes Through cAMP-Dependent Protein Kinase A

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ABSTRACT

Adhesion to type 1 collagen elicits different responses dependent upon whether the collagen is in fibrillar (gel) or monomeric form (film). Hepatocytes adherent to collagen film spread and proliferate, while those adherent to collagen gel remain rounded and growth arrested. To explore the role of potential intracellular inhibitory signals responsible for collagen gel-mediated growth arrest, cAMP-dependent protein kinase A (PKA) was examined in hepatocytes adherent to collagen film or gel. PKA activity was higher in hepatocytes on collagen gel than on film during G1 of the hepatocyte cell cycle. Inhibition of PKA using H89 increased cell spreading on collagen gel in an EGF-dependent fashion, while activation of PKA using 8-Br-cAMP decreased cell spreading on collagen film. PKA inhibition also restored ERK activation, cyclin D1 expression and G1-S progression on collagen gel, but had no effect on cells adherent to collagen film. Analysis of EGF receptor phosphorylation revealed that adhesion to collagen gel alters tyrosine phosphorylation of the EGF receptor, leading to reduced phosphorylation of tyrosine residue 845, which was increased by inhibition of PKA. These results demonstrate that fibrillar type 1 collagen can actively disrupt cell cycle progression by inhibiting specific signals from the EGF receptor through a PKA-dependent pathway.
INTRODUCTION

Adhesion to the extracellular matrix (ECM) is a key regulator of cell proliferation, differentiation, and apoptosis. Transmembrane adhesion receptors such as integrins link the ECM to the cytoskeleton, allowing both the biochemical and mechanical properties of the ECM to influence signal transduction cascades that regulate cell cycle progression (Assoian and Zhu 1997; Aplin et al., 1999; Giancotti and Ruoslahti 1999; Schwartz and Assoian 2001). While integrin-mediated adhesion is generally required for growth factor induced cell cycle progression, adhesion to certain ECMs can inhibit cell cycle progression. Growth inhibition by these ECMs likely plays a major role in maintaining tissue architecture and homeostasis, yet little is known about the mechanisms by which this occurs.

Adhesion to type I collagen can affect cell morphology, differentiation, and cell cycle progression, depending upon its structure. When polymerized into fibrils, as it is mostly found in vivo, type I collagen inhibits growth of a number of cell types including smooth muscle cells (Koyama, et al., 1996), melanoma cells (Henriet, et al., 2000), glomerular epithelial cells (Schocklmann, et al. 2000) and hepatocytes (Hansen and Albrecht 1999; De Smet, et al., 2001), while adhesion to monomeric collagen stimulates cell cycle progression under similar culture conditions. Unlike loss of adhesion, however, which blocks cell cycle progression and also induces apoptosis, adhesion to collagen gels promotes survival and increased differentiated function (Ben-Ze’ev, et al., 1988; Gomez-Lechon, et al, 1998).

Potential mechanisms by which adhesion to fibrillar type 1 collagen inhibits cell cycle progression have been described. Decreased cyclin D1 expression (Hansen and Albrecht 1999; Schocklmann, et al., 2000), and/or increased expression of cell cycle inhibitor proteins such as p21 or p27KIP (Koyama, et al, 1996; Henriet, et al., 2000) have been demonstrated in various cell types in response to adhesion to fibrillar collagen. The mechanisms by which adhesion to fibrillar collagen modulates the signaling pathways that determine the expression levels of cell cycle regulatory proteins, however, are not well defined.
In primary rat hepatocyte cultures, cells adherent to polymerized collagen gels maintain a rounded morphology, express lower levels of cyclin D1, and do not enter S-phase despite the presence of mitogenic quantities of EGF (Hansen and Albrecht, 1999). More recent results suggest that inhibition of EGF signaling by adhesion to collagen gel occurs in part by inhibition of ERK activation, which is required for cell cycle progression on monomeric collagen (Fassett, et al., 2003). One signaling molecule that is both regulated by adhesion and is inhibitory of EGF signaling is cAMP-dependent protein kinase (PKA). PKA can limit the activity of raf (Cook and McCormick 1993; Wu, et al., 1993), PAK (Howe and Juliano 2000), and the EGF receptor (Barbier, et al., 1999), all of which are important to mitogenic signaling. Furthermore, agents that increase cAMP levels or activate PKA in hepatocytes also decrease cell cycle progression (Gjertsen, et al., 1995; Mellgren, et al., 1997). Activation of PKA can also decrease cell spreading and disrupt the actin cytoskeleton (Lamb, et al., 1988; Clegg, et al., 1989; Amieux, et al., 2002). Importantly, PKA is activated in fibroblasts upon detachment from the ECM, and inhibition of PKA restores growth factor activation of ERK in suspended cells (Howe and Juliano 2000).

In this report, the potential role of PKA in growth arrest of hepatocytes by adhesion to polymerized type 1 collagen gel was investigated. It was demonstrated that PKA activity is differentially regulated by adhesion to polymerized or monomeric collagen, and that PKA activity plays an important role in cell shape, cyclin D1 expression, and cell cycle progression in response to EGF. The growth inhibitory effect of PKA activation by polymerized collagen appears to occur in part at the level of EGF receptor phosphorylation, suggesting a novel mechanism in which adhesion to different forms of ECM may control cell growth and morphology by activating a PKA-dependent pathway that inhibits specific EGF-dependent cell cycle events.
MATERIALS AND METHODS

Reagents: U0126 was purchased from Promega (Madison, WI). H89, myristoylated PKI, and 8-bromo cAMP were purchased from Calbiochem (La Jolla, CA). Antibodies used for Westerns include anti-rat cyclin E (cat# sc-481, Santa Cruz Biotechnology; Santa Cruz, CA) anti- phospho-tyrosine (cat #sc7020, Santa Cruz), anti-active-ERK, anti-ERK, anti-phosphotyr-845 EGF receptor, and anti-phosphotyr-1068 EGF receptor (cat#s9102, 9101,2231, 2234, respectively, Cell Signaling; Beverly, MA), anti-cyclin D1 (cat#06-137, Upstate Biotechnology; Lake Placid, NY), anti-EGF receptor (cat#06-847, Upstate Biotechnology), and anti-G3PDH (cat# 8245 100, Abcam Inc., Cambridge, UK).

Hepatocyte Culture. Primary rat hepatocytes were obtained by collagenase perfusion of adult Lewis rat liver (Seglen 1976), followed by purification through a Percoll gradient (Sigma Chemical Company, St. Louis, MO). Only cells from harvests yielding > 90% viability were used. Hepatocytes were plated at sub-confluent density (10-12,000 cells/ cm²) in serum-free William's medium E (Gibco, Grand Island, NY) with the following additives as previously described (Hansen and Albrecht 1999): epidermal growth factor (EGF) (10 ng/ml, Collaborative Research, Bedford, MA), insulin (20 mU/ml, Sigma), dexamethasone (5 nM, Sigma), sodium pyruvate (1 mM, Gibco), ascorbic acid (50 µg/ml, Gibco), and penicillin/streptomycin (100 U/ml, Irvine Scientific, Santa Ana, CA). Cultures were re-fed daily. Cultures were always performed in the presence of growth factors unless otherwise stated. “Time 0” samples represent freshly isolated cells in suspension at 4°C with minimal medium (no growth factors) prior to plating.

Collagen Substrate Preparation: Type I collagen ("Vitrogen 100", Collagen Biomaterials, Palo Alto, CA) was coated onto non-adhesive Petri dishes to produce either a monomeric coat of collagen referred to as collagen film, or a polymerized form referred to as collagen gel. Collagen film was prepared by coating dishes with collagen diluted in a basic, carbonate buffer (pH 9.4) as previously described (Hansen and Albrecht, 1999). To produce a collagen gel substrate, 50 µl NaOH was added to each ml of 5x Williams Media E. 1 part 5x Williams Media E (+NaOH),
were mixed with 4 parts Vitrogen 100 (final concentration ~2.4 mg/ml), added to a Petri dish at approximately 1 ml/10cm dish and incubated at 37°C for one hour. After coating, all plates were washed twice in PBS and incubated at least 20 minutes in 1% bovine serum albumin (BSA) in Williams E medium to block any non-coated sites. Hepatocyte attachment is equivalent on both substrates. Collagen gel overlays were accomplished by mixing 5 parts complete Williams E and 1 part Vitrogen 100 (final collagen concentration about 0.5 mg/ml). Media is removed from cells and replaced with overlay. Fresh media is added on top of the overlay after polymerization.

DNA Synthesis: DNA synthesis is measured by adding $[^3]H$-thymidine at 1.2 µCi/well (specific activity = 50Ci/mmol, ICN Biomedicals, Costa Mesa, CA) to 96-well plate cultures at the indicated hours after plating. $[^3]H$-thymidine incorporation into newly synthesized DNA was determined by harvesting cell lysates onto filter paper using a cell harvester (Brandel, Gaithersburg, MD) and quantitation by scintillation counting.

Protein isolation, immunoprecipitation, and western blot: Cultured hepatocytes were removed from the plates by washing once with ice cold PBS, then scraping in RIPA buffer (50mM Tris-HCl pH 7.4, 1% NP40, 150mM NaCl, 1mM EDTA, 1mM PMSF, 1ug/ml aprotinin, leupeptin, and pepstatin, 1mM sodium vanadate, 1mM NaF). Immunoprecipitation was carried out as previously described (Albrecht and Hansen, 1999). Western blot analysis was performed using approximately 30 µg of cellular protein per lane as previously described (Albrecht and Hansen 1999).

PKA assay: Assay for cAMP-dependent protein kinase A was performed as previously described (Day, et al., 1989). Briefly, cells were lysed, sonicated, centrifuged, and total protein determined. Equal protein was added to reaction mixture along with substrate (Kemptide, a synthetic peptide containing the consensus PKA phosphorylation sequence), and 5 µCi $^{32}$P ATP (3000Ci/mmol, Amersham). After 30°C incubation for 10 minutes, 25µl of reaction mixture was spotted onto Whatman P81 paper, washed extensively in 75 mM phosphoric acid, dried, and quantitated by scintillation. The PKA assay was also performed in the presence of PKI, a
peptide inhibitor of PKA. These values were similar to background counts obtained in the absence of Kemptide substrate, indicating the specificity of the assay (data not shown).

**Cell Size Measurements:** Hepatocytes were cultured for the indicated period of time, fixed with 4% paraformaldehyde/PBS pH 7.4, washed 3 times in PBS, then stained for 5 minutes with Coomassie blue/methanol solution. Cells were then washed five times in PBS and photographed measured using computerized image analysis with Adobe Photoshop 6.0. At least 50 randomly selected cells were analyzed and averaged for each condition.

**Scanning Electron Microscopy (SEM):** Hepatocytes were cultured on thermanox plastic cover slips (Nunc; Rochester, NY) that were coated with collagen film or collagen gel as described above. At the time of analysis, coverslips were washed 3 times in Williams E, fixed in 1% glutaraldehyde for 2 hours at 4°C, washed 3 times for 5 minutes with 0.1M Na cacodylate, treated with osmium tetroxide for 1 hour at room temperature, washed 3 times with diH2O, incubated in 50% ethanol for 10 minutes, washed in 70% ethanol for 10 minutes, and then stored until use. SEM was performed using a Hitachi S-4700 FEG scanning electron microscope (Hitachi; Pleasanton, Ca).
RESULTS

Interaction with polymerized collagen increases PKA activity.

Previously it was demonstrated that EGF-dependent activation of the p42/44 ERK pathway and G1-S progression is disrupted in hepatocytes adherent to polymerized (fibrillar) type I collagen gel, but not on monomeric type I collagen film (Fassett, et al., 2003). To determine if polymerized collagen gel had a dominant inhibitory effect on cell cycle progression, or if growth arrest was simply due to lack of sufficient positive ECM-generated signals, experiments were designed in which hepatocytes were first cultured on collagen film, which stimulates cell cycle progression, followed 12 hours later with an overlay of type I collagen gel. This “collagen sandwich” configuration has been described in the literature (Dunn, et al., 1989) and promotes a highly differentiated phenotype. Following addition of the gel overlay, the hepatocytes stopped spreading, but still remained more spread than the hepatocytes on collagen gel alone (Fig. 1A). Using computerized image analysis, the average spread area of overlaid cells was determined to be 41% (± 6%) the area of film, while gel was 31% (± 3%) the size of cells on film. DNA synthesis assay performed 54-74 hours after initial plating (42-62 hours after collagen gel overlay) demonstrated a significant inhibition of DNA synthesis in the presence of the gel overlay compared to cells plated on film over the same time period without overlay (Fig. 1B, p<0.01). These data indicate that collagen gel actively inhibits cell cycle progression even in cells already progressing through G1 phase, suggesting that fibrillar collagen interaction with hepatocytes induces a specific cell cycle inhibitor.

One signaling molecule that blocks mitogenic signaling upon activation in some cell types is the cAMP-dependent protein kinase (PKA). To investigate the possibility that PKA may play a role in different EGF signaling on distinct forms of collagen, the activity of PKA was measured at different times in hepatocytes adherent to monomeric collagen film, or polymerized collagen gel. As shown in Fig. 2, hepatocytes adherent to polymerized collagen have higher levels of PKA activity than those adherent to monomeric collagen and this higher activity lasts
throughout the first 48 hours of culture (i.e., throughout G1 phase of the hepatocyte cell cycle; p<0.05 for each timepoint tested). In addition, collagen overlay, when applied to hepatocytes on film 12 hours after plating, led to a significant increase in PKA activity at 24 hours compared to cells cultured for the same time period on film alone (p<0.05). The level of PKA activity induced by collagen gel overlay was similar to that measured in cells on collagen gel for the entire 24 hour culture.

**PKA regulates morphological response to EGF**

Because PKA levels appeared higher in the rounded cells on polymerized collagen than in spread cells on monomeric collagen, the effects of PKA activity on hepatocyte morphology were investigated by blocking PKA activity using the PKA specific inhibitor H89. Using an antibody specific for phosphorylated PKA substrates, a substantial reduction in phosphorylation in many cellular proteins is observed in the presence of H89, indicating its function in reducing PKA activity (data not shown). Surprisingly, treatment of hepatocytes with H89 at the time of plating dramatically increased cell spreading (Fig. 3A-3C). Conversely, treatment of cells adherent to collagen film with PKA agonist 8Br-cAMP had a similar effect as adhesion to collagen gel, causing cells to remain rounded and less spread (Fig. 3A, 3C). At 48 hours the difference between collagen film and gel was still evident. If only solitary cells were measured, the difference in size was even more pronounced (Fig. 3C inset at 48 hr), suggesting cell / cell adhesion can increase spreading across a basal layer of collagen gel. The ability to spread upon collagen gel under these conditions in response to PKA inhibition was dependent upon EGF, as H89 treated cells stimulated with EGF spread substantially by 48 hours, while H89 treated cells in the absence of EGF did not spread (Fig. 3B, 3C). It is also important to note that while H89-induced spreading on collagen gel was decreased by ~70% in the absence of EGF, spreading on collagen film was only diminished by ~30% in the absence of EGF (data not shown), suggesting that while EGF is important for spreading on collagen gel, adhesion to
collagen film can induce substantial spreading that is independent of EGF. Thus, while malleable gels such as collagen gels were traditionally thought to inhibit spreading due to the inability to resist cytoskeletal tension at adhesion sites, these results suggest that inhibition of cell spreading on type I polymerized collagen is due in part to induction of elevated PKA activity that inhibits EGF-induced spreading.

**Inhibition of PKA on collagen gel restores DNA synthesis**

It has long been recognized that the ability of the ECM to support cell spreading correlates with its ability to promote cell cycle progression (Folkman and Moscona, 1978; Ingber, et al., 1987; Mooney, et al., 1992; Hansen, et al., 1994). To determine the effects of PKA inhibition on cell cycle progression, DNA synthesis was measured in hepatocytes treated with varying concentrations of H89. Inhibition of PKA activity on collagen gel with moderate doses of H89 (1.5 - 6 μM) significantly increased DNA synthesis on collagen gel compared to DMSO controls on gel (p<0.005), and DNA synthesis levels were similar to that seen on film DMSO controls. However, inhibition of PKA on collagen film had no significant effect on G1-S progression until higher doses (6-12 μM) were used, and DNA synthesis actually decreased (Fig. 4A). The increase in DNA synthesis was not likely due to H89’s “non-specific” inhibition of other molecules, such as rho kinase (Leemhuis, et al., 2002), because treatment of cells on gel with rho kinase inhibitor Y2697 was unable to induce DNA synthesis of hepatocytes on collagen gel (not shown). To further test the specificity of PKA inhibition, a different inhibitor of PKA activity was utilized. A myristoylated form of PKI, a PKA-inhibitory peptide that blocks the substrate-binding site of PKA, was added to hepatocyte cultures on collagen gel for the first 24 hours of culture. This treatment also significantly increased hepatocyte DNA synthesis on collagen gel compared to controls (p<0.01 at 4 μg/ml; Fig. 4B), again to a level similar to that on film control (p>0.05). Because inhibition of PKA is able to restore EGF-dependent G1-S progression on collagen gel, but has little effect on collagen film, this is further evidence that
PKA-mediated inhibition of hepatocyte cell cycle progression is a response specific for polymerized and not monomeric collagen.

Hepatocytes in this system typically reach the growth factor restriction point around 44-48 hours on collagen film (Albrecht and Hansen, 1999). Removal of H89 at 24 hours after plating only slightly diminished DNA synthesis compared to H89 removal at 48 hrs, which corresponds to entry into S phase (Fig. 4C; not significant for film, p=0.047 for gel comparing 0-48hr to 0-24 hr), suggesting the inhibitory effects of PKA activity on cell cycle progression takes place during early and mid G1 of the cell cycle (0-24 hr). H89 also was not able to induce DNA synthesis in the absence of EGF, suggesting that H89 was restoring EGF signaling rather than an EGF-independent pathway to the cell cycle (Fig. 4C; p<0.01 comparing +EGF to −EGF for each condition).

Inhibition of PKA reduces collagen gel-mediated inhibition of p44/42 ERK and cyclin D1 expression

PKA is known to antagonize EGF signaling pathways and mitogenesis (Cook and McCormick 1993; Wu, et al., 1993). Previously we have shown that adhesion to polymerized collagen inhibited EGF-dependent G1-S progression and this inhibition was associated with lower levels of cyclin D1 expression (Hansen and Albrecht, 1999), due in part to decreased ERK activity in late G1 (around 36-48 hours after plating) (Fassett, et al., 2003). Over-expression of cyclin D1 (Hansen and Albrecht, 1999), or expression of activated mutants of the Ras signaling pathway were able to restore DNA synthesis on collagen gel (Fassett, et al., 2003). In smooth muscle cells, adhesion to fibrillar collagen gel does not effect cyclin D1 levels, but there is an increase in the levels of a cell cycle inhibitor protein p27KIP (Koyama, et al., 1996). To determine if PKA plays a role in collagen gel mediated inhibition of p42/44 ERK activity or regulates cyclin D1 or p27KIP expression, PKA was inhibited and these cell cycle regulators were analyzed by western blot. Both p42/44 ERK activation and cyclin D1 expression were increased on collagen
gel after inhibition of PKA (Fig. 5A). Blocking PKA for only the first 24 hours was sufficient for inducing an increase in ERK activity during late G1 and S. This level of ERK activation or cyclin D1 expression did not equal that found on collagen film however, suggesting that other signaling pathways to cyclin D1 may be active on collagen film that are not restored on collagen gel by PKA inhibition alone. p27Kip1 levels, lower on collagen gel than on collagen film at these timepoints, were increased by inhibition of PKA. Cyclin E levels or glyceraldehyde 3-phosphate dehydrogenase (G3PDH) levels, which are not significantly affected by adhesion to gel, also did not appear to be greatly effected by PKA inhibition. Treatment of hepatocytes with myristoylated PKI also was able to increase cyclin D1 expression on collagen gel, and had little effect on cyclin E (Fig. 5B). These results suggest that adhesion to collagen gel specifically blocks cyclin D1 through a PKA-dependent mechanism. Further analysis of the interaction between the upstream EGF signaling, collagen structure and PKA on cell cycle progression demonstrated that not all of the EGF dependent signaling pathways are disrupted by adhesion to collagen gel. For instance, EGF stimulation increases the levels of cyclin E and cdc2 expression above levels found in untreated cells (Fig. 5C). Both of these EGF-dependent events appeared unhindered by adhesion to polymerized collagen. However, EGF-dependent induction of cyclin A, another late G1/S phase cell cycle promoting gene, was blocked by adhesion to collagen gel, but was partially restored by H89 treatment, as was cyclin D1. Cdc2 tyrosine-15 phosphorylation and dephosphorylation, which are involved in the timing of mitosis (Krek and Nigg, 1991; Borgne and Meijer, 1996) also appeared to be inhibited by adhesion to collagen gel and were restored by H89 treatment (Fig. 5C). Thus, while not all EGF-dependent events are blocked by adhesion to polymerized collagen, a number of events critical to G1-S progression as well as progression out of S phase appear to be blocked by adhesion to collagen gel in a PKA-dependent fashion. This suggests that EGF is able to bind and activate the receptor to some degree, but some aspect of EGF signaling at the level of activation or downstream is disrupted.
Cyclin D1 may be induced by other signaling molecules, such as the small GTPase rac, independent of ERK (Welsh, et al., 2001). To determine if the increase in cyclin D1 in response to PKA inhibition was a result of restoring EGF signaling to ERK, cells were treated with H89 on collagen film or collagen gel for 48 hours, then treated with U0126, an inhibitor of MEK, the upstream activator of ERK, for the last 24 hours of culture (48-72 hrs). Protein lysates obtained at 72 hr and DNA synthesis between 48-72 hours was then measured. Results demonstrate that inhibition of MEK blocks cyclin D1 expression on collagen film as well as H89-mediated induction of cyclin D1 on collagen gel (Fig. 6A). Inhibition of MEK also reduced the DNA synthesis induced by PKA inhibition on collagen gel (Fig. 6B; p<0.01) as well as that normally seen on collagen film as previously described (Fig. 6B; p<0.01; Fassett, et al. 2003). These data demonstrate that PKA blocks EGF signaling to ERK and subsequent cyclin D1 expression and G1-S progression on collagen gel. Removal of EGF at 48 hr has no effect on DNA synthesis in the presence of H89 (data not shown), suggesting that progression into and possibly through S phase is dependent upon MEK during this 48-72 hr period, but no longer dependent upon EGF. EGF was required to drive the cell cycle to this point, and adhesion to collagen gel specifically blocks this progression through activation of PKA.

Tyrosine phosphorylation of EGF receptor is affected by adhesion to collagen film or gel.

There is evidence that integrin-mediated adhesion can activate the EGF receptor (EGFr) even in the absence of EGF (Moro, et al., 1998; Moro, et al., 2002). It has also been demonstrated that PKA can directly transmodulate the EGFr (Barbier, et al., 1999). Previous results from this lab suggest that inhibition of EGF signaling to ERK on polymerized collagen gel occurs upstream or at the level of ras activation (Fassett, et al., 2003). Thus it was of interest to determine if adhesion to polymerized collagen was affecting EGF signaling at the level of EGFr expression or activation. The results in Fig. 5 suggested some level of EGFr activity was persisting on collagen gel based on cyclin E expression, yet its activity required for cyclin D1
expression and activation of ERK appeared disrupted. To examine the ability of EGF to activate the receptor, immunoprecipitation (IP) followed by western blot analysis was performed. These results demonstrated no detectable difference in the amount of total phosphotyrosine-containing EGFr on gel or film over the first four hours in culture (Fig. 7A), suggesting that the EGFr signaling pathway was not compromised upon initial plating onto collagen gel. However, examination of a more extensive time course revealed that while overall phosphorylation of the EGFr is decreased on both film and gel at later times, a slightly higher level of EGFr phosphorylation is maintained for at least 24 hours on film (Fig. 7B, 7C first panel; p<.05 at 6-9 hours and 24 hours comparing gel to film).

Because the EGFr contains multiple tyrosine residues with different signaling functions, further analysis of specific tyrosine residues of the EGFr was also undertaken. Tyrosine 845 phosphorylation of the EGFr is mediated by Src in response to adhesion (Biscardi, et al., 1999; Moro, et al., 2002), and is also important for EGF-mediated DNA synthesis as well as DNA synthesis induced by trans-activating receptors that signal through the EGFr (Biscardi, et al., 1999; Tice, et al., 1999). Tyrosine 1068 is also phosphorylated in response to adhesion via integrins (Moro, et al., 2002), and is an autophosphorylation site for the EGFr (Downward, et al., 1984; Downward, et al., 1985). When cells were plated on collagen gel, phosphorylation of Tyr-845 never attained the same level of phosphorylation, and decreased more rapidly in comparison to cells plated on film, (p<.05 at all time points when comparing gel relative to film at same time point) whereas tyrosine 1068 phosphorylation appeared similar on both substrates (Fig. 8A,C). It is also evident that by 24 hours of culture, the overall levels of the EGFr are much reduced, as previously demonstrated in response to continuous stimulation (Gross, et al., 1983; Helin and Beguinot. 1991). Treatment of hepatocytes with H89 was able to increase both total EGFr phosphorylation (p<0.05 at 0.5 and 2.5 hr) and Tyr-845 phosphorylation on collagen gel (p<0.05 for 2.5 hr, not significant at 0.5 hr), but had little effect on Tyr-1068 (p>0.05), suggesting that PKA plays a specific role in the diminished phosphorylation of Tyr-845 in hepatocytes.
adherent to collagen gels (Fig. 8C; quantitation not shown). As this site has been demonstrated to be regulated by Src through an integrin-dependent mechanism (Moro, et al., 2002), it suggests that PKA may play a role in uncoupling the cooperative relationship between the EGFr and integrin signaling complexes in signaling through G1 towards cell cycle progression.

The differences in overall tyrosine phosphorylation of the EGFr as well as tyr 845 between film and gel appear to be greatest later in the culture period (between 9-24 hours). However, at the same time (~24 hours), overall phosphorylation as well as expression of the EGFr is down-regulated (Fig. 7B), and thus the role for EGFr phosphorylation in the late G1 ERK activity and cyclin D1 expression typically seen around 48 hours is not clear. To determine if the EGFr plays a role in late signaling to ERK and cyclinD1, EGF was removed after 12 hours of culture and the cells were incubated for an additional 36 hours in the presence or absence of H89 without EGF. H89 treatment from 0-48 hours of hepatocytes on collagen film did not alter ERK phosphorylation or cyclin D1 expression (data not shown). Figure 9A demonstrates that removal of EGF by 12 hours reduces the late G1 activation of ERK and cyclin D1 expression on collagen film (compare to control exposed to continuous EGF), suggesting a requirement for EGF beyond 12 hours of culture in order to obtain high levels of cyclin D1 expression. Re-introducing EGF at 48 hours on film strongly induces ERK phosphorylation, which is followed by increased cyclin D1 expression 12 hours later. On collagen gel, EGF re-introduced at this later stage did not induce strong ERK phosphorylation or cyclin D1 expression, but ERK phosphorylation and cyclin D1 expression was restored in cells on gel treated with H89 from 0-48 hours (Fig. 9A). This suggests that EGF signaling during these later times on film and gel are important for driving ERK activation and cyclin D1 expression, and elevated PKA activity reduces this late G1 EGFr signaling on collagen gel. Addition of EGF at 48 hours in the absence of prior EGF stimulation did not result in cyclin D1 expression 12 hours later (data not shown), suggesting that both early and late phases of EGF stimulation are important for inducing cyclin D1 expression.
Examination of EGFr phosphorylation during this late phase of signaling (Fig. 9B) demonstrates that following EGF re-addition, Tyr-845 and Tyr-1068 phosphorylation of the EGFr is inhibited on collagen gel relative to film (p<0.05 for Tyr-845, p<0.01 for Tyr-1068, quantitation not shown), but partially restored by inhibition of PKA (p<0.05 for Tyr-1068, p<0.001 for Tyr-845, gel vs. gel + H-89). It is also evident that re-expression of the EGFr after EGF re-introduction is stronger on collagen film than collagen gel, and this re-expression is slightly increased on gel in response to H89. Similar to the initial response to EGF, however, Tyr-1068 did not appear to be as strongly affected by H89 treatment, suggesting that inhibition of Tyr-845 phosphorylation is a specific target of adhesion signaling by collagen gel in a PKA-dependent manner. Because the phosphorylation of Tyr-845 is Src-dependent, its phosphorylation on gel +/- H89 was examined in the presence or absence of the Src inhibitor, PP2. The restoration of Tyr-845 phosphorylation, as well as ERK induction, by EGF re-addition is inhibited by PP2 (Fig. 9C), confirming the involvement of Src in Tyr-845 phosphorylation and downstream ERK activation.
DISCUSSION

Studies presented here reveal a novel ECM regulatory mechanism in which adhesion to fibrillar, but not monomeric, type I collagen actively blocks EGF signaling to ERK, cyclin D1 and DNA synthesis through a PKA-dependent mechanism. This mechanism helps to explain the consistent observation that, while adhesion to ECM is generally required for non-transformed cells to progress through the cell cycle in response to growth factors, not all ECMs support proliferation of all cell types. The differences in the abilities of certain ECMs to regulate proliferation or differentiation within the context of an entire organ or organism are likely to play an important role in maintaining tissue homeostasis in vivo. Defining specific molecular mechanism(s) of ECM-dependent regulation of growth and differentiation thus provide valuable insight into these processes.

Previous studies from this laboratory demonstrated that initial ERK activation following cell plating is similar on collagen film and gel, but a second sustained peak in ERK activity that occurs in late G1 phase on collagen film is absent on collagen gel (Fassett, et al., 2003). This second sustained activation of ERK is required for progression into S phase, whereas the initial peak of ERK activity is not required for cell cycle progression on a permissive matrix. Other studies demonstrate that sustained ERK activity is required for cyclin D1 expression and G1-S transition (Weber, et al., 1997; Roovers, et al., 1999; Bottazzi, et al., 2001) and is dependent upon adhesion and an intact cytoskeleton (Roovers, et al., 1999; Bottazzi, et al., 2001). Results presented here suggest that inhibition of PKA during early and mid-G1 was able to restore cell morphology and perhaps a cytoskeletal architecture that more efficiently transduced the late G1 MEK-dependent signals. Furthermore, the removal of EGF at 12 hours and subsequent loss of ERK activation and cyclin D1 expression suggests that at least two phases of EGF stimulation in early and late G1 are required to drive cyclin D1 expression. PKA inhibition by H89 during early and mid-G1, corresponding to the time during which PKA activity is higher on collagen gel, appears to prevent collagen gel-dependent inhibition of late ERK activation. The increased
activation of ERK and cyclin D1 expression around 48 hours in cells with H89 for either 24 or 48 hours suggests that fibrillar collagen inhibits cell cycle progression in hepatocytes by interfering with cell cycle competence, as defined in the competence/progression model of cell cycle control (Stiles, et al., 1979).

PKA has previously been shown to inhibit EGF signaling at a number of points by down-regulating the activity of the EGF receptor (Barbier, et al., 1999), inhibiting the activation of raf (Cook and McCormick, 1993; Wu, et al., 1993; Hu, et al., 1999), or inhibiting p21 activated kinase (PAK) (Howe and Juliano, 2000). Loss of adhesion activates PKA, which blocks growth factor signaling to ERK by inhibiting PAK (Howe and Juliano, 2000). PKA has also been shown to disrupt the actin cytoskeleton and decrease cell spreading (Lamb, et al., 1988; Clegg, et al., 1989; Amieux, et al., 2002). An intact cytoskeleton is a critical component of growth factor and adhesion dependent signaling to the G1-S transition (Assoian and Zhu, 1997; Bottazzi, et al., 2001; Schwartz and Assoian, 2001). When hepatocytes on collagen gel were treated with H89 to block PKA activity, EGF signaling to ERK and cyclin D1 was restored. Cell spreading was also associated with the increase in ERK activation and cyclin D1 expression in response to PKA inhibition, suggesting that PKA activity induced by adhesion to collagen gel may be involved in inhibiting morphological or cytoskeletal structures that promote ERK activation and cyclin D1 expression. H89 had no effect on cells adherent to film, demonstrating that PKA inhibitory activity is specific to collagen gel.

Adhesion even in the absence of EGF can signal through the EGF receptor (Moro, et al., 1998; Moro, et al., 2002). PKA has been shown to directly phosphorylate and inactivate the EGF receptor (Barbier, et al., 1999). The finding that adhesion to collagen gel increases PKA activity and decreases EGF receptor phosphorylation suggests that adhesion to fibrillar collagen acts in the opposite manner of growth-permissive adhesion, that is to directly suppress adhesion-dependent EGF signaling. Thus one difference between a growth supportive ECM
and one which inhibits growth may be its effect on PKA activity and subsequent effects on growth factor signaling and cell shape.

The increase in cell spreading, ERK activation, cyclins D1 and A expression, and DNA synthesis by PKA inhibition requires EGF, suggesting that the EGF-dependent signaling required for cell spreading and G1 progression is being blocked by PKA. However, not all EGF-dependent signaling was blocked by adhesion to collagen gel. Cyclin E and cdc2 expression were both increased similarly on collagen gel and film. Consistent with the findings that only some EGF-dependent signals were disrupted by adhesion to gel is the different pattern of phosphorylation of particular EGF receptor tyrosine residues in cells adherent to collagen film and gel. Tyr 1068 is an autophosphorylation site on the EGF receptor that is activated by integrin adhesion as well as EGF stimulation (Moro, et al., 2002). This site was activated by EGF similarly on collagen film or collagen gel. In contrast, Tyr 845, which is also activated by adhesion and EGF stimulation (Moro, et al., 2002), showed reduced phosphorylation in cells attached to collagen gel. Tyr 845 phosphorylation of the EGF receptor is carried out by Src (Sato, et al., 1995), which associates with the EGF receptor in response to adhesion (Moro, et al., 2002). It has also been demonstrated that Tyr 845 is important for EGF-induced DNA synthesis (Biscardi, et al., 1999) as well as zinc-induced Ras activation (Wu, et al., 2002), and that Src and the EGF receptor cooperate to induce cellular transformation (Tice, et al., 1999). An increase in Tyr 845 phosphorylation in response to H89 suggests that PKA activity on collagen gel may disrupt interaction between the EGF receptor and Src, but have less effect on EGF-induced autophosphorylation. It is also possible that the increased ability of cells to spread upon collagen gel in response to H89 may relate to increased integrin activation and subsequent integrin /EGF receptor interactions, suggesting an indirect effect of PKA upon the EGF receptor.

The mechanism by which either adhesion to type I collagen fibrils or loss of adhesion activates PKA is not known, nor is it clear if PKA activation occurs through a similar mechanism
in each situation. In general, ECM-mediated cell cycle inhibition can result from biochemical and/or physical properties of the ECM/cell contact. For example, adhesion to laminin through $\alpha_2\beta_1$ integrins fails to seed the formation of cytosolic signaling complexes and cytoskeletal organization important for growth factor signaling in endothelial cells, while $\alpha_5\beta_1$-mediated adhesion to fibronectin does promote proliferative signaling in the same cells (Mettouchi, et al., 2001). Different integrins, as well as non-integrin receptors such as DDRs (Vogel, et al., 1997; Shrivastava, et al., 1997), bind to distinct regions within type I collagen, depending on whether the collagen is in native or denatured conformation (Gullberg, et al., 1990; Gullberg, et al., 1992). Thus these or other specific receptors may play a role in differential signaling by collagen conformation.

Mechanical or spatial properties of the matrix can also affect cell cycle progression and differentiation, at least in part by its ability to support cell spreading and a growth permissive cytoskeletal organization. Adhesion to islands of ECM in which the spreading area is limited (Huang, et al., 1998), or adhesion to malleable ECMs that do not resist cytoskeletal tension (Mochitate, et al., 1991; Rosenfeldt and Grinnell, 2000; Wang, et al., 2000) will also inhibit progression through the G1 phase of the cell cycle, similar to loss of adhesion. Activation of PKA could result from a lack of resistance to cytoskeletal tension on collagen gel. Loss of tension in fibroblasts embedded within a mechanically loaded collagen matrices results in increased cAMP levels, loss of cytoskeletal organization, and disrupted growth factor signaling (Mochitate, et al., 1991; Rosenfeldt and Grinnell, 2000). A key similarity between these conditions and loss of adhesion is the disruption of cytoskeletal organization permissive for growth factor signaling. It is possible that a particular cytoskeletal organization, mediated by adhesion, plays a role in preventing inhibition of growth factor signaling by PKA, since PKA and its subunits associate with a number of proteins that anchor it to the actin cytoskeleton (Diviani and Scott, 2001). Rigid substrates that promote cell spreading and focal adhesion formation will typically support growth factor signaling. This is dependent upon cytoskeletal integrity, as
chemical disruption of the cytoskeleton on otherwise permissive ECM substrates will block cell cycle progression in G1 (Iwig, et al., 1995; Zhu and Assoian, 1995; Bohmer, et al., 1996; Bhadriraju and Hansen, 2004). Actin disruption by cytochalasin D treatment of cells blocks FAK activation in response to adhesion, but not integrin-dependent EGF receptor phosphorylation (Moro, et al., 2002). Furthermore, myosin-dependent contractility is required for G1 events such as ERK activation and cyclin D1 expression even in non-contractile cells such as hepatocytes (Bhadriraju and Hansen, 2004). Thus, cytoskeletal integrity, including acto-myosin contractility, which are responsive to the mechanical nature of the ECM, play a key role in growth-permissive intracellular signaling, and may be involved in the differential activation of PKA.

While these results demonstrate that addition of collagen gel overlay can increase PKA activity and decrease DNA synthesis, it is not clear whether this is a result of switching from rigid collagen film adhesions to new adhesions formed between previously unoccupied integrins with compliant collagen adhesions, or from activation of a collagen fibril-activated growth inhibitory receptor. In either case, adhesion to polymerized collagen can induce a dominant inhibitory signal that disrupts growth factor signaling towards G1-S progression. It has also been demonstrated in vivo that over-expression of type I collagenase in rat livers leads to cell proliferation (Nishio, et al., 2003), suggesting collagen may play a role in maintaining stasis under otherwise normal conditions. Whether PKA plays a role in this mechanism in vivo is not known. As type I collagen is the most abundant protein in the human body, it will be important to determine the mechanism by which PKA is activated by adhesion to collagen fibrils, and also determine if PKA is activated in other cell types by type I collagen fibrils. As PKA can promote cell proliferation in some cell types, while inhibiting the growth of others, the role of PKA in adhesion to type I collagen could be important for understanding fibrotic diseases, where type I collagen production increases and alters the architecture and cell population of the fibrotic tissue (Seyer, et al., 1977; Murata, et al., 1984). Because activation of PKA could be a result of biochemical or mechanical signaling from fibrillar collagen, understanding the mechanism by
which PKA is activated will require separating the potential effects of fibril-specific ligands from the role of substrate malleability on PKA activity.

In conclusion, these results demonstrate that type I collagen structure plays a strong role in EGF signaling in hepatocytes. Data presented here demonstrate that this differential regulation of cell cycle progression is due at least in part to a novel ECM structure-dependent up-regulation of PKA activity and subsequent PKA-dependent alteration of EGF receptor phosphorylation and resulting inhibition of key G1 phase cell cycle events. These results could have important implications in understanding the role of adhesion to type I collagen fibrils in fibrotic diseases as well as in maintenance of homeostasis by type I collagen.
REFERENCES


Gullberg, D., Gehlsen, K. R., Turner, D. C., Ahlen, K., Zijenah, L. S., Barnes, M. J., and K. (1992) Analysis of \( \alpha_1 \beta_1, \alpha_2 \beta_1, \) and \( \alpha_3 \beta_1 \) integrins in cell-collagen interactions: identification of conformation dependent \( \alpha_1 \beta_1 \) binding sites in collagen type I. EMBO J. 11: 3865-73.


FIGURE LEGENDS

Figure 1. Collagen gel actively inhibits DNA synthesis.

(A) Hepatocytes were plated on collagen film or gel in the presence of growth factors. 12 hours later, a solution of type I collagen in William’s E medium was overlaid onto cells in a subset of the film plates, which formed a layer of polymerized collagen on top of the cells. Photos were taken of cultures at 48 hours. (B) [³H]Thymidine incorporation was assessed as a measurement of DNA synthesis 54-74 hours after initial plating. Data presented is mean +/- S.D. of quadruplicate samples from one representative experiment. (** = p<0.01)

Figure 2. PKA activity is increased on polymerized collagen.

(A) PKA activity was measured in hepatocytes adherent to collagen film or collagen gel at the indicated time points. T=0 (black bar) indicates freshly isolated cells prior to plating. PKA activity was determined by measuring incorporation of radiolabeled phosphate into a PKA substrate peptide (Kemptide) added to cell lysates and subtracting background counts obtained in the absence of substrate. (B) PKA assay was performed as above, with the addition of the overlay condition, in which hepatocytes were plated onto collagen film for 12 hours, followed by collagen gel overlay. PKA activity was assessed 24 hours after initial plating for all conditions (12 hours after overlay addition). Error bars represent S.D. from duplicate samples of representative experiments (from 6 PKA assay experiments and 3 overlay experiments) (*=p<0.05; NS=not significant)

Figure 3. Inhibition of PKA increases cell spreading on polymerized collagen.

(A) Hepatocytes were plated on collagen film with or without the PKA agonist, 8Br-cAMP (1 mM) and on collagen gel with or without the PKA inhibitor H89, then at 24 hr, cells were either photographed using phase contrast microscopy with Varel optics, or processed for scanning
electron microscopy (SEM). (B) Hepatocytes were photographed at 48 hours after culture on film, on collagen gel, on collagen gel in the presence of 3 µM H89, or on collagen gel with 3 µM H89, but in the absence of EGF. (C) Cell areas at 48 hr for the above conditions were analyzed by computer image analysis of at least 50 randomly selected cells per condition. Inset: Cell area at 48 hr when only solitary cells were measured.

**Figure 4. Inhibition of PKA activity increases DNA synthesis on collagen gel.**

(A) Hepatocytes were cultured on collagen film or collagen gel in Williams’s E medium containing EGF in the presence of increased amounts of H-89, and DNA synthesis was measured by incorporation of [³H]-thymidine between 52 -72 hours. (NS=not significant; **=p<0.05, compared to matrix/DMSO controls “-“) (B) Hepatocytes adherent to collagen gel were cultured with increasing amounts of myristoylated PKI for 0-24 hours after plating and DNA synthesis was measured by thymidine incorporation between 52 and 72 hours. (NS=not significant; **=p<0.01) (C) Hepatocytes adherent to collagen film or collagen gel were exposed to 3µM H89 for either 24 or 48 hours in the presence or absence of EGF. [³H]-Thymidine incorporation was measured between 48 and 72 hours. (NS=not significant; *p=0.047; **=p<0.01; N.D.=not determined)

**Figure 5. Inhibition of PKA activity restores EGF signaling to ERK and cyclin D1.**

(A) Hepatocytes were cultured in the presence or absence of 3µM H89 for the first 24 hours of plating. Cell lysates were collected at 52 and 72 hours after plating, and expression of the indicated proteins was analyzed by western blot. (B) Hepatocytes were cultured on collagen gel and incubated with myristoylated PKI (1µg/ml) for the first 24 hours of culture. Cell lysates were collected at 60 hours and analyzed by western blot. (C) Hepatocytes were cultured on film with
or without EGF, or on gel with EGF in the presence or absence of 3μM H89 from 0-48 hours, and lysates were collected at the indicated times for western blot analysis.

**Figure 6.** *Increase in cyclin D1 by PKA inhibition is dependent upon MEK activity.*

(A) Hepatocytes were cultured for 48 hours with 3μM H89 or equivalent concentration of DMSO, then treated between 48 and 72 hours with DMSO or 25 μM U0126. Cell lysates were collected at 72 hours and analyzed for cyclin D1 expression or G3PDH by western analysis. (B) Hepatocytes were treated as in (A) and DNA synthesis was measured between 48 and 72 hours.

**Figure 7.** *Polymerized collagen inhibits EGF receptor phosphorylation.*

(A) To analyze EGF receptor phosphorylation on collagen film or gel and in response to PKA modulation, hepatocytes were cultured up to 4 hours on collagen film or gel, followed by immunoprecipitation with anti-phosphotyrosine antibody and western blot analysis with anti-EGFr antibody. (B) A more extensive timecourse of EGFr phosphorylation was examined from 2-24 hr on collagen film (F) or gel (G) in culture, analyzed by anti-phosphotyrosine western blot using antibodies for total phospho-tyrosine and total EGF receptor. (C) Densitometry of bands from at least three different blots as in 7B was performed and compared relative to the maximal value (*p<0.05 comparing film and gel).

**Figure 8.** *Polymerized collagen inhibits phosphorylation of specific tyr residue in EGFr in a PKA-dependent manner.*

(A) EGFr phosphorylation on specific tyr residues (Tyr-845 and Tyr-1068) was examined from 15 minutes through 24 hr on collagen film (F) or gel (G) in culture. (B) Densitometry of bands from at least three different blots as in 8A was performed and compared relative to the maximal
value. (*p<0.05 comparing film and gel) (C) Hepatocytes were treated with H89 or DMSO for thirty minutes prior to addition of EGF for 30 minutes or 2.5 hr. Lysates were analyzed by western blot for p-tyrosine, tyrosine residue 845, tyrosine residue 1068, and total EGF receptor.

Figure 9. EGFr phosphorylation and subsequent cell cycle events are reduced in hepatocytes on gel in response to additional EGF at later timepoints.

(A) Hepatocytes were cultured for 12 hours with EGF on film or gel in the presence or absence of H89. After 12 hours of treatment, the media was removed and replaced with media (+/- H89) free of EGF for 36 hours. New media was added with or without EGF at 48 hours, and lysates were collected at the indicated time points and analyzed for phospho-ERK, cyclin D1, and G3PDH by western blot. Controls exposed to EGF for 48 hr are also shown ("48"). (B) Tyrosine residues 1068 and 845, and total EGF receptor levels were analyzed in lysates collected after 48 hours of continuous EGF treatment (control, "C"), or 36 hours after EGF withdrawal with or without re-addition of EGF. H89 was present for only the first 48 hours. (C) The role of Src in EGFr Tyr-845 phosphorylation on film or gel +/- PKA inhibitor was examined by culturing hepatocytes as in 9B in the presence or absence of Src inhibitor, PP2.
### A

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**Cyclin D1**

**G3PDH**

### B

Bar chart showing DNA synthesis (% of FILM) with H-89 and U0126 treatments.

- **H-89**: - - + + + +
- **U0126**: - + - + + +

**FILM**

**GEL**

- **P-value**: **,**
- **P-value**: *