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Integrin Binding and Cell Spreading on Extracellular Matrix Act at Different Points in the Cell Cycle to Promote Hepatocyte Growth

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This study was undertaken to determine the importance of integrin binding and cell shape changes in the control of cell-cycle progression by extracellular matrix (ECM). Primary rat hepatocytes were cultured on ECM-coated dishes in serum-free medium with saturating amounts of growth factors (epidermal growth factor and insulin). Integrin binding and cell spreading were promoted in parallel by plating cells on dishes coated with fibronectin (FN). Integrin binding was separated from cell shape changes by culturing cells on dishes coated with a synthetic arg-gly-asp (RGD)-peptide that acts as an integrin ligand but does not support hepatocyte extension. Expression of early (junB) and late (ras) growth response genes and DNA synthesis were measured to determine whether these substrata induce G_0-synchronized hepatocytes to reenter the growth cycle. Cells plated on FN exhibited transient increases in junB and ras gene expression (within 2 and 8 h after plating, respectively) and synchronous entry into S phase. Induction of junB and ras was observed over a similar time course in cells on RGD-coated dishes, however, these round cells did not enter S phase. The possibility that round cells on RGD were blocked in mid to late G_1 was confirmed by the finding that when trypsinized and replated onto FN-coated dishes after 30 h of culture, they required a similar time (12–15 h) to reenter S phase as cells that had been spread and allowed to progress through G_1 on FN. We have previously shown that hepatocytes remain viable and maintain high levels of liver-specific functions when cultured on these RGD-coated dishes. Thus, these results suggest that ECM acts at two different points in the cell cycle to regulate hepatocyte growth: first, by activating the G_0/G_1 transition via integrin binding and second, by promoting the G_1/S phase transition and switching off the default differentiation program through mechanisms related to cell spreading.

INTRODUCTION

Cell adhesion is an important component in the growth activation pathway along with growth factors and nutrients. Both in vivo and in vitro, the extracellular matrix (ECM) works in concert with soluble growth factors to produce a complete proliferative response. Nontransformed cells cultured in suspension in the presence of growth factors fail to undergo DNA synthesis (MacPherson and Montagnier, 1964; Benecke *et al.*, 1978), and the loss of "anchorage-dependence" is a hallmark of cellular transformation (Stoker *et al.*, 1968; Shin *et al.*, 1975). ECM also has been shown to modulate cell sensitivity to soluble mitogens and regulate cell proliferation in vitro (Ingber *et al.*, 1987; Ingber, 1990; Moo-

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ney *et al.*, 1992a) as well as in vivo (Ingber and Folkman, 1988; Talhouk *et al.*, 1992). Yet, whereas the role of soluble growth factors in mitogenesis has been well defined, the mechanism by which adhesion promotes cell growth remains unclear.

Many ECM molecules such as fibronectin (FN) contain the arg-gly-asp (RGD) amino acid sequence to which cell surface integrin receptors bind specifically (Ruoslahti and Pierschbacher, 1987). Occupancy and clustering of integrins recently have been shown to activate intracellular chemical signaling pathways associated with the G_0/G_1 transition that are turned on early during growth activation by soluble mitogens, including Na+/H+ exchange (Ingber *et al.*, 1990; Schwartz *et al.*, 1991), inositol lipid turnover (McNamee *et al.*, 1993), and protein tyrosine phosphorylation (Guan *et al.*, 1991; Kornberg *et al.*, 1991). Cell attachment to ECM (Lee *et al.*, 1984; Dike and Farmer, 1988; Mooney *et al.*, 1992a) and integrin binding (Adams and Watt, 1989; Werb *et al.*, 1989) also have been shown to induce gene expression, apparently as a result of activation of specific ECM-sensitive regulatory elements (Schmidhauser *et al.*, 1990). Thus, the ECM is involved in cell growth at the molecular level, regulating specific chemical signals involved in the growth activation pathway.

At the same time, cell shape changes (i.e., spreading) that commonly accompany cell binding to ECM also appear to be required for the growth of many different types of anchorage-dependent cells (Folkman and Moscona, 1978; Watt *et al.*, 1988; Ingber, 1990). These observations raise the possibility that, in addition to chemical signaling, binding of ECM molecules to integrins may regulate growth by some form of "mechanical" signaling (Ingber, 1991, 1993a,b). For example, when cells are cultured on malleable ECM gels that cannot support cell spreading, growth is inhibited and tissue-specific functions are maintained (Emerman and Pitelka, 1977; Li *et al.*, 1987; Ben-Ze'ev *et al.*, 1988). However, the same ECM molecules induce proliferation and cause loss of differentiated features when coated on dishes or organized in a rigid structure on which cells can physically extend (Opas, 1989; Bucher *et al.*, 1990). Variations in the density at which ECM molecules are presented also influence cell shape and growth in a coordinated manner (Ingber, 1990; Mooney *et al.*, 1992a). For example, hepatocyte attachment to a high density (1000 ng/cm^2) of FN or laminin leads to cell spreading, histone mRNA expression, and DNA synthesis. In contrast, a low density (1 ng/cm^2) of the same ECM molecule that cannot support cell extension suppresses growth and maintains near normal levels of liver-specific gene expression and protein secretion (Mooney *et al.*, 1992a). Thus, the ability of the ECM to promote cell spreading, independent of its chemical specificity, also appears to be a critical determinant for switching between growth and differentiation programs. Determination of the specific role of cell spreading, however, has been difficult because many experimental methods used to enhance cell spreading also increase the density of immobilized integrin ligand (Ingber, 1990; Farrell and Greene, 1992; Mooney *et al.*, 1992a). Cell binding to a high density of ECM ligand has been shown to activate intracellular chemical signaling pathways independently of cell spreading, by promoting local clustering of integrin receptors on the cell surface (Schwartz *et al.*, 1991; McNamee and Ingber, 1993; McNamee *et al.*, 1993).

The present study was designed to determine whether these two components of ECM signaling, direct chemical signaling, and indirect mechanical signaling associated with changes in cell shape and structure act at different points in the cell cycle to promote cell growth. To test this hypothesis, hepatocyte cell cycle progression was examined under conditions in which integrin binding could be promoted in the presence of a constant amount of soluble growth factors with or without associated cell shape changes. Using this approach, we now show that cell binding to a high density of immobilized integrin ligand, without changes in cell shape, activates chemical signaling mechanisms that are sufficient for induction of growth response genes associated with both the G_0/G_1 transition and the mid G_1 phase of the cell cycle. However, these cells do not grow. For hepatocytes to enter S phase and proliferate, ECM molecules must also promote cell spreading and activate associated mechanical signaling mechanisms.

MATERIALS AND METHODS

Cell Isolation and Culture

Rat hepatocytes were obtained by collagenase perfusion of adult Lewis rat liver followed by purification through a Percoll (Sigma Chemical, St. Louis, MO) gradient, as previously described (Aiken *et al.*, 1990). Cell viability was determined using trypan blue exclusion; only cell preparations yielding ≥85% viability were used. FN (human serum fibronectin, Boehringer Mannheim, Indianapolis, IN) or RGD-peptide (Peptite-2000, Telios, La Jolla, CA) was coated on petri dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) using a carbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate pH 9.4), and nonspecific binding sites were blocked using 1% bovine serum albumin, as previously described (Mooney *et al.*, 1992a). Freshly isolated hepatocytes were kept on ice in medium without growth factors until the experiment began; cells were plated immediately after medium containing mitogens was added. Hepatocytes were plated (10,000 cells/cm^2) in chemically-defined medium consisting of William's medium E (GIBCO, Grand Island, NY), insulin (20 mU/ml, Sigma), epidermal growth factor (10 ng/ml, Collaborative Research, Bedford, MA), dexamethasone (5 nM, Sigma), sodium pyruvate (20 mM, GIBCO), ascorbic acid (50 μg/ml, GIBCO), and penicillin/streptomycin (100 U/ml, Irvine Scientific, Santa Ana, CA) and refed daily. Photographic images of adherent glutaraldehyde-fixed cells were recorded on a Nikon Diaphot (Garden City, NY) inverted microscope under Hoffman optics using Kodak (Rochester, NY) T-Max 100 film.

Cell Growth

DNA synthesis was measured by quantitating incorporation of [^{3}H]-thymidine using autoradiography as described (Ingber, 1990). [^{3}H]-thymidine (1 μCi/ml) was added from 24–48 h after cell plating.

Nuclear labeling indices were determined by counting 100 cells from four randomly selected regions and calculating the percentage of cells with labeled nuclei. Photographic images were recorded as described above. In the replating experiments, cells were initially cultured on 150-mm petri dishes coated with either FN or RGD. After 30 h cells were trypsinized, washed in phosphate-buffered saline (PBS), and replated (5×10^3 cells/well) in defined medium on FN-coated 96-well plates (500 ng FN/well, Immunolon II, Dynatech, Alexandria, VA). To determine the time course of entry into S phase, different wells containing replated cells were pulsed with [^{3}H]-thymidine (2 μCi/ml) every 3 h. DNA synthesis was measured in the adherent cells by quantitating incorportation of [^{3}H]-thymidine into trichloroacetic acid-precipitable material using scintillation counting.

RNA Analysis

Total cellular RNA was obtained using lysis and precipitation with a LiCl/urea solution as described (Auffray and Rougeon, 1980). Briefly, for nonadherent controls (t = 0), an aliquot of freshly isolated cells was pelleted at 4°C and resuspended in 3 M LiCl/6 M urea. Adherent cells were washed twice with cold PBS to remove nonadherent cells, 3 M LiCl/6 M urea was added to culture plate, and the cell lysis solution was scraped off the plate. After further purification with phenol/chloroform extractions and ethanol precipitation, equal amounts of purified RNA were separated on formaldehyde agarose gels and transferred onto nitrocellulose. Ethidium bromide staining of gels assured equal loading of RNA in each lane. cDNA labeled with [^{32}P]-dCTP using the random primer method was hybridized to the blot overnight as previously described (Mooney *et al.*, 1992a) and placed on X-ray film for autoradiography. Mouse junB cDNA and rat v-ras (Kirsten) cDNA were kindly provided by Drs. James Darnell (Rockefeller University, NY) and Stephen Farmer (Boston University School of Medicine, Boston, MA).

RESULTS

Intact Fibronectin Is Required for Hepatocyte Spreading

Dishes coated with a high density (1000 ng/cm^2) of FN or RGD-peptide readily promoted attachment of freshly isolated hepatocytes; however, they differed greatly in their ability to promote cell spreading. Cells extended out small processes within 2 h and appeared highly flattened and spread by 24 h on FN (Figure 1). In contrast, cells only spread minimally on RGD (Figure 1). Cells maintained these poorly spread forms even after several days in culture, as previously described (Mooney *et al.*, 1992a,b). Importantly, the lack of hepatocyte spreading on dishes coated with RGD-peptide was not because of inefficient immobilization of the peptide because capillary endothelial cells and vascular smooth muscle cells spread normally on these dishes (unpublished data).

Integrin Binding Is Sufficient to Induce the G_0/G_1 Transition

A hallmark of growth activation by soluble mitogens is the expression of immediate early growth response genes, such as members of the Jun family of protooncogenes, within 1–2 h after stimulation (Ryder *et al.*, 1988). The protein products of the Jun family are transcription factors that are required for cell cycle progression (Kovary and Bravo, 1991), and their expression is indicative of the passage of quiescent cells through the G_0/G_1 transition. junB mRNA levels were thus used as an indicator of cell cycle entry in freshly isolated hepatocytes that normally exist in vivo in a synchronized G_0 state. Levels of immediate early growth response genes, such as junB, have been shown to be slightly elevated in freshly isolated hepatocytes, apparently as a result of collagenase perfusion (Rana *et al.*, 1991). However, Northern analysis revealed that cell attachment to high FN resulted in an increase in junB mRNA levels above this baseline, with maximal expression being observed ~2 h after plating (Figure 2A). Similar induction of junB expression was also observed at 2 h, when cells were plated on dishes coated with a high density of synthetic RGD-peptide (Figure 2B), even though cell spreading was not promoted. This induction appeared to be specific and not because of a generalized induction of gene expression. For example, expression of ras, another growth-associated gene that is normally associated with later stages of cell cycle progression, remained low at these same early time points (Figure 3). These results indicate that integrin binding, independent of cell shape changes, is sufficient to support hepatocyte entry into the cell cycle. This result is consistent with past studies that demonstrate that integrin clustering is alone sufficient to activate chemical signaling pathways associated with the G_0/G_1 transition in other cell types (Schwartz *et al.*, 1991).

Cell Spreading Is Required for G_1 Progression and S Phase Entry

We have previously shown that hepatocyte attachment to a high density of laminin induces histone gene

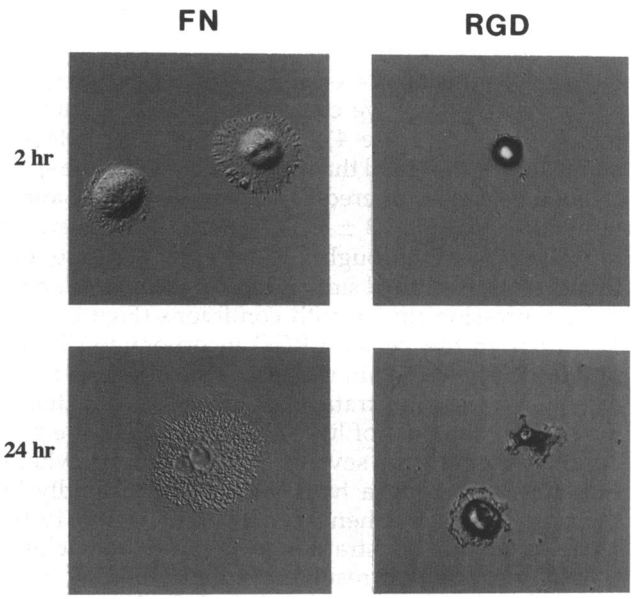

Figure 1. Cell spreading on different ECM substrata. Freshly isolated hepatocytes were cultured for 2 or 24 h on dishes coated with FN or a synthetic RGD-peptide, fixed in glutaraldehyde, and photographed under Hoffman Optics (×160).

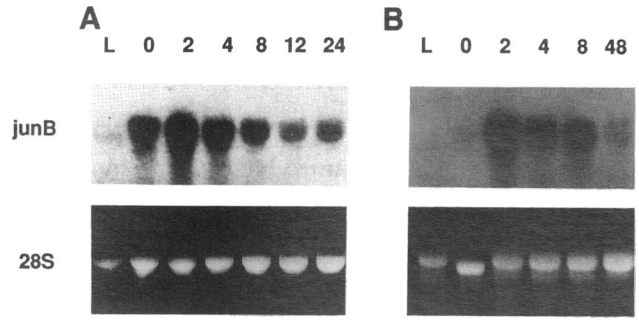

Figure 2. Autoradiograph of a Northern blot showing the time course of junB expression in hepatocytes plated on FN (A) vs. RGD (B). Hepatocytes were cultured for the indicated number of hours, and total RNA was assayed for expression of junB protooncogene mRNA (junB). The loading of the gels was determined by visualizing the 28S rRNA band using ethidium bromide (28S). L, RNA from whole liver; 0, RNA from freshly isolated hepatocytes before plating.

expression and promotes DNA synthesis, whereas cells on low laminin remain quiescent and maintain high levels of expression of differentiation-specific genes (Mooney *et al.*, 1992a). To determine if FN and RGD-coated dishes differentially regulate progression through G_1, we measured the expression of the ras protooncogene. ras mRNA levels are normally elevated during mid-G_1 in nontransformed cells including hepatocytes both when cultured in vitro (Goyette *et al.*, 1984) and during liver regeneration in vivo (Kruijer *et al.*, 1986). Northern analysis revealed that spread cells on FN and round cells on RGD both exhibited increased levels of ras mRNA beginning ~8 h after plating (Figure 3). In other words, cell binding to a high density of the RGD-peptide stimulated hepatocytes to progress to mid-G_1, independently of cell shape.

To determine if FN or RGD differentially regulate entry into S phase, we carried out [^{3}H]-thymidine autoradiography (Figure 4). Quantitation of nuclear labeling indices revealed that most (61 ± 4%) of the spread cells on high FN entered S phase between 24 and 48 h. In contrast, only 13 ± 4% of cells on RGD were able to fully progress through G_1 and enter S phase, even though cells exhibited similar induction of junB and ras gene expression under both conditions (Figures 2 and 3). Although few cells on RGD incorporated [^{3}H]-thymidine, they did remain viable. In fact, hepatocytes cultured on these substrata that prevent cell extension maintain high levels of liver-specific functions (e.g., albumin secretion) for several days in culture, whereas cells that spread on a high ECM density rapidly lose their differentiated phenotype (Mooney *et al.*, 1992b). These results demonstrate that hepatocyte attachment to RGD peptide and resultant integrin binding induce hepatocytes to progress through mid-G_1, but it does not support S phase entry. Apparently, cell spreading must act at some point in mid- to late G_1 to promote subsequent cell cycle progression.

To confirm that hepatocytes were blocked in mid-to late G1, we carried out a replating experiment. Entry into S phase has been reported to occur between 24-30 h in primary adult rat hepatocytes (Tomomura *et al.*, 1987). Thus, cells that were adherent to FN- or RGD-coated dishes for 30 h (a point near the G_1/S phase border) were trypsinized, replated onto FN-coated dishes, and pulsed every 3 h with [^{3}H]-thymidine. Cells that were allowed to spread and progress through G_1 on FN for 30 h before replating onto new FN dishes required ~12–15 h before they reentered S phase (Figure 5). This lag period before S phase reentry is similar to that caused by use of trypsin in a past cell cycle study when synchronized fiboblasts were removed from dishes and replated on tissue culture substrata (Campisi and Medrano, 1983). If cells adherent to RGD for 30 h remained in G_0, then they should require ≥24 h to enter S phase when replated onto FN. The time for required for reentry would increase to 36–39 h, if the time lag because of exposure to trypsin is also included. In contrast, cells trypsinized from RGD dishes and replated onto FN exhibited nearly identical kinetics of S phase entry as those as on FN (Figure 5); only 12–15 h were required before the onset of DNA synthesis. These results in combination with the ras data confirm that cell binding to integrin ligands is sufficient without any cell shape change to induce hepatocytes to progress through mid- to late G_1 phase of the cell cycle. However, to progress further through G_1 and enter S phase, cell spreading also must be promoted.

DISCUSSION

Cell adhesion to ECM plays a critical role in the control of cell proliferation and differentiation. ECM molecules are thought to exert their effects on cell function by binding to transmembrane receptors on the cell surface,

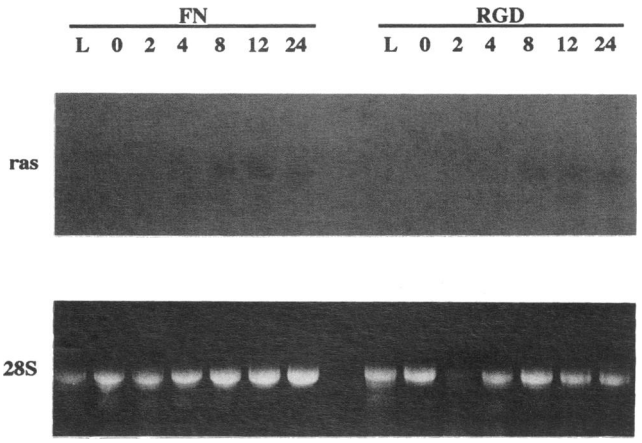

Figure 3. Autoradiograph of a Northern blot showing the time course of ras expression in hepatocytes cultured on FN or RGD peptide. Labeling is the same as indicated in Figure 2.

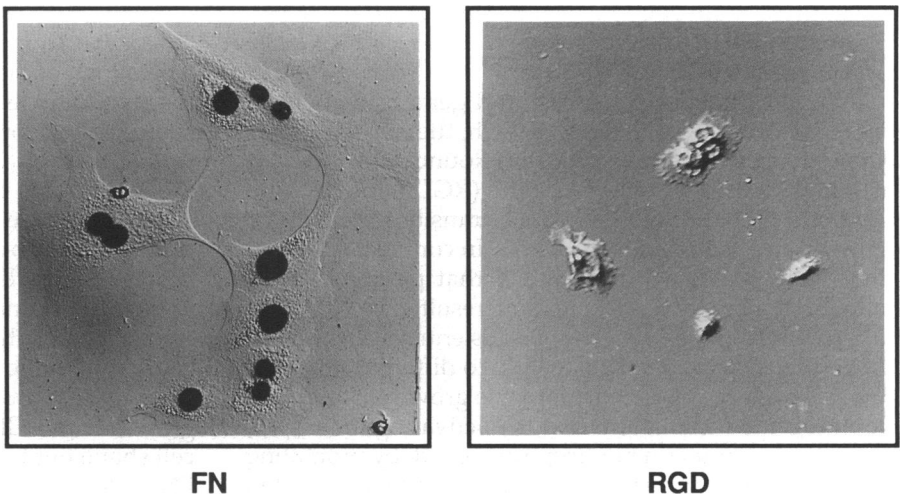

Figure 4. [^{3}H]-thymidine autoradiograph of cells cultured on FN or RGD-peptide and viewed under Hoffman optics. Nuclei covered with black grains indicate cells that synthesized DNA during the labeling period (24–48 h after plating).

such as integrins, and activating intracellular chemical signaling pathways (Hynes, 1992; Schwartz, 1992; Juliano and Haskill, 1993). Previous studies also have shown that cell attachment to immobilized integrin ligands activates early immediate growth response genes (Dike and Farmer, 1988; Haskill *et al.*, 1988; Dhawan and Farmer, 1990). In general, a high density of immobilized ECM ligand was found to be required for both induction of growth response genes (Farrell and Greene, 1992) and S phase entry (Mooney *et al.*, 1992a). However, in these past studies, it was not possible to discriminate whether raising the coating density of the immobilized ECM molecule promoted cell growth and gene activation by increasing the local density of bound integrin receptors on the cell surface (i.e., receptor clustering) or by promoting cell spreading. This is an important point for clarification because integrin clustering is sufficient to activate many chemical signaling pathways, independently of cell shape changes (Schwartz *et al.*, 1991; McNamee and Ingber, 1993).

In the present study, we were able to discriminate between these two potential regulatory mechanisms by examining cell cycle events within cells attached to dishes coated with high local densities of attachment molecules (FN and RGD-peptide) that bind integrins to a similar degree but differ in their ability to promote cell spreading. Our results confirm that integrin binding acts directly to activate chemical signaling mechanisms in hepatocytes. Cell attachment to dishes coated with a high density of either intact FN or a synthetic peptide containing the RGD cell binding site alone induced similar increases in the expression of early and late growth response genes (junB and ras, respectively), even though cell extension was only observed on the FN-coated dishes. The ability of both integrin ligands (FN and RGD) to activate growth-associated genes when presented at a high density is likely related to their ability to promote integrin clustering and release chemical second messengers. This interpretation is based on our past finding that FN-coated microbeads that promote integrin clustering (Plopper and Ingber, 1993) activate chemical signaling pathways (e.g., Na+/H+ exchange, inositol lipid synthesis) to the same degree as dishes coated with a high FN density (Schwartz *et al.*, 1991). In contrast, cell binding to low FN or soluble ligands that promote integrin occupancy alone (e.g., RGD peptides, anti-integrin antibody Fab fragments) failed to

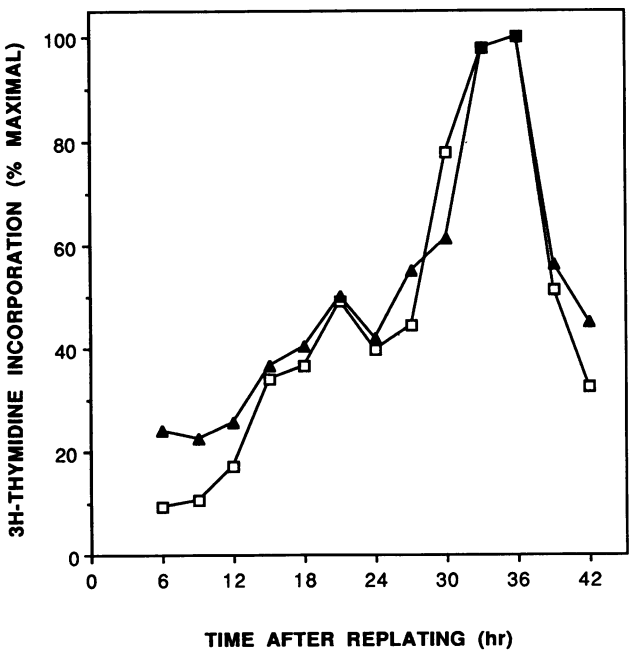

Figure 5. Time course of hepatocyte reentry into S phase after trypsinization and replating. □, cells cultured on FN for 30 h, trypsinized, and replated on FN; ▲, cells cultured on RGD for 30 h, trypsinized, and replated on FN. Each data point represents triplicate samples with each triplicate consisting of cells within four pooled culture wells.

elicit these transduction events (Ingber *et al.*, 1990; Schwartz *et al.*, 1991; McNamee *et al.*, 1993; McNamee and Ingber, 1993).

Whereas chemical signaling and gene activation by integrins may be required for growth, the present results show that they are not sufficient. Round cells attached to a high density of integrin ligand (RGD peptide) were found to pass through the G_0/G_1 transition, only to be growth-arrested in mid- to late G_1. In contrast, substrata that were coated with intact FN that promoted both integrin binding and cell spreading resulted in activation of growth response genes as well as entry into S phase. ECM therefore appears to act at two different points in the cell cycle to regulate hepatocyte growth in response to mitogenic stimulation: first, by activating the G_0/G_1 transition via integrin binding and second, by promoting the G_1/S phase transition through mechanisms related to cell spreading. Thus, these results represent a first step toward placing the long recognized "phenomenon" of cell shape–dependent growth control (Folkman and Moscona, 1978; Ben-Ze'ev *et al.*, 1980, 1988; Spiegelman and Ginty 1983; Watt *et al.*, 1988; Ingber, 1990; Singhvi *et al.*, 1994) in the context of current molecular views of cell cycle regulation.

The mechanism of G_0/G_1 transition seen in cells attached to FN and RGD in the present study may involve direct intracellular signaling through integrins, acting in concert with signaling pathways directed through growth factor receptors. For example, soluble mitogens, cell adhesion to FN, and integrin clustering all have been shown to activate the Na+/H+ antiporter, a signaling event that appears to be required for entry into S phase in many cells, including hepatocytes (Ingber *et al.*, 1990; Hansen *et al.*, 1992). In certain cell types, integrin occupancy (i.e., rather than clustering) and associated signaling events appear to be sufficient to induce expression of differentiation-specific genes (Adams and Watt, 1989). In fact, hepatocytes remain highly differentiated when cultured on dishes coated with a *low* ECM density (1 ng/cm^2 versus 1000 ng/cm^2 used in the present study) that promote integrin ligation but only minimal receptor clustering and chemical signal activation (Ingber *et al.*, 1990; Schwartz *et al.*, 1991). However, we have previously shown that hepatocytes also maintain high levels of expression of liver-specific functions (e.g., albumin secretion) when cultured on RGD-coated dishes under the same conditions used in the present study (Mooney *et al.*, 1992b). Thus, if integrin occupancy is also sufficient to switch on differentiation genes in hepatocytes, then this differentiation-inducing signal is not turned off when integrin clustering is promoted and cells are induced to progress through the G_0/G_1 transition. Apparently, to switch off differentiation and turn on growth in hepatocytes, cell spreading must also be promoted, as suggested by previous studies (Ben Ze'ev *et al.*, 1988; Mooney *et al.*, 1992a; Singvhi *et al.*, 1994).

Analysis of changes in the expression of the ras protoocogene and the replating experiments revealed that activation of integrin-mediated chemical signaling mechanisms was sufficient to induce hepatocytes to pass completely through the G_0/G_1 transition and progress through mid-G_1. However, only cells that were bound to FN and able to change their shape and spread also entered S phase. One possible caveat is that although RGD peptides bind integrins, they lack other regions of the FN molecule that could be responsible for transmitting additional growth signals. For example, fibroblasts require the heparin-binding domain of FN for cell spreading to occur (Izzard *et al.*, 1986; Streeter and Rees, 1987). Thus, FN may promote different effects on growth than RGD peptide not because of differences in cell shape but because of the presence of regions of the FN molecule that bind heparan sulfate–containing proteoglycans on the cell surface. Ligation of these proteoglycans, in turn, could be responsible for activating separate chemical signaling mechanisms that may be required for growth (Woods and Couchman, 1992a,b). However, our hepatocytes also will not enter S phase when cultured on substrata that are coated with whole ECM proteins (e.g., laminin or FN), if the substrata are engineered so that cell spreading is limited to an area that is similar in size to that exhibited by cells on RGD-coated dishes in the present study (Singhvi *et al.*, 1994). In other words, hepatocyte binding to a high local density of an *intact* ECM molecule will not promote S phase entry, if cell spreading is prevented. Taken together, these results suggest that cell shape functions as a physiological control element and acts independently of initial integrin receptor binding events to regulate hepatocyte growth.

Cell shape appears to exerts its effects on cell cycle progression by acting at some point during mid- to late G_1, after induction of ras and before S phase entry. Studies in fibroblasts also map an adhesion-sensitive growth restriction point to late G_1 (Otsuka and Moskowitz, 1975; Guadagno and Assoian, 1991). Specifically, fibroblast adhesion regulates expression of cyclin A (Guadagno *et al.*, 1993), which together with its catalytic subunits, the cdks, express kinase activity that is required for the G_1/S phase transition. In contrast, our results suggest that activation of the G_1/S transition may not be triggered by adhesion alone, but more specifically, by the ECM's ability to cluster integrin receptors, activate chemical signaling mechanisms, and promote cell spreading.

Although adhesion appears to be required for induction of early cell cycle events in primary hepatocytes and some fibroblasts (Dike and Farmer, 1988), it is not in other cell lines (Guadagno and Assoian, 1991). Transformed cells that grow independent of anchorage become capable of constituitively expressing the signals normally induced by attachment to ECM (Schwartz *et al.*, 1990; Guan and Shalloway, 1992; Schwartz and

Ingber, 1994). The lack of a requirement of adhesion for activation of growth response genes observed in certain established cell lines therefore may be associated with the progressive decrease in anchorage requirements for growth and increased rate of spontaneous transformation that often accompanies extensive cell passaging.

How cell shape changes might regulate biochemical events that are required for entry into S phase remains unknown. However, many elements of the cell's metabolic machinery that are required for growth signaling and DNA replication appear to be immobilized on insoluble scaffolds within the cytoskeleton and nucleus (Ingber, 1993b). Integrins physically interconnect ECM with the actin cytoskeleton and transmit mechanical signals across the cell surface via this molecular bridge (Wang *et al.*, 1993). For this reason, altering cell-ECM contacts changes the mechanical force balance within the cell and results in coordinated changes in cell, cytoskeletal, and nuclear form (Ingber *et al.*, 1987; Hansen and Ingber, 1992; Sims *et al.*, 1992; Ingber, 1993a). Architectural rearrangements within the intracellular lattice could alter association of immobilized enzymes with their substrates or inhibitors and thereby alter biochemical reactions and chemical signaling cascades within the cytoplasm and nucleus. Mechanical expansion of nuclear pores, as occurs when nuclei extend within spreading cells (Feldherr and Akin, 1993), also could augment nuclear transport of molecules (e.g., growth factors, DNA replication complexes) that are required for initiation of DNA synthesis (Hansen and Ingber, 1992). In other words, cell cycle progression could be promoted in late G_1 as a result of the changes in cytoskeletal and nuclear structure that accompany hepatocyte spreading.

In conclusion, the results presented in this report demonstrate that the ECM acts at two distinct points in the cell cycle to regulate growth. Cell binding to high densities of integrin ligands induces early G_0-G_1 events, most likely through integrin clustering. Neither interaction with non-RGD binding ECM receptors (e.g., proteoglycans) nor cell spreading is required for this activation. Once cells have progressed into G_1, the ability of the ECM to promote cell spreading is required for full progression through G_1 and entry into S phase. These cell shape changes are also required to turn off what may essentially be viewed as a "default" differentiation program in these cells (Mooney *et al.*, 1992a,b). Studies are currently underway to determine precisely where in mid- to late G_1 growth arrest occurs in the absence of cell extension and to determine the mechanism by which cell spreading promotes DNA synthesis and shuts off differentiation.

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REFERENCES

Adams, J.C., and Watt, F.M. (1989). Fibronectin inhibits the terminal differentiation of human keratinocytes. Nature *340*, 307–309.

Aiken, J., Cima, L., Schloo, B., Mooney, D., Johnson, L., Langer, R., and Vacanti, J.P. (1990). Studies in rat liver perfusion for optimal harvest of hepatocytes. J. Pediatr Surg. *25*, 140–145.

Auffray, C., and Rougeon, F. (1980). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem. *107*, 303–314.

Benecke, B.-J., Ben-Ze'ev, A., and Penman, S. (1978). The control of mRNA production, translation and turnover in suspended and reattached anchorage-dependent fibroblasts. Cell *14*, 931–939.

Ben-Ze'ev, A., Farmer, S.R., and Penman, S. (1980). Protein synthesis requires cell-surface contact while nuclear events respond to cell shape in anchorage-dependent fibroblasts. Cell *21*, 365–372.

Ben-Ze'ev, A., Robinson, G.S., and Farmer, S.R. (1988). Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. Proc. Natl. Acad. Sci. USA *85*, 2161–2165.

Bucher, N.L.R., Robinson, G.S., and Farmer, S.R. (1990). Effects of extracellular matrix on hepatocyte growth and gene expression: Implications for hepatic regeneration and the repair of liver injury. Semin. Liver Dis. *10*, 11–19.

Campisi, J., and Medrano, E.E. (1983). Cell cycle perturbations in normal and transformed fibroblasts caused by detachment from the substratum. J. Cell. Physiol. *114*, 53–60.

Dhawan, J., and Farmer, S.R. (1990). Regulation of α_1(I)-collagen gene expression in response to cell adhesion in swiss 3T3 fibroblasts. J. Biol. Chem. *265*, 9015–9021.

Dike, L.E., and Farmer, S.R. (1988). Cell adhesion induces expression of growth-associated genes in suspension-arrested fibroblasts. Proc. Natl. Acad. Sci. USA *85*, 6792–6796.

Emerman, J.T., and Pitelka, D.R. (1977). Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. In Vitro *13*, 316–328.

Farrell, R.E., Jr., and Greene, J.J. (1992). Regulation of c-*myc* and c-Ha-*ras* oncogene expression by cell shape. J. Cell. Physiol. *153*, 429–435.

Feldherr, C.M., and Akin, D. (1993). Regulation of nuclear transport in proliferating and quiescent cells. Exp. Cell Res. *205*, 179–186.

Folkman, J., and Moscona, A. (1978). Role of cell shape in growth control. Nature *273*, 345–349.

Goyette, M., Petropoulos, C.J., Shank, P.R., and Fausto, N. (1984). Regulated transcription of c-Ki-*ras* and c-*myc* during compensatory growth of rat liver. Mol. Cell. Biol. *4*, 1493–1498.

Guadagno, T.M., and Assoian, R.K. (1991). G_1/S control of anchorage-independent growth in the fibroblast cell cycle. J. Cell Biol. *115*, 1419–1425.

Guadagno, T.M., Ohtsubo, M., Roberts, J.M., and Assoain, R.K. (1993). A link between cyclin A expression and adhesion-dependent cell cycle progression. Science *262*, 1572–1575.

Guan, J.-L., and Shalloway, D. (1992). Regulation of pp125^{FAK} both by cellular adhesion and by oncogenic transformation. Nature *358*, 690–692.

Guan, J.-L., Trevithick, J.E., and Hynes, R.O. (1991). Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. Cell Regul. *2*, 951–964.

Hansen, L.K., and Ingber, D.E. (1992). Regulation of nucleocytoplasmic transport by mechanical forces transmitted through the cytoskeleton. In: Nuclear Trafficking, ed. C. Feldherr, Orlando, FL: Academic Press, 71–86.

Hansen, L.K., Schwartz, M.A., Lechene, C., and Ingber, D.E. (1992). Fibronectin and laminin differ in their ability to activate the Na+/H+-antiporter in hepatocytes. J. Cell. Biochem. *16F* (Suppl.), 157.

Haskill, S., Johnson, C., Eierman, D., Becker, S., and Warren, K. (1988). Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. J. Immunol. *14*, 1690–1694.

Hynes, R.O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. Cell *69*, 11–25.

Ingber, D.E. (1990). Fibronectin controls capillary endothelial cell growth by modulating cell shape. Proc. Natl. Acad. Sci. USA *87*, 3579–3583.

Ingber, D.E. (1991). Integrins as mechanochemical transducers. Curr. Opin. Cell Biol. *3*, 841–848.

Ingber, D.E. (1993a). Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. J. Cell Sci. *104*, 613–627.

Ingber, D.E. (1993b). The riddle of morphogenesis: a question of solution chemistry or molecular cell engineering? Cell *75*, 1249–1252.

Ingber, D.E., and Folkman, J. (1988). Inhibition of angiogenesis through inhibition of collagen metabolism. Lab. Invest. *59*, 44–51.

Ingber, D.E., Madri, J.A., and Folkman, J. (1987). Endothelial growth factors and extracellular matrix regulate DNA synthesis through modulation of cell and nuclear expansion. In Vitro Cell. & Dev. Biol. *23*, 387–394.

Ingber, D.E., Prusty, D., Frangioni, J.V., Cragoe, E.J., Lechene, C., and Schwartz, M.A. (1990). Control of intracellular pH and growth by fibronectin in capillary endothelial cells. J. Cell Biol. *110*, 1803–1811.

Izzard, C.S., Radinsky, R., and Culp, L.A. (1986). Substratum contacts and cytoskeletal reorganization of BALB/c 3T3 cells on a cell-binding fragment and heparin-binding fragments of plasma fibronectin. Exp. Cell Res. *165*, 320–336.

Juliano, R.L., and Haskill, S. (1993). Signal transduction from the extracellular matrix. J. Cell Biol. *120*, 577–585.

Kornberg, L.J., Earp, H.S., Turner, C.E., Prockop, C., and Juliano, R.L., (1991). Signal transduction by integrins: Increased protein tyrosine phosphorylation caused by clustering of β_1 integrins. Proc. Natl. Acad. Sci. USA *88*, 8392–8396.

Kovary, K., and Bravo, R. (1991). The jun and fos protein families are both required for cell cycle progression in fibroblasts. Mol. Cell. Biol. *11*, 4466–4472.

Kruijer, W., Skelly, H., Botteri, F., Putten, H.v.d., Barber, J.R., Verma, I.M., and Leffert, H.L. (1986). Proto-oncogene expression in regenerating liver is simulated in cultures of primary adult rat hepatocytes. J. Biol. Chem. *261*, 7929–7933.

Lee, E.Y.-H., Parry, G., and Bissell, M.J. (1984). Modulation of secreted proteins of mouse mammary epithelial cells by the collagenous substrata. J. Cell Biol. *107*, 1589–1598.

Li, M.J., Aggeler, J., Farson, D.A., Hatier, C., Hassell, J., and Bissell, M.F. (1987). Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA *84*, 136–140.

MacPherson, I., and Montagnier, L. (1964). Agar suspension culture for selective assay of cells transformed by polyoma virus. Virology *23*, 291–294.

McNamee, H.P., and Ingber, D.E. (1993). Integrin-dependent activation of phosphoinositide kinases occurs within the focal adhesion complex. Mol. Biol. Cell *4*, 362a.

McNamee, H.P., Ingber, D.E., and Schwartz, M.A. (1993). Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. J. Cell Biol. *121*, 673–678.

Mooney, D.J., Hansen, L.K., Vacanti, J.P., Langer, R., Farmer, S.R., and Ingber, D.E. (1992a). Switching from differentiation to growth in hepatocytes: control by extracellular matrix. J. Cell. Phys. *151*, 497–505.

Mooney, D.J., Hansen, L.K., Vacanti, J.P., Langer, R., and Ingber, D.E. (1992b). Induction of hepatocyte differentiation by the extracellular matrix and an RGD-containing synthetic peptide. Mat. Res. Soc. Symp. Proc. *252*, 199–204.

Opas, M. (1989). Expression of the differentiated phenotype by epithelial cells in vitro is regulated by both biochemistry and mechanics of the substratum. Dev. Biol. *131*, 281–293.

Otsuka, H., and Moskowitz, M. (1975). Arrest of 3T3 cells in G1 phase in suspension culture. J. Cell. Physiol. *87*, 213–220.

Plopper, G., and Ingber, D.E. (1993). Rapid induction and isolation of focal adhesion complexes. Biochem. Biophys. Res. Commun. *193*, 571–578.

Rana, B., Xie, Y.-H., Bucher, N.L.R., and Farmer, S.R. (1991). Cell-ECM interactions regulate expression of immediate early growth gene in hepatocytes in culture. J. Cell Biol. *115*, 446a.

Ruoslahti, E., and Pierschbacher, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science *238*, 491–497.

Ryder, K., Lau, L.F., and Nathans, D. (1988). A gene activated by growth factors is related to the oncogene v-*jun*. Proc. Natl. Acad. Sci. USA. *85*, 1487–1491.

Schmidhauser, C., Bissell, M.J., Myers, C.A., and Casperson, G.F. (1990). Extracellular matrix and hormones transcriptionally regulate bovine beta-casein 5′ sequences in stably transfected mouse mammary cells. PNAS (USA) *87*, 9118–9122.

Schwartz, M.A. (1992). Transmembrane signalling by integrins. Trends Cell Biol. *2*, 304–308.

Schwartz, M.A., and Ingber, D.E. (1994). Integrating with integrins. Mol. Biol. Cell *5*, 389–393.

Schwartz, M.A., Lechene, C., and Ingber, D.E. (1991). Insoluble fibronectin activates the Na+/H+ antiporter by clustering and immobilizing integrin $\alpha 5\beta 1$, independent of cell shape. Proc. Natl. Acad. Sci. USA *88*, 7849–7853.

Schwartz, M.A., Rupp, E.E., Frangioni, J.V., and Lechene, C. (1990). Cytoplasmic pH and anchorage independent growth induced by v-Ki-ras, v-src, or polyoma middle T. Oncogene *5*, 55–58.

Shin, S., Freedman, V.H., Risser, R., and Pollack, R. (1975). Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage-independent growth in vitro. Proc. Natl. Acad. Sci. USA *72*, 4435–4439.

Sims, J., Karp, S., Ingber, D.E. (1992). Altering the cellular mechanical force balance results in integrated changes in cell, cytoskeletal, and nuclear shape. J. Cell Sci. *103*, 1215–1222.

Singhvi, R., Kumar, A., Lopez, G., Stephanopoulos, G.N., Wang, D.I.C., Whitesides, G.M., and Ingber, D.E. (1994). Engineering cell shape and function. Science *264*, 696–698.

Spiegelman, B.M., and Ginty, C.A. (1983). Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. Cell *35*, 657–666.

Stoker, M., O'Neill, C., Berryman, S., and Waxman, V. (1968). Anchorage and growth regulation in normal and virus transformed cells. Int. J. Cancer. *3*, 683–693.

Streeter, H.B., and Rees, D.A. (1987). Fibroblast adhesion to RGDS shows novel features compared with fibronectin. J. Cell Biol. *105*, 507–515.

Talhouk, R.S., Bissell, M.J., and Werb, Z. (1992). Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. J. Cell Biol. *118*, 1271–1282.

Tomomura, A., Sawada, N., Sattler, G.L., Kleinman, H.K., and Pitot, H.C. (1987). The control of DNA synthesis in primary cultures of hepatocytes from adult and young rats: interactions of extracellular matrix components, epidermal growth factor, and the cell cycle. J. Cell. Physiol. *130*, 221–227.

Wang, N., Butler, J.P., and Ingber, D.E. (1993). Mechanotransduction across the cell surface and through the cytoskeleton. Science *260*, 1124–1127.

Watt, F.M., Jordan, P.W., and O'Neill, C.H. (1988). Cell shape controls terminal differentiation of human epidermal keratinocytes. Proc. Natl. Acad. Sci. USA *85*, 5576–5580.

Werb, Z., Tremble, P.M., Behrendtsen, O., Crowley, E., and Damsky, C.H. (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. *109*, 877–889.

Woods, A., and Couchman, J.R. (1992a). Heparan sulfate proteoglycans and signalling in cell adhesion. Adv. Exp. Med. Biol. *313*, 87–96.

Woods, A., and Couchman, J.R. (1992b). Protein kinase C involvement in focal adhesion formation. J. Cell Sci. *101*, 277–290.