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Fibrillar Collagen Inhibits Arterial Smooth Muscle Proliferation through Regulation of Cdk2 Inhibitors

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Summary

Arterial smooth muscle cells (SMCs) are arrested in the G1 phase of the cell cycle on polymerized type I collagen fibrils, while monomer collagen supports SMC proliferation. Cyclin E–associated kinase and cyclin-dependent kinase 2 (cdk2) phosphorylation are inhibited on polymerized collagen, and levels of the cdk2 inhibitors $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ are increased compared with SMCs on monomer collagen. $p27^{Kip1}$ associates with the cyclin E–cdk2–$p21^{Cip1/Waf1}$ complex in SMCs on polymerized collagen. Monovalent blocking antibodies to $\alpha 2$ integrins, integrins that mediate adhesion to both forms of collagen, mimic these effects on monomer collagen. Furthermore, polymerized collagen rapidly suppresses p70 S6 kinase, a possible regulator of $p27^{Kip1}$. Thus, fibrillar collagen specifically regulates early integrin signaling that may lead to up-regulation of cdk2 inhibitors and inhibition of SMC proliferation.

Introduction

Regulation of cell proliferation is a key event in normal development, pathophysiological responses to injury, and tumorigenesis. A number of peptide growth factors are capable of stimulating proliferation of target cells that express specific cell surface receptors. However, the presence of the growth factor and its receptor is not necessarily sufficient to induce cell proliferation. The nature of the local environment, particularly the extracellular matrix and intercellular interactions, can dramatically regulate the response to a given growth factor (Martin and Sank, 1990; Sporn and Roberts, 1990).

A majority of cell–cell and cell–matrix interactions are mediated by specific membrane receptors of the integrin family of proteins (Hynes, 1992). Occupancy and clustering of integrins can activate intracellular signaling pathways (Hynes, 1992; Juliano and Haskill, 1993; Plopper et al., 1995) and induce transcription factors (Qwarnström et al., 1994; Rana et al., 1994) and subsequent gene expression (Juliano and Haskill, 1993). Many of the same pathways are stimulated by growth factors. It has been suggested that cell attachment and spreading are essential for traverse of cells through the G1 phase of the cell cycle (Folkman and Moscona, 1978; Guadagno et al., 1993; Hansen et al., 1994; Fang et al., 1996) and that this process may be mediated by integrins. The G1/S phase transition appears to be regulated by activation of cyclin-dependent kinase 2 (cdk2), which is complexed with cyclin A and with cyclin E (Sherr, 1993), together with proteins that bind to the cdk–cyclin complex and inhibit its activity (Hunter and Pines, 1994; Peter and Herskowitz, 1994; Sherr and Roberts, 1995). However, the effects of integrin signaling and cytoskeletal structure on the regulation of cyclins, cdks, and cdk inhibitors are poorly understood.

Vascular smooth muscle cells (SMCs) normally reside in the media of the artery, have a low proliferative index, and are surrounded by a meshwork of several extracellular matrix components, including collagen types I, III, and IV and laminin (Thyberg et al., 1990). However, in the process of atherogenesis, SMC proliferation is increased in the forming neointima and the innermost part of the underlying media (Rekhter and Gordon, 1995). Within lesions, SMCs also synthesize novel matrix components and together with macrophages degrade and modify the extracellular matrix (Thyberg et al., 1990; Galis et al., 1994). In the present study, we examine the effects of structurally distinct forms of type I collagen, monomer versus polymerized fibrils, and show that polymerized collagen regulates early integrin signaling distinct from monomer collagen and has a profound inhibitory effect on SMC proliferation and specific cell cycle regulatory molecules.

Results

Polymerized Collagen Suppresses DNA Synthesis and Decreases Cyclin E–Associated Kinase Activity and Cyclin A Expression

Our working hypothesis proposes that degradation or structural alteration of extracellular matrix is necessary for SMC proliferation in vivo. To investigate how the structure of type I collagen affects SMC proliferation, we prepared two forms, monomer collagen (coated on plastic in acetic acid) and polymerized collagen fibrils, and examined the effects of each on platelet-derived growth factor (PDGF)-BB- and fetal calf serum (FCS)-stimulated SMC proliferation. When SMCs are cultured on polymerized collagen, basal and growth factor–stimulated [^{3}H]thymidine incorporation into DNA of SMCs is more than 10-fold lower than on monomer collagen (Figure 1A) and is associated with a reduction in cell number 3 days after treatment with growth factors (Figure 1B). The inhibition of SMC DNA synthesis by polymerized collagen is not dependent on the concentration of collagen, since 0.1–2.0 mg/ml concentrations were similarly effective (data not shown). Cell cycle analysis of SMCs on polymerized collagen reveals that 24 hr after treatment with FCS only 8.5% of the cells are in the S phase fraction and 82.7% of the cells are arrested in G1. In contrast, on monomer collagen, 32.3% of the cells are in the S phase fraction 24 hr after treatment with FCS. This suppressive effect of polymerized

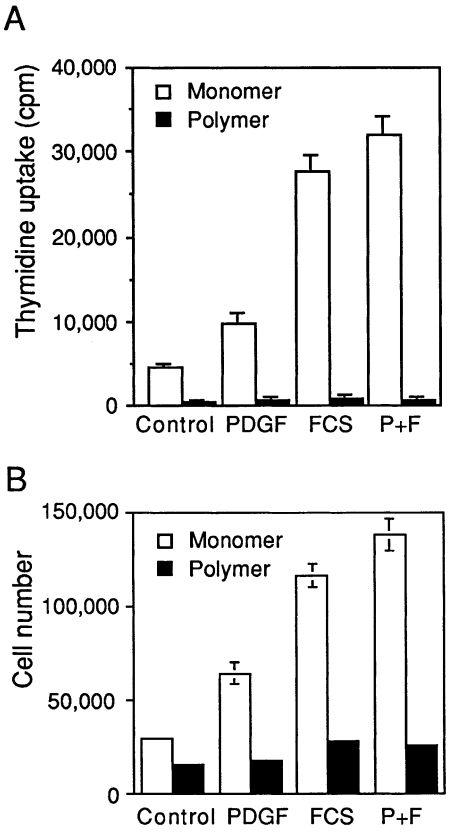

Figure 1. Polymerized Collagen Inhibits DNA Synthesis and Cell Proliferation

Human SMCs were synchronized in 1% PDS for 48 hr and were replated on either monomer or polymerized collagen. Vehicle (control), PDGF, fetal calf serum (FCS), or PDGF plus FCS (P+F) was added 24 hr after replating. (A) DNA synthesis ([^{3}H]thymidine incorporation into DNA at 20 hr after stimulation) and (B) cell number (3 days after stimulation) were determined. Each column represents mean ± SD (n = 3).

collagen is maintained for up to 48 hr in the presence of growth factors (data not shown), suggesting that the cell cycle is not delayed, but rather cells on polymerized collagen are arrested in G1.

Because polymerized collagen induced cell cycle arrest in G1, we examined whether polymerized collagen affects cyclin E– and cyclin A–associated kinase activities, known regulators of the G1/S transition (Sherr, 1993). On monomer collagen, either PDGF or FCS causes an increase in cyclin E–associated kinase activity and cyclin A protein (Figure 2). In contrast, polymerized collagen suppresses both basal and growth factor (PDGF or FCS) stimulated cyclin E–associated kinase activities without suppressing protein levels of cyclin E. Induction of cyclin A protein by growth factors is also abrogated by polymerized collagen, and PDGF or FCS fails to activate cyclin A–associated kinase (data not shown). Cyclin D1 protein, a major cyclin D species expressed in human SMCs, is also slightly suppressed on polymerized collagen. However, PDGF can increase the level of cyclin D1 on polymerized collagen comparable to that on monomer collagen (data not shown).

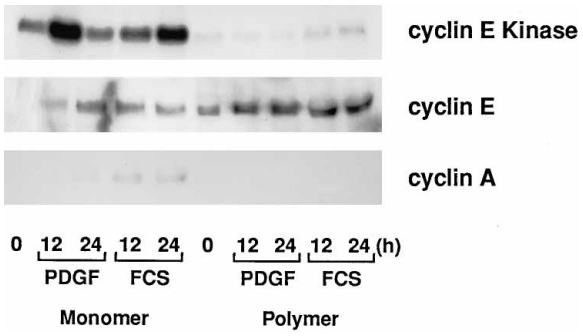

Figure 2. Polymerized Collagen Prevents the Accumulation of Cyclin E–Associated Kinase Activity in Human SMCs

Cyclin E complexes were immunoprecipitated from cell lysates prepared from SMCs cultured on monomer or polymerized collagen for 24 hr (0) and stimulated with PDGF or fetal calf serum (FCS) for an additional 12 or 24 hr. Kinase activity was measured using histone H1 as the substrate as described. Cell lysates were immunoblotted sequentially with antibodies to cyclin E and cyclin A.

Polymerized Collagen Suppression of Cyclin E–Cdk2 Kinase Activity Correlates with a Lack of Cdk2 Phosphorylation

To examine further the mechanism of suppression of cyclin E–associated kinase activity on polymerized collagen, we took advantage of the fact that attachment and spreading of quiescent human SMCs on monomer collagen in the absence of growth factors support significant levels of basal DNA synthesis. After plating SMCs on monomer collagen, [^{3}H]thymidine incorporation begins as early as 12–18 hr and is maximal at 24–30 hr (Figure 3A). In striking contrast, [^{3}H]thymidine incorporation is not observed when SMCs are cultured on polymerized collagen. Analysis of the cell cycle distribution revealed that 24 hr after plating, a significant proportion of the SMCs are in S phase on monomer collagen, whereas on polymerized collagen the SMCs are arrested in G1 (Figure 3B).

Examination of cell cycle molecules demonstrates that when human SMCs are plated on monomer collagen, cyclin E–associated kinase activity is increased by 3.5-fold at 12 hr, whereas on polymerized collagen, cyclin E–associated kinase is not activated at timepoints up to 24 hr (Figure 3C). In spite of the lack of activation of cyclin E–associated kinase on polymerized collagen, cyclin E protein is induced, and its level at each timepoint is slightly higher than on monomer collagen. Cyclin A expression follows the cyclin E–associated kinase activity and is induced on monomer collagen. In contrast, on polymerized collagen cyclin A induction and activation of its associated kinases do not occur.

Cdk2 protein levels are similar in cells cultured on monomer or polymerized collagen (Figure 3C). Furthermore, analysis of cdk2 in cyclin E immunoprecipitates shows no significant quantitative differences between cells on monomer versus polymerized collagen (Figure 4A and Table 1). On monomer collagen, a faster migrating form of cdk2 appears as early as 12 hr and predominates at 24 hr (Figure 3C). On polymerized collagen, only the slower migrating form of cdk2 is observed at timepoints up to 24 hr. The faster migrating form of

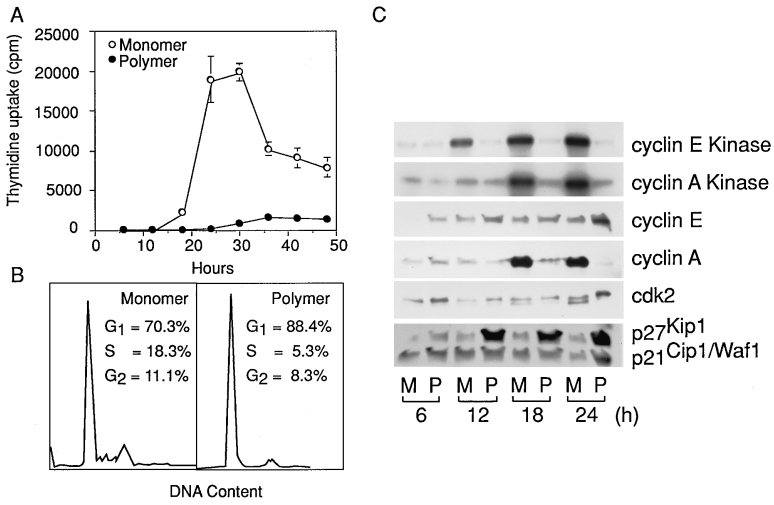

Figure 3. Up-Regulation of $p27^{Kip1}$ and $p21^{Cip1/Waf1}$, and Lack of Cdk2 Phosphorylation in SMCs Arrested in G1 on Polymerized Collagen

(A) Kinetics of basal DNA synthesis ([^{3}H]thymidine incorporation into DNA at the indicated times after plating, mean ± SD, n = 4); (B) cell cycle distribution for cells plated for 24 hr; and (C) cyclin E– and cyclin A–associated kinase activities, cdk2 phosphorylation, and levels of cdk inhibitors were determined for SMCs following plating on monomer (M) or polymerized (P) collagen. Cyclin E and cyclin A activities were determined from immunoprecipitated complexes. Cell lysates were analyzed by Western blot analysis sequentially with antibodies to cyclins, cdk2, and the cdk inhibitors.

cdk2 is phosphorylated on Thr-160 (activating) (Gu et al., 1992). We therefore examined the cdk-activating kinase (CAK) activity that is responsible for its phosphorylation (Morgan, 1995). CAK activity was measured by incubating bacterially expressed glutathione S-transferase (GST)–cdk2 and H6–cyclin A with lysates obtained from cells cultured on monomer versus polymerized collagen for 0–24 hr. When 0.5 μg of exogenous cdk2 and cyclin A were incubated with 50 μg of cell lysates, exogenous cdk2 was equally activated by either lysate (data not shown), indicating that CAK is abundantly expressed and thus not significantly regulated by polymerized collagen. To evaluate whether inactivation of cdk2 by polymerized collagen could be the result of inhibitory phosphorylation on Tyr-15 (Morgan, 1995), cyclin E–cdk2 immunoprecipitates from cells on polymerized collagen were incubated with mouse cdc25M2, the tyrosine phosphatase responsible for dephosphorylation of cdk2 on Tyr-15. Treatment with this phosphatase did not increase the activity of cyclin E–cdk2 from cells on polymerized collagen, although it slightly increased the activity from cells on monomer collagen (data not shown).

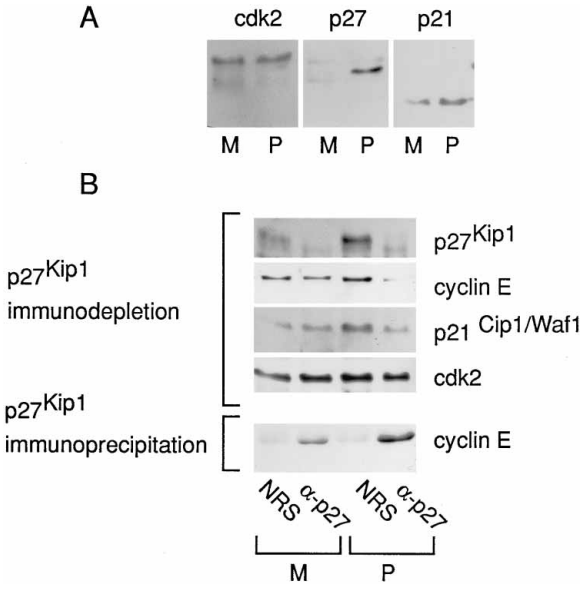

Figure 4. Increased Association of $p27^{Kip1}$ with Cyclin E–Cdk2 Complexes in Human SMCs Cultured on Polymerized Collagen

Quiescent human SMCs were cultured on monomer (M) or polymerized (P) collagen for 12 hr and the following levels determined by immunoblotting: (A) $p27^{Kip1}$, $p21^{Cip1/Waf1}$, and cdk2 bound to cyclin E complexes determined after immunoprecipitation with anti–cyclin E; (B) the levels of $p27^{Kip1}$, cyclin E, $p21^{Cip1/Waf1}$, and cdk2 remaining in the supernatant following immunoprecipitation with anti-$p27^{Kip1}$ antibodies ($p27^{Kip1}$ immunodepletion), and cyclin E association with $p27^{Kip1}$ by immunoprecipitation with anti-$p27^{Kip1}$ antiserum (represented as α-p27), or normal rabbit serum (NRS).

Polymerized Collagen Up-Regulates the Cdk2 Inhibitors $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ and Their Association with Cyclin E–Cdk2

To investigate how polymerized collagen inhibits cyclin E–cdk2 activity and cdk2 phosphorylation, we examined the regulation of two candidates for cdk2 inhibition, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (Hunter and Pines, 1994; Peter and Herskowitz, 1994; Sherr and Roberts, 1995). Immunoblot analysis showed that $p27^{Kip1}$ protein is up-regulated as early as 6 hr after plating SMCs on polymerized collagen (Figure 3C). $p27^{Kip1}$ up-regulation and suppression of cdk2 kinase activity were also observed in cells cultured on 0.1 mg/ml polymerized collagen. The result was not

Table 1. Quantitation of the Levels of Cdk2, $p27^{Kip1}$, and $p21^{Cip1/Waf1}$ Complexed with Cyclin E

Collagen	Time (hr)	Cdk2	$p27^{Kip1}$	$p21^{Cip1/Waf1}$
Monomer	12	81	27	83
Polymer	12	113	111	119
Monomer	24	100	28	95
Polymer	24	95	85	120

Quiescent SMCs were replated on monomer or polymerized collagen and cultured for 12 or 24 hr. Cyclin E was immunoprecipitated from 200 μg of cell lysates, and cdk2 and the ckd inhibitors were quantitated by immunoblotting using recombinant cdk2, $p27^{Kip1}$, and $p21^{Cip1/Waf1}$ proteins as standards. All values are expressed as fmols.

dependent on the concentration of collagen (data not shown). Quantitative immunoblot analysis of $p27^{Kip1}$, using recombinant protein as a standard, revealed that the level of $p27^{Kip1}$ on polymerized collagen is 3-fold higher than on monomer collagen at both 12 and 24 hr after plating. $p21^{Cip1/Waf1}$ levels are also modestly increased on polymerized collagen (1.5-fold at 12 hr and 1.8-fold at 24 hr) as compared with monomer collagen (Figure 3C; data not shown). No significant difference in the levels of cdk2 inhibitors was observed between cells immediately after trypsinization (0 hr) and cells cultured on monomer collagen for 6 hr (data not shown).

Consistent with the increase in $p27^{Kip1}$ observed in lysates from cells on polymerized collagen, the amount of the inhibitor in cyclin E–immunoprecipitates from these cells 12 hr after plating is significantly greater than on monomer collagen (Figure 4A). In contrast, cdk2 and $p21^{Cip1/Waf1}$ protein levels are comparable in the same cyclin E–immunoprecipitates from cells cultured on monomer and polymerized collagen. Immunoprecipitation using an anti-$p27^{Kip1}$ antibody indicates that larger amounts of cyclin E molecules are present in $p27^{Kip1}$ immunoprecipitates on polymerized collagen compared with monomer collagen (Figure 4B). Furthermore, almost all of the cyclin E in lysates of SMCs on polymerized collagen is coimmunodepleted with $p27^{Kip1}$. In contrast, on monomer collagen, large proportions of cyclin E are not immunoprecipitated by the $p27^{Kip1}$ antibody. On polymerized collagen, significant amounts of $p21^{Cip1/Waf1}$ are also coimmunodepleted with $p27^{Kip1}$. Cdk2 is abundantly expressed in SMCs either on monomer or on polymerized collagen. Thus, cdk2 is not immunodepleted either by anti-$p27^{Kip1}$ (Figure 4B) or by anti–cyclin E antibodies (data not shown).

To evaluate further the molar ratio between cdk2 and cdk inhibitors complexed with cyclin E, immunoprecipitation with anti–cyclin E antibodies was followed by quantitative immunoblot analysis using recombinant GST–cdk2, $p27^{Kip1}$, and $p21^{Cip1/Waf1}$ as standards (Table 1). After 12 hr on monomer collagen, the ratio of $p21^{Cip1/Waf1}$/cdk2 is about 1.0, while the ratio of $p27^{Kip1}$/cdk2 is 0.33. In contrast, on polymerized collagen, the ratio of $p27^{Kip1}$/cdk2 is increased to about 1.0 and $p21^{Cip1/Waf1}$/cdk2 is maintained at about 1.0. Similar molar ratios between inhibitors and cdk2 are maintained up to 24 hr after plating.

Formation of Focal Adhesion Sites Is Decreased and Cell Spreading Is Delayed on Polymerized Collagen

We next investigated whether alterations in the interaction of the cells with collagen are involved in the regulation of $p27^{Kip1}$ levels by polymerized collagen. As previously described (Skinner et al., 1994), the $\alpha2\beta1$ integrin is the principal collagen receptor expressed in cultured human arterial SMCs and is required for their migration. When SMCs are cultured for 24 hr on polymerized collagen, $\alpha2$ and $\beta1$ integrin expression is not dramatically regulated, while $\alpha3$ expression is significantly decreased (data not shown). An alternate collagen receptor, $\alpha v\beta3$, is not detected in these human SMCs (Skinner et al., 1994), and levels of $\alpha v\beta3$ are not altered on monomer or polymerized collagen (data not shown). The attachment efficiency of SMCs on both monomer and polymerized collagen is similar, and SMC attachment to either form of collagen is inhibited by a blocking (P1H5), but not by a nonblocking (P1H6), anti-$\alpha2$ monoclonal antibody (Table 2). Similar results are obtained with another blocking antibody to $\alpha2$ (P1E6) and a blocking antibody to $\beta1$ (P4C10) integrin (data not shown).

Table 2. $\alpha2$ Integrin-Mediated Attachment of Human SMCs to Monomer and Polymerized Collagen

	Percentage of Attached Cells		
Collagen	15 min	30 min	60 min
Monomer			
Control	52.4 ± 0.59	67.4 ± 3.4	79.0 ± 1.4
P1H5	13.5 ± 1.4	36.6 ± 4.9	53.0 ± 1.2
P1H6	53.2 ± 4.6	60.4 ± 2.3	80.9 ± 1.7
Polymer			
Control	59.7 ± 3.2	73.4 ± 4.5	79.8 ± 1.4
P1H5	15.6 ± 0.91	38.2 ± 0.26	51.4 ± 1.8
P1H6	56.3 ± 4.6	69.1 ± 2.2	81.9 ± 0.6

SMCs incubated for 2 days in 1% PDS were suspended with diluted trypsin, washed with medium containing 1% PDS, and treated for 30 min with blocking (P1H5) and nonblocking (P1H6) anti-$\alpha2$ integrin antibody. After incubation, the cells were cultured on monomer or polymerized collagen and nonattached cells were counted after 15, 30, or 60 min. Each value shows the percentage of attached cells (mean ± SD, n = 3).

Although equivalent attachment is observed, SMC spreading is delayed on polymerized collagen as compared with monomer collagen (Figure 5A). This suggests that organization of focal adhesion components may be delayed, suppressed, or altered. To address these possibilities, focal adhesion formation was examined by fluorescence immunocytochemistry and confocal microscopy. The number of focal adhesion sites, as determined by vinculin staining, is markedly decreased in cells cultured for 3 hr on polymerized collagen, compared with cells on monomer collagen (Figure 5B). The decrease in focal adhesion formation on polymerized collagen is associated with a decrease in formation of actin stress fibers (Figure 5B). Such changes in cytoskeletal organization are relatively specific to actin because microtubule organization (determined by α-tubulin immunostaining) is comparable on either collagen preparation (Figure 5B).

Anti-$\alpha2$ Integrin Fab Fragments Up-Regulate the Cdk2 Inhibitors

To determine whether integrin-mediated formation of focal adhesion sites and cell spreading regulate cyclin E–associated kinase activity and the level of cdk2 inhibitors, we used the blocking anti-$\alpha2$ integrin antibody P1H5 to inhibit the formation of focal adhesion sites in cells on monomer collagen. In contrast with treatment with nonblocking P1H6 Fab fragments, incubation of SMCs with P1H5 Fab fragments inhibits formation of focal adhesion sites (determined by vinculin staining) and actin stress fibers, with no observable effects on microtubule organization (Figure 6A). This effect of P1H5 Fab fragment is similar to the response observed on polymerized collagen (Figure 5B). The effects of the blocking anti-$\alpha2$ integrin antibody are not observed when intact immunoglobulin G (IgG) is used (data not

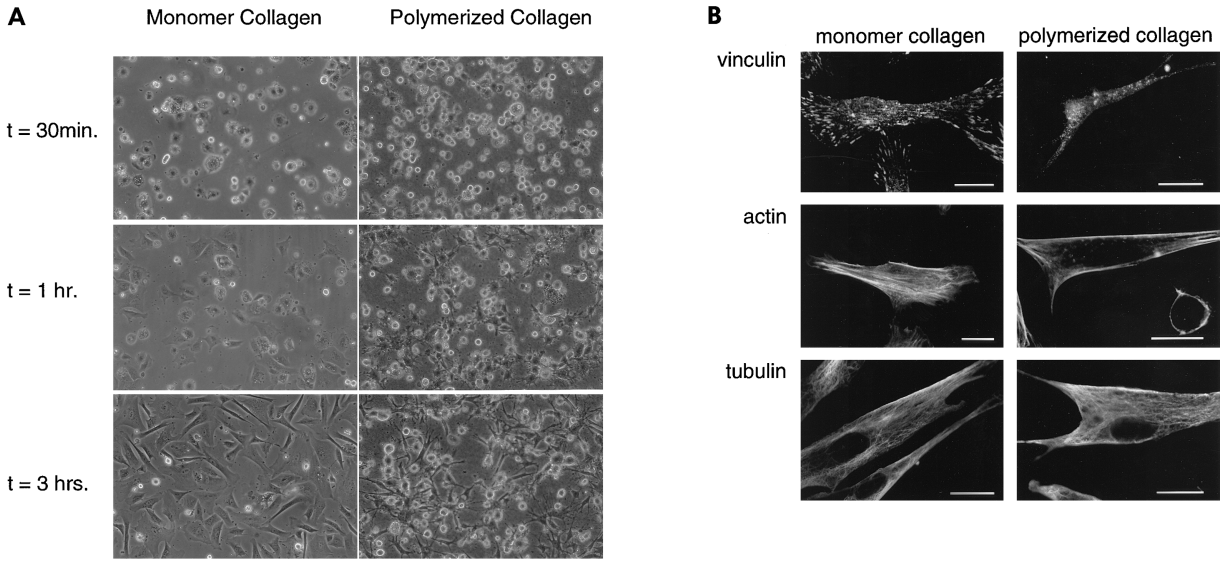

Figure 5. Cell Spreading and Focal Adhesion Formation Is Suppressed on Polymerized Collagen

(A) Cell shape was evaluated using phase-contrast microscopy, and (B) focal adhesion complexes and actin stress fibers were determined as shown by vinculin, actin, and tubulin staining and evaluated by confocal microscopy. Each bar represents 25 μm.

shown). The lack of an effect of intact IgG was not due to a difference in access of IgG to the integrin, since comparable levels of IgG were associated with cells on monomer collagen as observed with suspended cells (data not shown). When the cells are incubated on monomer collagen with blocking P1H5 Fab fragments, cyclin E–associated kinase activation is inhibited 12 hr after plating (Figure 6B), while the nonblocking anti-α2 integrin P1H6 Fab has no effect. Importantly, cells treated with P1H5 Fab fragments, but not with intact IgG, have significantly higher $p27^{Kip1}$ levels compared with control or P1H6-treated cells (Figure 6B and data not shown).

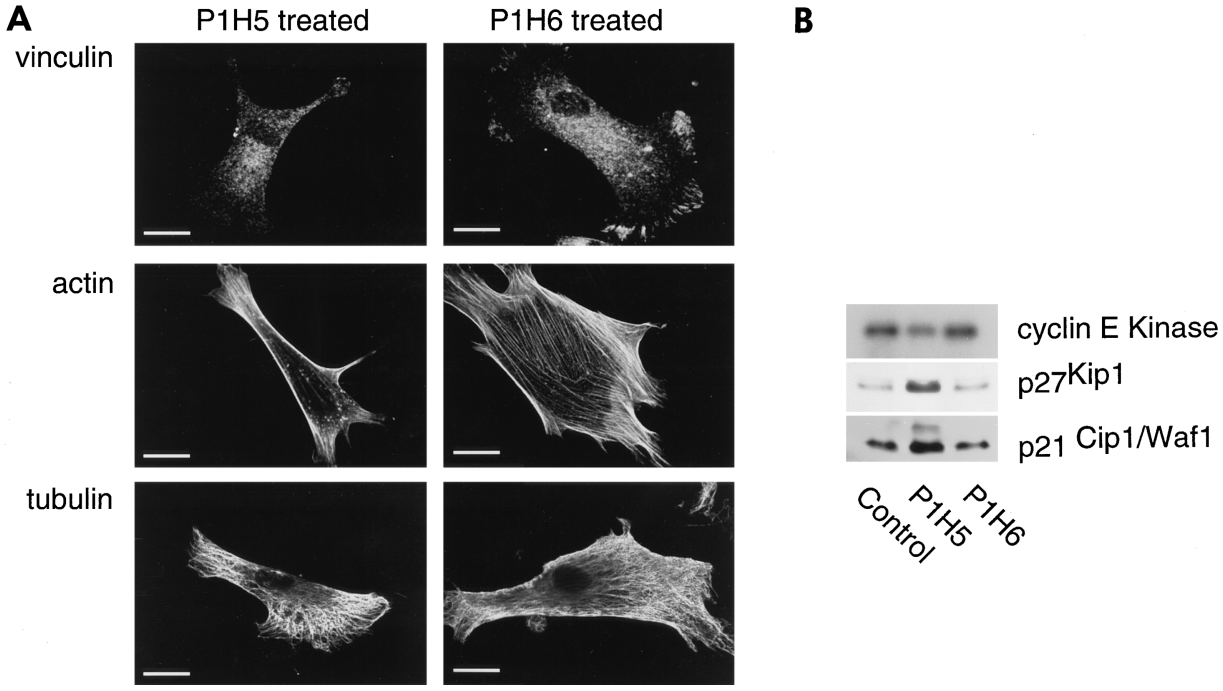

Figure 6. Blocking Fab Fragments against α2 Integrin Inhibit Focal Adhesion Site Formation, Up-Regulate Cdk2 Inhibitors, and Abrogate Activation of Cyclin E–Associated Kinase

For SMCs plated on monomer collagen following treatment with Fab fragments of blocking (P1H5) or nonblocking (P1H6) anti-α2 integrin antibodies (5 μg/200,000 cells), the following were evaluated: (A) focal adhesion formation 3 hr after plating as determined by staining for vinculin, tubulin, and actin staining (each bar represents 25 μm); and (B) cyclin E–associated kinase activity and cdk2 inhibitors levels in cell lysates obtained 12 hr after plating.

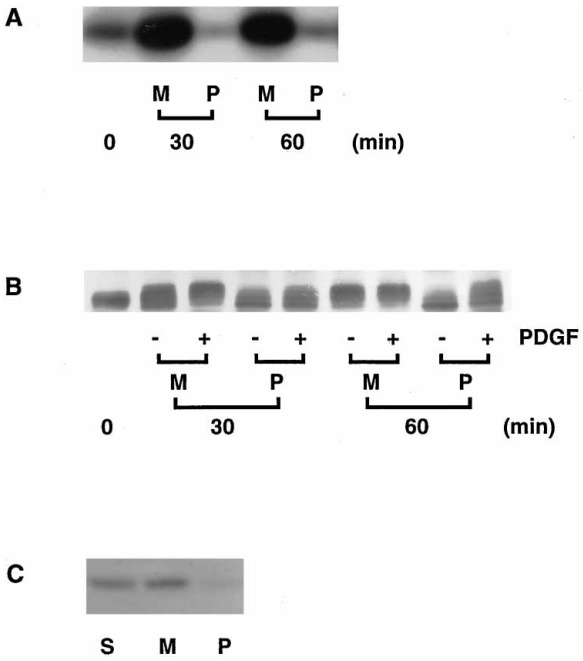

Figure 7. Polymerized Collagen Regulation of p70^{S6K}

Quiescent SMCs were cultured on monomer (M) versus polymerized collagen (P) for the indicated times or kept in suspension for 30 min (S). In some experiments, PDGF (1 nM) was included when the cells were plated. Cell lysates were prepared, and p70^{S6K} was immunoprecipitated using an anti-p70^{S6K} antibody. p70^{S6K} activity (A and C) was monitored by determining the levels of phosphorylation of the ribosomal protein S6. The phosphorylation status of p70^{S6K} (B) was analyzed by immunoblotting following immunoprecipitation. Slower migrating bands correspond to phosphorylated forms of p70^{S6K}.

p21$^{Cip1/Waf1}$ is also modestly increased in cells treated with P1H5 Fab fragments.

Polymerized Collagen Rapidly and Profoundly Suppresses p70 S6 Kinase Activities

An increasing body of literature shows that levels of p27^{Kip1} and cdk2 activity may be regulated by upstream activation of p70 S6 kinase (p70^{S6K}; Morice et al., 1993; Albers et al., 1993; Nourse et al., 1994). Thus, we asked whether p70^{S6K} activation is altered on polymerized collagen. As shown in Figure 7A, when SMCs are plated on polymerized collagen, p70^{S6K} activity is completely suppressed within 30 min, in dramatic contrast with cells on monomer collagen, where p70^{S6K} is further activated. Immunoblot analysis following immunoprecipitation of p70^{S6K} suggests that its phosphorylation is inhibited by polymerized collagen. p70^{S6K} molecules with reduced mobility in SDS electrophoresis corresponding to phosphorylated forms are decreased in SMCs on polymerized collagen (Figure 7B). p85^{S6K} is also immunoprecipitated by the same antibody and is similarly regulated by polymerized collagen (data not shown). Although PDGF is able to increase both the kinase activity (data not shown) and phosphorylation of p70^{S6K} (Figure 7B) on polymerized collagen, the maximal activity on polymerized collagen is much lower than the basal level on monomer collagen. Of note, the levels of p70^{S6K} activity are not significantly different in cells kept in suspension or plated on monomer collagen for 30 min (Figure 7C). Furthermore, phosphorylation of p70^{S6K} is only slightly inhibited by treatment of SMCs on monomer collagen with cytochalasin D (10 μM), where formation of actin stress fibers and cell spreading are inhibited (data not shown). In contrast with the modulation of p70^{S6K} by polymerized collagen, MAP kinase kinase and MAP kinase activities (p42 and p44) show no significant differences 0.5–6 hr after plating SMCs on monomer or polymerized collagen (data not shown).

Discussion

Fibrillar Collagen Inhibits Human Arterial SMC Proliferation by Inactivation of Cyclin E–Cdk2 Kinase Activity

Adhesion and spreading of normal diploid cells are prerequisite for proliferation and for cells to respond to growth factors (Folkman and Moscona, 1978). However, adhesion of fibroblasts and SMCs to type I fibrillar collagen results in a dramatic reduction in cell proliferation as compared with growth on tissue culture plastic (Schor, 1980; Rhudy and McPherson, 1988). This study identifies some of the molecular mediators responsible for SMC arrest in G1 when plated on polymerized collagen but not on monomer collagen.

It has been proposed that cdk activities control the G1/S transition in mammalian cells. Cyclin E and its associated kinase, cdk2, accumulate in late G1 (Sherr, 1993), suggesting a role for this cyclin in regulating the transition to S phase. Although polymerized collagen blocks the activation of cyclin E–associated kinase, it does not inhibit accumulation of cyclin E or its association with cdk2. However, cdk2 phosphorylation is significantly reduced by polymerized collagen, and accumulation of cyclin A is strongly inhibited. The phosphorylation of cdk2 on Thr-160 is required for kinase activity (Gu et al., 1992), and is catalyzed by the upstream kinase, CAK, which consists of cdk7 and its regulatory partner, cyclin H (Morgan, 1995). The lack of regulation of CAK in the present study agrees with the concept that CAK activity is expressed throughout the cell cycle and is not rate limiting (Morgan, 1995). Together, these results suggest that inhibition of active cyclin E–associated kinase complexes, possibly together with regulation of cyclin A expression, are the likely targets in prevention of DNA synthesis in SMCs on polymerized collagen.

Polymerized Collagen Shifts the Balance of Cdk2 Inhibitors in Cyclin E–Cdk2 Complexes toward Inhibition

A family of cyclin-dependent kinase inhibitors plays a major role in the cell cycle machinery (Hunter and Pines, 1994; Peter and Herskowitz, 1994; Sherr and Roberts, 1995). Two molecules, p21$^{Cip1/Waf1}$ and p27^{Kip1}, directly inhibit cdk2 activity and prevent its phosphorylation on Thr-160 in vitro (Aprelikova et al., 1995). p27^{Kip1} has recently been shown to mediate cell cycle arrest in response to transforming growth factor β (Koff et al., 1993), rapamycin (Nourse et al., 1994), and cAMP (Kato et al., 1994). On the other hand, p21$^{Cip1/Waf1}$ appears to be involved in radiation-induced cell cycle arrest (Dulić et al., 1994; Brugarolas et al., 1995).

In this study of human SMCs on polymerized collagen, $p27^{Kip1}$ protein is rapidly up-regulated and its induction precedes suppression of cyclin E–associated kinase activity. $p21^{Cip1/Waf1}$ levels are also modestly increased. Our data suggests that $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ are involved in inactivation of the cyclin E–cdk2 complex in SMCs on polymerized collagen. On monomer collagen, cyclin E complexes with cdk2, which leads to CAK-induced phosphorylation of cdk2 as early as 12 hr after plating. $p21^{Cip1/Waf1}$ also associates with this complex as early as 12 hr, but appears incapable of suppressing CAK-induced cdk2 phosphorylation and cdk2 activity. It has been shown that cyclin–cdk–$p21^{Cip1/Waf1}$ complexes retain activity with a low ratio of inhibitor to cdk (Zhang et al., 1994), conditions observed in this study on monomer collagen. In contrast, on polymerized collagen, $p27^{Kip1}$ is induced simultaneously with cyclin E, and both $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ appear to assemble with cyclin E–cdk2, resulting in a high ratio of inhibitors to cdk2. This complex formation occurs as early as 12 hr and, thus, may be responsible for preventing the CAK-induced cdk2 phosphorylation on polymerized collagen.

$p70^{S6K}$ as a Possible Target for Polymerized Collagen-Mediated Cell Cycle Regulation

Polymerized collagen suppresses a specific signaling pathway, $p70^{S6K}$, which has been shown to be associated with mitogenesis (Lane et al., 1993). Activation of $p70^{S6K}$ is mediated by a signaling pathway distinct from that of the serine/threonine kinases p42/p44 MAP kinase and $p90^{rsk}$ (Ballou et al., 1991). Accordingly, although polymerized collagen inhibits $p70^{S6K}$ activity, it has no effect on MAP kinase kinase and MAP kinase activities. Similar to polymerized collagen, rapamycin is a potent inhibitor of mitogenesis and a selective suppressor of $p70^{S6K}$ (Chung et al., 1992; Graves et al., 1995). Activation of $p70^{S6K}$ in human SMCs on monomer collagen is completely inhibited by rapamycin (data not shown). This rapamycin-sensitive pathway appears to be essential for up-regulation of $p27^{Kip1}$, which leads to inhibition of cyclin–cdk complexes (Morice et al., 1993; Albers et al., 1993; Nourse et al., 1994). Rapamycin (10 nM) also completely inhibits PDGF-stimulated DNA synthesis, suppresses cyclin E–associated kinase activity, and induces $p27^{Kip1}$ in human SMCs on monomer collagen (data not shown). Thus, there are marked similarities between the effects of polymerized collagen and rapamycin on signaling pathways that lead to cell cycle regulation.

Polymerized collagen-mediated inactivation of $p70^{S6K}$ is associated with rapid dephosphorylation of the enzyme, raising the possibility that polymerized collagen may regulate a phosphatase upstream of $p70^{S6K}$. Preliminary observations show that the effect of polymerized collagen on $p70^{S6K}$ dephosphorylation is blocked by a protein phosphatase inhibitor calyculin A (data not shown). Thus, a protein phosphatase may mediate the action of polymerized collagen.

Integrin-Mediated Suppression of Cyclin E–Cdk2 Activity

The attachment efficiency of human SMCs to monomer or polymerized collagen is similar and is mediated primarily by $\alpha2\beta1$ integrins. We have demonstrated that suppression of cyclin E–cdk2 activity and induction of cdk2 inhibitors by polymerized collagen is mimicked by monovalent Fab fragments of a blocking antibody to $\alpha2$ integrin, but not by Fab fragments of a nonblocking antibody. The intact divalent form of the same blocking IgG fails to mimic the effects. Dependence of antibody blocking on valency has also been shown for anti-$\beta1$ integrin antibodies in which monovalent Fab fragments inhibited collagen-induced increases in 1,2-diacylglycerol formation (DAG), while divalent IgG enhanced the increases in DAG (Cybulsky et al., 1993). Coincident with suppression of cyclin E–cdk2 activity and increase in cdk inhibitors by Fab fragments of the blocking antibody to $\alpha2$ integrin is an apparent decrease in focal adhesion sites and actin stress fibers with no effect on microtubule organization, characteristics also observed in SMCs on polymerized collagen. Taken together, these data suggest that integrin-mediated focal adhesion formation can regulate both cyclin E–associated kinase activity and cdk2 inhibitor levels.

Cell Shape–Dependent and Cell Shape–Independent Effects of Polymerized Collagen

The level of DNA synthesis in normal diploid cells is tightly coupled to cell attachment, shape, and spreading (Folkman and Moscona, 1978; Hansen et al., 1994), which can be controlled either by substratum adhesiveness or by crowding of neighboring cells. As cells are brought from an extremely flat shape toward a spheroidal shape, fewer cells synthesize DNA. Two studies recently demonstrated that suspended cells fail to activate cyclin E–cdk2 kinase activity and are linked to increased association of the cdk inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (Fang et al., 1996; Zhu et al., 1996). In our studies, SMC density and attachment are similar on both monomer and polymerized collagen. However, on polymerized collagen the cells spread less well and have fewer focal adhesions. The delay in cell spreading on polymerized collagen is associated with induction of cdk2 inhibitors and suppression of cyclin E–associated kinase activity. These data do not distinguish whether cell shape "controls" up-regulation of the cdk inhibitors or whether lack of focal adhesion sites (possible inhibition of integrin clustering) is the regulating factor.

A likely molecular basis for focal adhesion site regulation of cdk inhibitor levels could be altered recruitment of signaling and cytoskeletal molecules to sites of integrin binding. A hierarchy of transmembrane actions has been identified in human diploid fibroblasts in which signaling molecules localize to cell–matrix contact sites after integrin aggregation in the absence of occupancy (Miyamato et al., 1995). However, recruitment of cytoskeletal molecules to focal adhesion sites is extremely limited without occupancy and associated phosphorylation. A decrease in focal adhesion sites and actin stress fibers was observed in SMCs cultured on polymerized collagen, or on monomer collagen when treated with anti-integrin Fab fragments. It has been suggested that many of the signaling molecules induced by integrins and growth factors may function on insoluble cytoskeletal scaffolds (Plopper et al., 1995). These cytoskeletal platforms are probably very different in cells on monomer versus polymerized collagen.

The ability of polymerized collagen to inhibit $p70^{S6K}$ implies that its regulation of cdk inhibitors is not solely an indirect effect of cell shape. Disruption of actin filaments by cell suspension or by treatment of the cells on monomer collagen with cytochalasin D does not markedly inhibit $p70^{S6K}$ activity. Since polymerized collagen suppresses $p70^{S6K}$ activity to levels below those of suspended cells, this suppression appears to be an active process. Thus, it seems unlikely that the effects of polymerized collagen can be explained solely by a lack of integrin signaling and formation of focal adhesions. Furthermore, SMCs appear to require integrin-mediated signaling (either cell–cell or cell–matrix interactions) for their survival. When human SMCs are kept in suspension and cell–cell interaction is prevented, more than 50% become apoptotic within 24 hr (data not shown), whereas no apoptosis is observed in SMCs on polymerized collagen. Thus, polymerized collagen appears to induce integrin signaling distinct from monomer collagen dependent upon physical characteristics (rather than chemical), which may lead to up-regulation of cdk2 inhibitors and prevent SMC proliferation.

In Vivo Implications of Fibrillar Collagen-Mediated Suppression of Cyclin E–Cdk2 Activity

Adhesion-dependent signaling provides the basis for highly localized signals from the matrix, which may control patterns of morphogenesis and growth (Adams and Watt, 1993; Gumbiner, 1996). Moreover, signals generated by adhesion receptors can regulate signal transduction pathways stimulated by locally released growth factors (Martin and Sank, 1990; Sporn and Roberts, 1990). The capacity of polymerized collagen to inhibit cell proliferation has been appreciated for some time (Schor, 1980; Martin and Sank, 1990) and is consistent with a low proliferative index of SMCs in the media of the normal artery wall (Rekhter and Gordon, 1995). Our findings provide a molecular basis for this observation by demonstrating regulation of cyclin E–cdk2 activity and cdk2 inhibitors. Our studies further predict that the capacity of SMCs to respond to mitogenic stimuli in intact arteries versus sites of inflammation and atherogenesis, where the state of the matrix is perturbed, may be highly regulated by changes in the extracellular matrix.

Experimental Procedures

Growth Factors and Antibodies

Recombinant human PDGF-BB was provided by F. Hoffmann–La Roche (Basel, Switzerland). Polyclonal antisera for human cyclin A, cyclin E, and $p27^{Kip1}$ were generated in the laboratory of one of the authors (J. M. R.). The following antibodies were generously provided as indicated or purchased: monoclonal antibodies for human cyclin A (BF683) and cyclin E (HE172 and HE12) provided by E. Harlow and N. Dyson (Massachusetts General Hospital Cancer Center, Charlestown, MA); polyclonal antibody for human cdk2 (Santa Cruz Biotechnology Incorporated, Santa Cruz, CA); monoclonal antibody for PSTAIR sequence provided by M. Yamashita (Hokkaido University, Sapporo, Japan); monoclonal antibody for human $p21^{Cip1/Waf1}$ (Oncogene Science, Manhasset, NY); monoclonal antibodies directed against $\alpha 2$ (P1H5, P1H6, and P1E6), $\alpha 3$ (P1B5), and $\beta 1$ (P4C10) integrin subunits provided by W. G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA); monoclonal antibody specific for $\alpha 1$ integrin (TS2/7.1.1; hybridoma cells from American Tissue Culture Collection, Rockville, MD); monoclonal antibody detecting $\alpha v\beta 3$ integrins provided by M. Ginsberg (Scripps Research Institute, La Jolla, CA); monoclonal antibody for chicken vinculin (Calbiochem-Novabiochem Corp., La Jolla, CA); monoclonal antibody for α-tubulin (Amersham, Buckinghamshire, England); rabbit polyclonal antibody against $p70^{S6K}$ provided by E. G. Krebs (University of Washington, Seattle, WA). Fab fragments of monoclonal antibodies against $\alpha 2$ integrin receptor were prepared by papain cleavage of protein G–Sepharose-bound IgG (Coulter and Harris, 1983). Purified IgG and Fab fragments of P1H5 were titrated in the Boyden Chamber chemotaxis assay with the SMCs to determine effective blocking concentrations (Skinner et al., 1994).

Recombinant Proteins

Recombinant proteins of H6–human $p27^{Kip1}$ and H6–human $p21^{Cip1/Waf1}$ were prepared in the laboratory of one of the authors (J. M. R.). Expression vectors and recombinant proteins for H6–human cyclin A, GST–human cdk2, and GST–mouse cdc25M2 expressing cDNA were provided by R. Y. C. Poon and T. Hunter (The Salk Institute, San Diego, CA).

Cell Cultures

Human newborn (13 days) arterial SMCs were obtained from the thoracic aorta as described previously (Bornfeldt et al., 1994). Cell viability was checked by fluorescein diacetate–propidium iodine staining (Jones and Senft, 1985) and was always greater than 95%. All experiments were repeated at least twice.

Collagen Matrices

SMCs were cultured on the surface of the collagen preparations. Polymerized collagen fibrils (1.0–2.0 mg/ml final concentration) were prepared by neutralizing the collagen solution (Vitrogen 100, Celtrix, Santa Clara, CA) with 1/6 volume of 7× Dulbecco's modified Eagle's medium (DMEM) concentrate and diluting to a final volume with 1× DMEM to which human plasma-derived serum (PDS) was added at a final concentration of 1.0%. Gels formed following incubation of this solution (0.15 ml/cm^2) at 37°C for a minimum of 1 hr. To coat with monomer collagen (Vitrogen 100), dishes were soaked in 0.5% acetic acid for 20 min at 60°C, rinsed with distilled water, and incubated with 0.1 mg/ml of collagen solution in 0.1 M acetic acid for at least 3 hr at room temperature and then washed and stored in DMEM.

Measurement of DNA Synthesis and Proliferation

DNA synthesis of SMCs was evaluated by [^{3}H]thymidine incorporation assays. Cells, which had been cultured in 1% PDS/DMEM for 2 days, were plated in 1% PDS/DMEM onto 24-well trays coated with monomer or polymerized collagen. Cells were labeled with 2 μCi/ml [^{3}H]thymidine (Amersham) for the last 2 hr. Growth factors were added 24 hr after the cells were plated onto the collagen preparations. To harvest cultures on polymerized collagen, they were digested with 2.5–5 mg/ml type I collagenase in DMEM for 30–60 min until the cells dispersed completely; for cultures on monomer collagen, cells were washed twice with phosphate-buffered saline (PBS: 145 mM NaCl, 5 mM KCl, 10 mM sodium phosphate [pH 7.4]) and digested with 0.01% trypsin, 0.1 mM EDTA. Dispersed cells were washed once with PBS, precipitated with 10% trichloroacetic acid (TCA), transferred to glass fiber filters (Ahlstrom, Mt. Holly Spring, PA), and washed twice with 5% TCA, and then radioactivity was measured. Cell proliferation was measured by determining cell number in a cell counter (Coulter) following fixation in Holley's fixative (3.7% formaldehyde, 86 mM NaCl, 106 mM Na_2SO_4).

Measurement of Cyclin-Dependent Kinase and CAK Activity

Cells were quickly rinsed with PBS and sonicated in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 5 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell lysates were clarified by centrifugation at 27,000 × g for 30 min, and aliquots were analyzed for lactate dehydrogenase (LDH) activity (measured using the Bio-Analytics LDH assay kit, Palm City, FL), and samples were adjusted to equivalent LDH levels. Preliminary experiments revealed that LDH levels correlated with cellular protein levels. Cell lysates were precleared

with immobilized protein G (ImmunoPure: Pierce, Rockford, IL), incubated with monoclonal anti–cyclin E or cyclin A antibody (50–100 μl/sample, respectively) for 2–4 hr on ice, precipitated with 10–50 μl of immobilized protein G (50% slurry), washed three times with lysis buffer containing 0.5% NP-40 and twice with kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4), and then incubated at 30° for 30 min in 30 μl of kinase buffer containing 20 μM [γ-^{32}P]ATP (Amersham) and 1 μg of histone H1 (Boehringer, Mannheim, Federal Republic of Germany). The reactions were stopped by the addition of 2× Laemmli sample buffer, and samples were analyzed by SDS–PAGE followed by autoradiography or cutting substrate bands from the gels to measure the radioactivity by scintillation counting. CAK activity was measured as described (Poon et al., 1993) using bacterially produced H6–cyclin A and GST–cdk2.

Protein Analysis

For detecting cyclins and cdks, cell lysates were separated on 12.5%–15% SDS–PAGE. The proteins were transferred to Immobilon membrane (Millipore Corporation, Bedford, MA), immunoblotted with antibodies, using a horseradish peroxidase–conjugated second antibody, and developed using the enhanced chemiluminescence method (Amersham). Cyclin–cdk complexes was detected by immunoprecipitation with antibodies to cyclin E and $p27^{Kip1}$, followed by immunoblotting with specific antibodies. Quantitation of cdk2, $p27^{Kip1}$, and $p21^{Cip1/Waf1}$ was determined by immunoblot analysis using recombinant proteins as standards.

Flow Cytometry Analysis for Integrins and Cell Cycle Distribution

SMCs maintained for 2 days in 1% PDS/DMEM were replated and cultured in 1% PDS/DMEM for 24 hr on monomer or polymerized collagen. Cells were suspended after either trypsin or collagenase digestion. Expression of integrin receptors or cell cycle distribution, respectively, was determined by flow cytometry as previously described (Skinner et al., 1994) and following staining of DNA with 4,6-diamidino-2-phenylindole.

Attachment Assay

SMCs were preincubated in suspension with antibodies for α2 integrins (5 μg/200,000 cells) for 30 min at 37°C before plating. Cells (50,000 cells/well) were plated on 24-well dishes coated with monomer or polymerized collagen and unattached cells were counted.

Immunocytochemistry and Phalloidin Staining of Actin Filaments

SMCs were plated on coverslips or chamber slides and fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized in 0.5% Triton X-100 in PBS for 10 min, and washed serially in PBS, 50 mM NH_4Cl/PBS, and PBS. Thereafter, the cells were blocked in 1.0% BSA/PBS for 1 hr and incubated with primary antibodies in 0.1% BSA/PBS for 1 hr at room temperature, and subsequent incubation with biotin-labeled or FITC-labeled secondary antibodies. Actin filaments were visualized with 0.2 μM FITC- or TRITC-labeled phalloidin (Sigma).

Measurement of $p70^{S6K}$, MAP Kinase Kinase, and MAP Kinase Activity

$p70^{S6K}$ activity was measured from the cell lysates (Bornfeldt et al., 1994) by immunoprecipitation followed by measurement of the phosphorylation of ribosomal protein S6 as described previously (Graves et al., 1995). The gel-mobility shift of $p70^{S6K}$ was measured to estimate phosphorylation of the enzyme following immunoprecipitation, 10% SDS–PAGE, blotting onto nitrocellulose filters, and detection with the $p70^{S6K}$ antibody and alkaline phosphatase-conjugated secondary antibody. MAP kinase kinase and MAP kinase activities were measured as previously described (Bornfeldt et al., 1994).

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