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Modulation of Cyclin Dependent Kinase Inhibitor Proteins and ERK1/2 Activity in Allylamine-Injured Vascular Smooth Muscle Cells

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Abstract Chronic oxidative injury by allylamine (AAM) induces proliferative vascular smooth muscle cell (vSMC) phenotypes in the rat aorta similar to those seen in rodent and human atherosclerotic lesions. The proliferative advantage of AAM vSMC compared to control cells is maintained with serial passage of the cells and the advantage is nullified when AAM cells are seeded on a collagen substrate. In this study, we evaluate the potential role of cyclin dependent kinase inhibitors, p27 and p21, and mitogen activated protein (MAP) kinases, ERK1/2, in mediating the proliferative advantage of AAM stressed vSMC over control cells on plastic or collagen substrates. p27 levels in randomly cycling cells were comparable in both cell types irrespective of the substrate. In contrast, basal levels of p21 were 1.9 ± 0.3 ($P < 0.05$)-fold higher in randomly cycling AAM cells seeded on plastic compared to controls, a difference that was lost on a collagen substrate. Following G_0 synchronization, basal levels of both p27 and p21 were higher in AAM cells seeded on plastic compared to controls (1.7 ± 0.2 and 2.0 ± 0.3-fold, respectively, $P < 0.05$), but these differences were lost upon mitogenic stimulation. Pyrrolidine dithiocarbamate (PDTC) decreased p27 and p21 levels in cycling AAM cells relative to controls in a substrate-dependent manner. AAM cells seeded on plastic exhibited enhanced ERK1/2 activation upon mitogenic stimulation; seeding on collagen nullified this advantage. The duration of ERK1/2 activation was prolonged in AAM cells independently of the seeding substrate. We conclude that substrate-dependent acquisition of proliferative phenotypes following repeated cycles of AAM injury correlates with modulation of the cyclin dependent kinase inhibitors, p27 and p21. J. Cell. Biochem. 91: 1248–1259, 2004.

Key words: atherosclerosis; extracellular matrix; free radicals; signal; transduction; vascular smooth muscle cells

The development of atherosclerosis involves multiple changes in the vascular wall, including damage to endothelial cells and vascular smooth muscle cells (vSMC), recruitment and activation of inflammatory cells, proliferation of vSMC in response to mediators, and accumulation of lipids and matrix proteins. While the contributing cell types and characteristics of the plaque are well characterized, molecular mechanisms responsible for the initiation and propagation of vascular pathology remain elusive. De-differentiation of vSMC from native contractile phenotypes to highly proliferative phenotypes is a key step in atherogenesis. Such phenotypic changes arise from generation of reactive oxygen species (ROS) and the subsequent imbalance in cellular redox status [Griendling et al., 2000].

Allylamine (AAM), a selective cardiovascular toxicant, promotes the development of SMC

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lesions similar to those found in atherosclerotic vessels, and has been characterized as a model for chemically induced atherosclerosis [Cox and Ramos, 1990]. In SMCs from the large arteries, AAM is metabolized by semicarbazide sensitive amine oxidase (SSAO) to H_2O_2 and acrolein, a reactive aldehyde. One of the molecular mechanisms involved in AAM-induced vascular injury is the generation of ROS [Cox and Ramos, 1990; Ramos and Parrish, 1995; Ramos, 1999; Allen and Tresini, 2000]. ROS, including superoxide and hydrogen peroxide, are important signaling molecules [Griendling et al., 2000], functioning as second messengers to multiple intracellular signaling cascades, many of which are important for cell growth and differentiation. Through activation of signaling cascades and redox-sensitive transcription factors, many genes with important functional roles in the physiology and pathophysiology of vascular cells are induced/repressed. SMCs derived from aortas of rats exposed chronically to AAM (AAM cells) maintain a proliferative advantage after serial propagation in vitro over cells derived from control rats [Cox and Ramos, 1990; Parrish and Ramos, 1997; Wilson et al., 2002]. It is proposed that phenotypic modulation of AAM cells to a proliferative state involves genetic reprogramming of growth signaling induced by oxidative chemical injury.

A role for oxidative stress in the proliferative advantage of AAM cells is evidenced by increased activity of AP-1, and NF-κB transcription factors in AAM injured vSMC [Parrish and Ramos, 1997]. Inhibition of NF-κB by pyrrolidine dithiocarbamate (PDTC) selectively compromises proliferation of AAM cells, while seeding of AAM cells on a collagen matrix decreases inducible NF-κB binding upon mitogen stimulation. This suggests the participation of integrin-mediated signaling in the proliferative dysregulation of these oxidatively stressed vSMC. Along with transcription factor alterations, the proliferative advantage of AAM cells is characterized by altered expression of integrin-associated proteins and extracellular matrix interactions [Parrish and Ramos, 1997; Wilson et al., 2002]. The proliferative phenotype of AAM cells is modulated in a matrix-specific manner. AAM cells adhered to plastic, fibronectin, or laminin maintain a proliferative advantage that is nullified when AAM cells are seeded on collagen [Parrish and Ramos, 1997]. This observation led us to hypothesize that AAM injury alters expression and activity of cell cycle regulatory proteins and that manifestation of increased proliferation is modulated by the extracellular matrix in the localized environment.

In this study, we test the hypothesis that chronic oxidative injury of vSMC by repeated cycles of AAM dosing results in alterations of matrix-dependent p21 and p27 protein levels and MAP kinase activation. Evidence presented here indicates that the proliferative advantage of AAM cells is mediated through substrate-dependent upregulation of p27 and p21 levels. MAP kinase activity may serve as a key focal point for altered regulation in atherogenesis. These results support the hypothesis that chronic oxidative stress induced by AAM modulates matrix-dependent intracellular signaling events and cell cycle regulation contributing to the proliferative phenotypes of vSMC associated with atherosclerosis.

MATERIALS AND METHODS

Materials

Cell culture reagents were purchased from Gibco/Invitrogen (Carlsbad, CA). Control cell lysates were purchased from Upstate Biotechnologies (Lake Placid, NY). Type I collagen (rat tail collagen) coated plates were obtained from Becton Dickinson (Franklin Lakes, NJ). All other reagents for SDS–PAGE and Western blotting were from Bio-Rad (Hercules, CA). General laboratory chemicals were purchased from Sigma (St. Louis, MO).

Antibodies

Primary antibodies were purchased from New England Biolabs, Beverly, MA (rabbit polyclonal anti-phosphorylated ERK1/2), Upstate Biotechnologies (rabbit polyclonal anti-total ERK1/2), Santa Cruz Biotechnologies, Inc., Santa Cruz, CA (rabbit polyclonal anti- p21, p27, and cdk2) and Advanced Immunochemical Incorporated, Long Beach, CA (mouse monoclonal anti-GAPDH). Secondary antibodies were donkey anti-rabbit and donkey anti-mouse horseradish peroxidase conjugated IgGs obtained from Jackson, West Grove, PA.

Cell Culture

vSMC were isolated by successive enzymatic digestion of the aortas from adult male Sprague–Dawley rats gavaged with AAM-HCl

(70 mg/kg—AAM cells) or tap water (control cells) daily for 20 days as described [Cox and Ramos, 1990]. Subcultures were prepared by trypsinization of subconfluent cultures and maintained as outlined [Cox and Ramos, 1990]. Cell cultures were grown to 70% confluence at which time they were washed with phosphate buffered saline (PBS) followed by mitogen restriction (0.5% or 0.1% fetal bovine serum (FBS, Hyclone Logan, UT, in Medium 199) for 48 h. Quiescent cultures were then stimulated by addition of Medium 199 containing 10% FBS or pretreated with 100 nM PDTC or 25 μM U0126, a selective MEK kinase inhibitor (New England Biolabs), followed by the addition of Medium 199 containing 10% FBS, with or without PDTC or UO126.

Immunoblotting. Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.4), 50 mmol/L NaCl, 50 mmol/L NaF, 50 mmol/L EDTA, 20 mmol/L Na pyrophosphate, 1% Triton X-100, 1% protease inhibitor cocktail (Calbiochem, San Diego, CA), and 1% phosphatase inhibitor cocktail (Calbiochem). The lysate was frozen, thawed, and cleared by centrifugation. Protein concentration was determined using a BCA protein assay (Pierce Chemical, Rockford, IL).

Prior to electrophoresis, protein (2–10 μg) was added to sample buffer (500 mmol/L Tris-HCl (pH 6.8), 75% glycerol, 50 mmol/L dithiothreitol (Calbiochem), 0.05% bromophenol blue, and 5% SDS) and boiled for 5 min. Proteins were separated by SDS–PAGE and electroblotted to a nitrocellulose membrane. Membranes were blocked and incubated with appropriate primary antibody followed by secondary antibody incubation. Antibody signal was detected using Supersignal® reagent (Pierce Chemical) and membrane exposure to X-ray film. Phosphorylated ERK1/2 antibodies were removed from the membranes with Restore® (Pierce Chemical) stripping reagent and reprobed with anti-ERK1/2 antibody to verify loading. Membranes reacted for p27 and p21 were stripped and reprobed with anti-GAPDH antibody. Densitometry was performed on signals detected on the developed film using Multianalyst software® (Bio-Rad).

Propidium Iodide Staining of Ethanol Fixed Cells

Ethanol fixation and propidium iodide (Calbiochem) staining of cell suspensions was followed as outlined previously [Rodgers, 1998]. Briefly, cells were trypsinized and washed in PBS + 1% bovine serum albumin (BSA, USBiochemical, Piscataway, NJ) followed by fixation in cold ethanol. Propidium iodide staining was accomplished by centrifugation of the fixed cell suspension and removal of ethanol followed by washing with PBS + 1% BSA. Propidium iodide (50 μg/ml prepared in 0.038 M sodium citrate pH 7.0) and RNase A (1 mg/ml prepared in 10 mM Tris-HCl, pH 7.5, Worthington Biochemicals, Lakewood, NJ) were added to resuspended cells. The cell suspension was incubated at 37°C for 30 min. Samples were immediately analyzed using a FACS Calibur apparatus (Becton Dickinson) with ModFit software (Verity Software House, Topsham, ME) for data analysis.

Statistical Analysis

Statistical significance was assessed by Student's unpaired two-tailed *t*-test or analysis of variance followed by comparison of group averages by Fisher's LSD analysis, using the StatView statistical program (SAS Institutes, Inc., Cary, NC).

RESULTS

Differential Regulation of p27 and p21 in Cycling Control and AAM Cells Seeded on Plastic or Collagen

Oxidatively stressed vSMC isolated from AAM-treated rats are characterized by enhanced mitogenic sensitivity and increased rates of proliferation when seeded on plastic or fibronectin substrates, but not collagen [Wilson et al., 2002]. To evaluate the involvement of cyclin dependent kinase inhibitors in the regulation of matrix-modulated proliferation, protein lysates were harvested from control and AAM cells subjected to medium containing 0.5% FBS for 48 h. No significant differences in p27 levels were observed under any of the experimental conditions examined (Fig. 1A,B). p21 protein was increased in AAM cells seeded on plastic compared to control cells (1.9 ± 0.3-fold, $^*P < 0.05$), while comparable levels of p21 were observed in control and AAM cells on a collagen substrate (Fig. 1A,C). Elevated p21 levels were observed in control cells grown on collagen compared to growth on plastic. These findings are consistent with the loss of proliferative advantage of AAM cells compared to control cells when seeded on a collagen substrate [Wilson et al.,

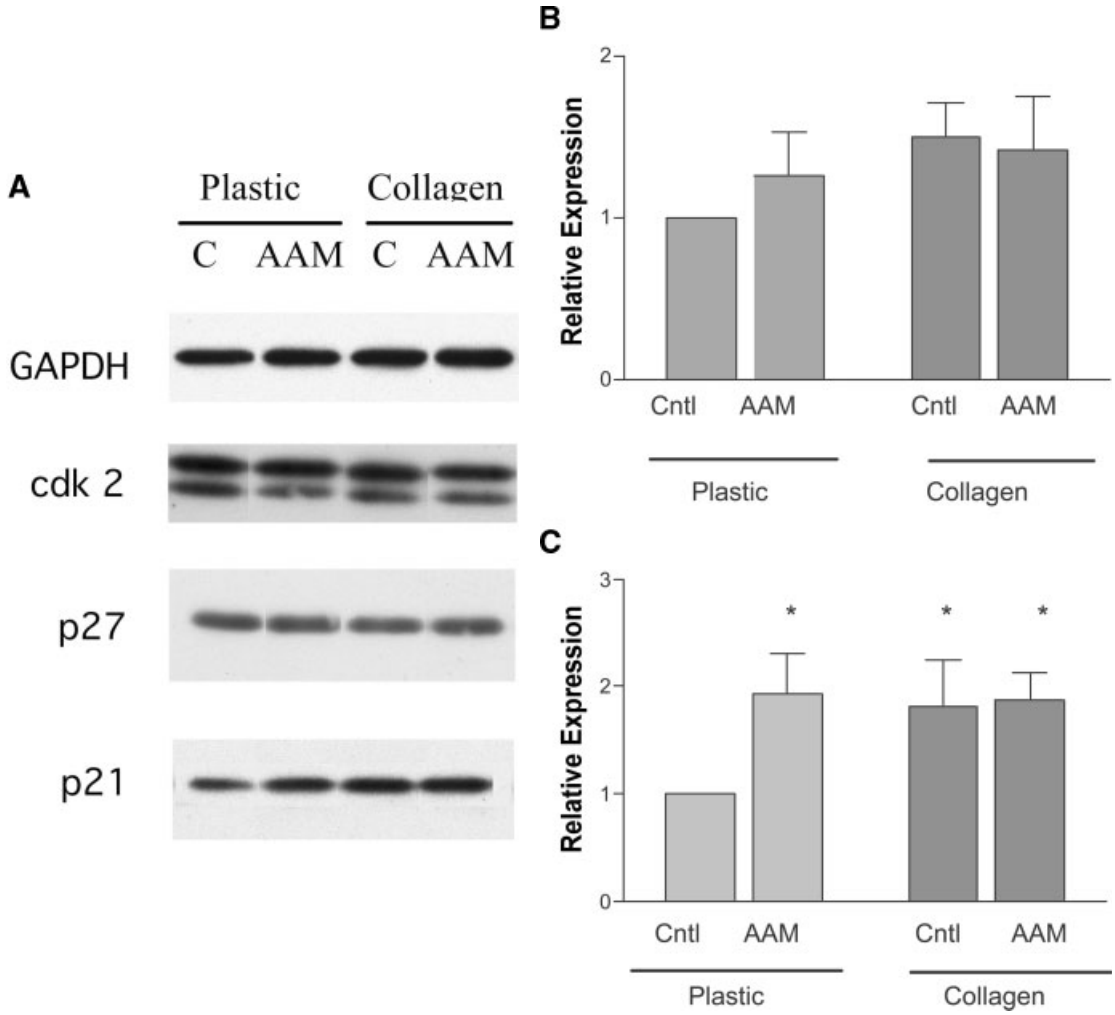

Fig. 1. Differential regulation of p21 and p27 in cycling control and AAM cells cultured on plastic or collagen. Control and AAM cells were grown to 70% confluence. Following serum deprivation (0.5% FBS) for 48 h, cell lysates were prepared. **A**: Western blots of p27 and p21 with control blots of GAPDH (p36) and cdk2 (p34) are shown (Cntl, control; AAM, allylamine). **B**, **C**: Graph of the average fold changes in p27 and p21, respectively, compared to control cells on plastic. Data are presented as the average ± SEM derived from representative blots from three independent experiments (*$P < 0.05$ vs. control, plastic).

2002]. Membranes were stripped and reprobed with anti-GAPDH and cdk2 to confirm equal protein loadings. Flow cytometric analysis indicated that cells grown on plastic were not synchronized in G_0; the percentages of cells in G_0/G_1 and S were 62.6 and 21.7 for control cells, and 58.4 and 20.2 for AAM cells, respectively.

Expression of p27 Following Serum Stimulation in G_0 Synchronized Control and AAM Cells Seeded on Plastic or Collagen

In order to test the effect of G_0 synchronization (0 h) on p21 and p27 expression, G_0/G_1 synchrony was achieved by serum restriction in 0.1% FBS for 48 h and confirmed with propidium iodide staining of fixed cell suspensions. The total percentage of G_0/G_1 synchronized control and AAM cells was 80.7 and 79.6, respectively, with only 10.5 and 6.6 of the populations in S phase. Confirmation of cell cycle progression was accomplished by addition of 10% serum to G_0 synchronized cells for 18 h, which reduced the G_0/G_1 population and caused proportional increases in S phase cells (Control $G_0/G_1 = 44.2$, $S = 55.8$; AAM $G_0/G_1 = 33.7$, $S = 66.2$). AAM cells showed a greater increase in cells proceeding to S compared to control cells (an average of 14% more AAM cells moved to S phase) and a 25% increase in DNA synthesis as determined by BrdU labeling (Data not shown).

p27 levels were higher (1.8 ± 0.2-fold increase, *$P < 0.05$) in G_0 synchronized AAM cells seeded on a plastic substrate relative to control

(Fig. 2A,B 0 h). On a collagen substrate, p27 levels in AAM cells were comparable to levels in control cells, and p27 levels for both cell types were elevated (3.2 ± 0.2 and 2.8 ± 0.1 for control and AAM cells $^{*}P < 0.05$, respectively) over those observed when control cells were grown on plastic. A marked decrease in p27 levels was observed after serum stimulation as cells progressed through G_1 when cells were grown on either substrate. However, this difference was most pronounced on a collagen substrate (Fig. 2A,B, 9 h, $^{+}P < 0.05$). Interestingly, no differences in the levels of p27 were observed between the two cell types following serum stimulation irrespective of the seeding substrate. These results suggest that increased p27 in AAM cells during G_0 may play a role in regulation of the proliferative advantage of AAM injured vSMC on plastic.

To determine if a functional link exists between expression of p27 cdk inhibitors and NF-κB signaling in the regulation of AAM vSMC proliferation, protein lysates were harvested from AAM and control cells following mitogenic stimulation of growth arrested cultures seeded on plastic or collagen in the presence or absence of 100 nM PDTC. Similar to the effect of seeding cells on a non-permissive collagen substrate, PDTC pre-treatment increased p27 levels by 2.5 ± 0.13-fold ($^{*}P < 0.05$) in G_0 synchronized control cells seeded on a plastic substrate. There was no effect of PDTC pre-treatment on p27 levels in G_0 synchronized AAM cells on either matrix and in G_0 synchronized control cells seeded on a collagen substrate (Fig. 2A,C, 0 h +PDTC). Serum stimulation for 9 h decreased p27 expression in both cell types grown on either substrate. PDTC treatment caused a further reduction in the serum induced decrease of p27 levels in AAM cells on both seeding substrates, but this effect was most dramatic when AAM cells were seeded on collagen. (Fig. 2A,B, 9 h +PDTC, $^{\wedge}P < 0.05$).

Expression of p21 Following Serum Stimulation in G_0 Synchronized Control and AAM Cells Seeded on Plastic or Collagen

To determine if there were differences in p21 levels in control and AAM cells in response to G_0 synchronization and release by serum, protein lysates were prepared from control and AAM cells seeded on either plastic or collagen and processed for Western blot analysis. An increase in p21 (2.0 ± 0.3-fold) was observed in G_0 synchronized AAM cells on plastic compared to control cells, and the levels of p21 for both control and AAM cells were increased in G_0 synchronized cells seeded on collagen compared to control cells on plastic (1.8 ± 0.2 and 1.8 ± 0.1 for control and AAM, respectively, $^{*}P < 0.05$) (Fig. 2C,D, 0 h). Although serum stimulation increased p21 levels in control and AAM cells on both substrates, it was particularly evident on plastic (Fig. 2C,D, 9 h).

The effect of PDTC on p21 expression levels in G_0 synchronized and cycling cells was determined for both control and AAM cells seeded on plastic or collagen. Pre-treatment with PDTC resulted in increased p21 expression (2.1 ± 0.2-fold) in control cells on plastic (Fig. 2C,D 0 h +PDTC, $^{*}P < 0.05$). No difference was observed in p21 expression in AAM cells seeded on plastic compared to untreated cells. There was no change in expression in p21 in either cell type in response to PDTC pretreatment when cells were adhered to collagen. PDTC had no effect on the serum-induced increase in p21 levels of control cells seeded on either substrate (Fig. 2C,D 9 h +PDTC). In AAM cells, treatment with PDTC attenuated the serum induced increase in p21 expression levels on both substrates (Fig. 2C,D, plastic and collagen, 9 h +PDTC, $^{\#}P < 0.05$ and $^{\wedge}P < 0.05$, respectively). These results are consistent with an increase in p21 levels in proliferating vSMC and that increased p21 expression in AAM cells is modulated by oxidant-dependent mechanisms as demonstrated by the selective decrease in p21 expression in serum-stimulated AAM cells by PDTC.

Differential ERK1/2 Activation of Control and AAM Cells Seeded on Plastic or Collagen

To determine if ERK1/2 activation corresponded with matrix-dependent regulation of proliferation in AAM cells, protein lysates were harvested from AAM and control cells following time dependent FBS stimulation of mitogen-restricted cultures grown on plastic and collagen. ERK1/2 activation peaked 15 min post-stimulation in both control and AAM cells, as determined by Western blot analysis using anti-phosphorylated ERK1/2 antibodies (Fig. 3A). Peak activation was increased in AAM cells versus controls when cells were grown on plastic, and this difference was lost in cells grown on a collagen substrate. Comparable ERK1/2

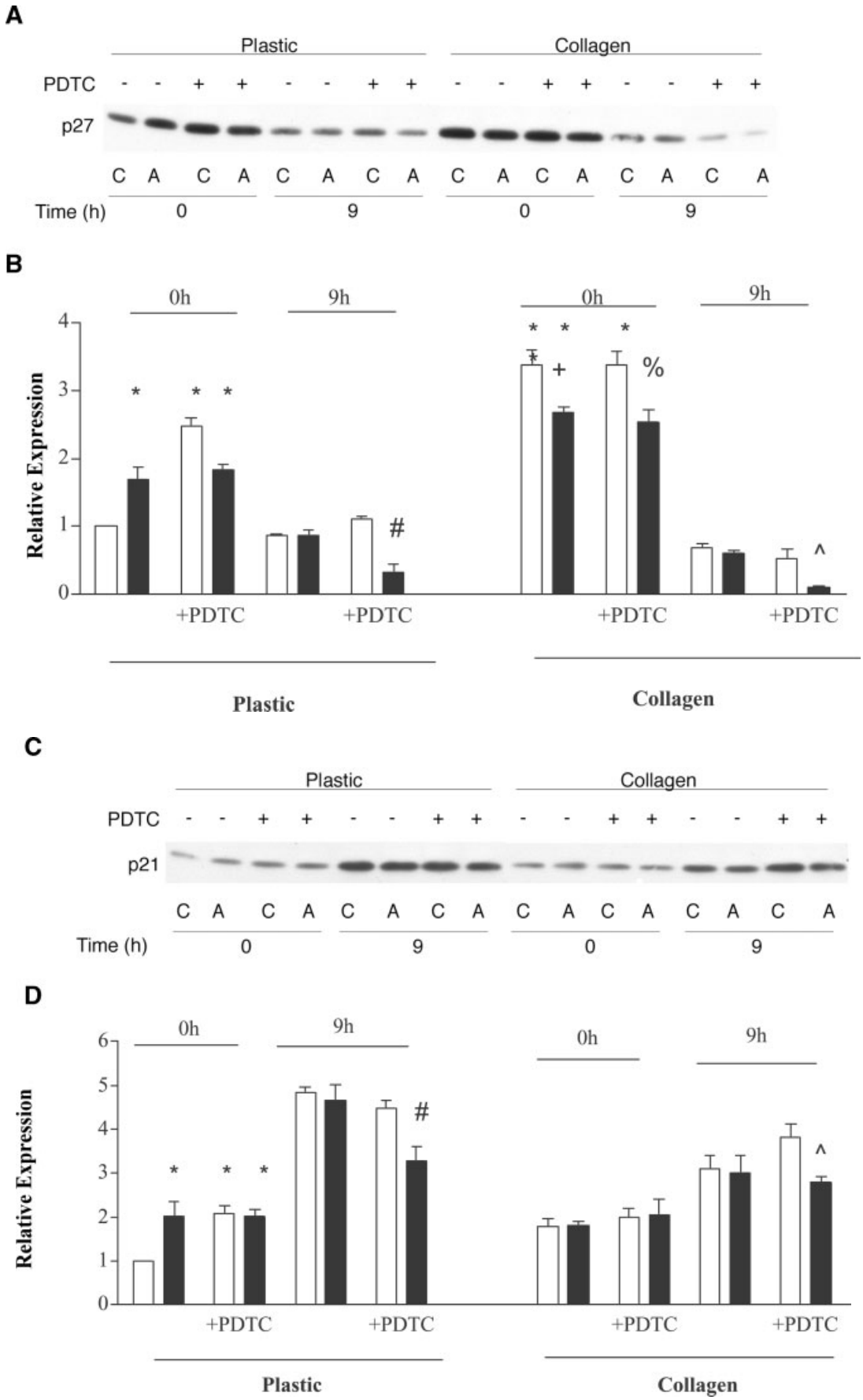
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Fig. 2.

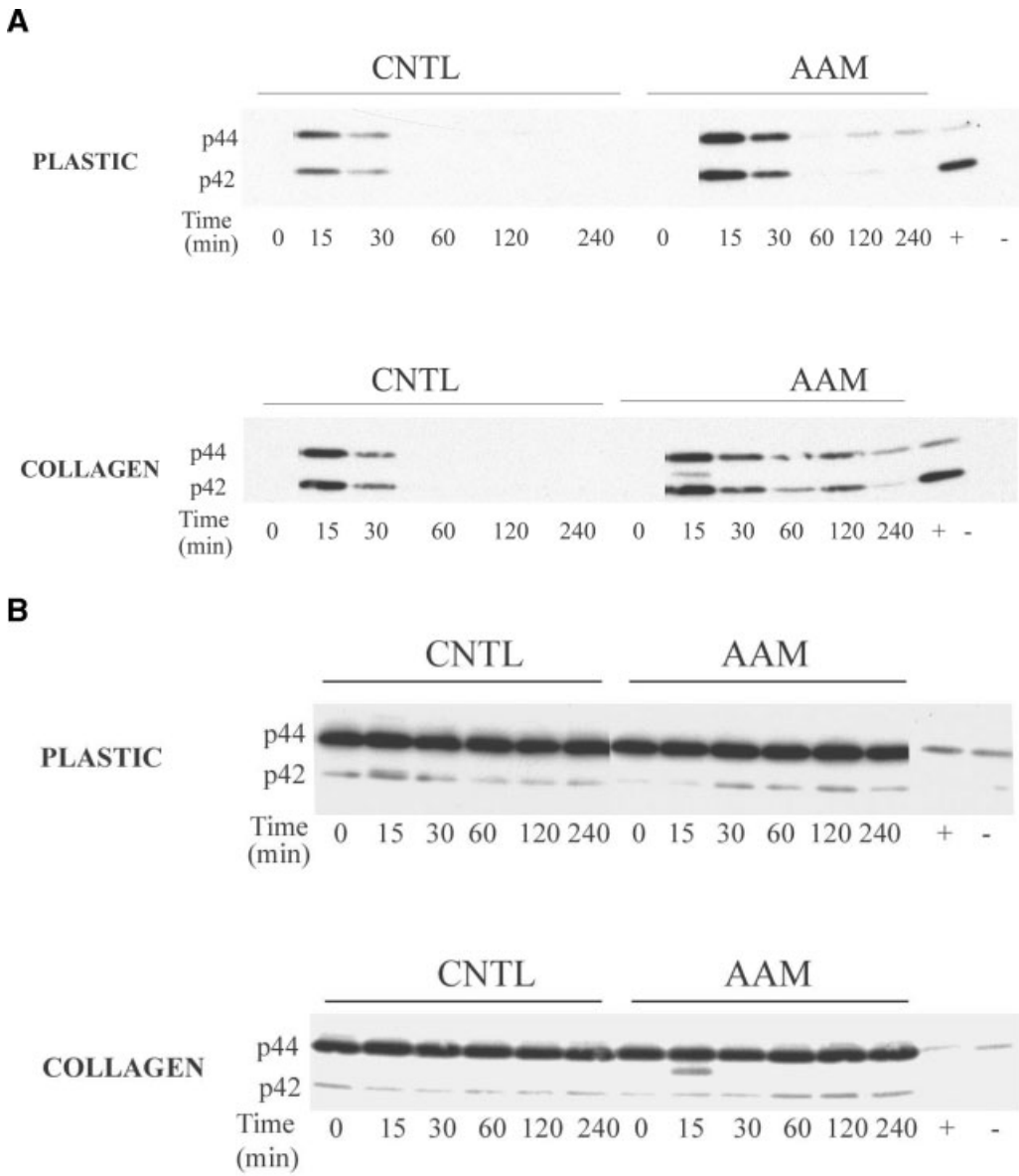

Fig. 3. Differential ERK1/2 activation of control and AAM cells cultured on plastic or collagen. Control and AAM cells were grown to 70% confluence. Following serum deprivation for 48 h, cultures were stimulated with media containing 10% FBS. Cell lysates were prepared at 0, 15, 30, 60, 120, and 240 min following exposure to serum. (CNTL, control lysates; AAM, allylamine lysates; + positive control, serum stimulated 3T3 lysates; − negative control, nonstimulated 3T3 lysates) **A**: Representative immunoblots (n = 3) of anti-phosphorylated ERK1/2 on plastic and collagen are shown. **B**: Immunoblots of anti-total ERK1/2 on plastic and collagen are shown. Membrane was stripped and reprobed with anti-total ERK1/2 to verify equal ERK1/2 loadings.

Fig. 2. (*Overleaf*) Differential expression of p27 and p21 following serum stimulation and pyrrolidine dithiocarbamate (PDTC) treatment of synchronized control and AAM cells cultured on plastic or collagen. Control and AAM cells were grown to 70% confluence. Following serum deprivation (0.1% FBS for 48 h), 2 h pretreatment (media change only or 100 nM PDTC) was followed by serum stimulation with or without 100 nM PDTC. Cell lysates were prepared at 0 h (following pretreatment) and 9 h. **A**: Western blot of p27 is shown and is representative of at least two independent experiments (C, control; A, allylamine). **B**: Graphic representation of fold changes in of p27 expression for control (white) and AAM (black) cells compared to control cells on plastic at time 0. **C**: Representative immunoblot of p21 (C, control; A, allylamine). **D**: Graphic representation of fold changes in expression of p21 for each experimental condition in control (white) and AAM (black) cells compared to control cells on plastic at 0 time. Data represent the average of multiple determinations ± SEM (*$P < 0.05$ vs. control, plastic, 0 h, $^{\#}P < 0.05$ vs. control, plastic, 0 h +PDTC, $^{+}P < 0.05$ vs. control, collagen, 0 h, $^{\%}P < 0.05$ vs. control, collagen, 0 h +PDTC, ^$P < 0.05$ vs. control collagen, 9 h +PDTC.

loadings were confirmed by stripping and reprobing membranes with anti-total ERK1/2 (Fig. 3B).

As both the degree and duration of ERK1/2 activation are important in cell growth and proliferation, evaluation of ERK1/2 activation following prolonged mitogen stimulation was examined. AAM cells maintained increased ERK1/2 activation for a longer duration than control cells when grown on either plastic or collagen. Equal ERK1/2 loading was confirmed by stripping and reprobing membranes with anti-total ERK1/2 (Fig. 3A,B).

Effect of a MEK1/2 Inhibitor on p27 and p21 Expression in Control and AAM Cells Seeded on Plastic or Collagen

Given the observed differences in matrix dependent alterations in peak ERK1/2 activation in AAM cells, we investigated a possible functional link between ERK1/2 activation and cdk inhibitor expression. Protein lysates were harvested from AAM and control cells seeded on either plastic or collagen following serum stimulation for 9 h in the presence or absence of 25 μM U0126, a MEK1/2 inhibitor. MEK1/2 inhibition increased p27 levels in both control and AAM cells irrespective of the seeding substrate (Fig. 4). No differences in p27 induction were observed between the two cell types, suggesting that differences observed in p27 expression in oxidatively injured vSMC are not dependent on MAP kinase activation. In contrast, cell type-specific differences in p21 expression were observed following inhibition of MEK1/2. U0126 reduced p21 expression in both cell types, but the response was markedly enhanced in AAM cells, and preferentially observed on a restrictive collagen substrate. These data suggest the involvement of ERK1/2 activity in matrix dependent modulation of p21 levels in oxidatively injured vSMC.

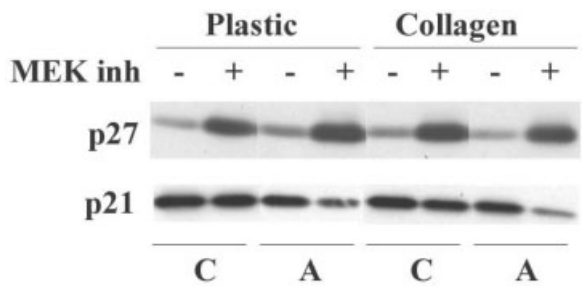

Fig. 4. Effect of MEK 1/2 inhibition on p21 and p27 expression in control and AAM cells cultured on plastic or collagen. Representative immunoblots of p21 and p27 protein lysates are shown (n = 3). Control and AAM cells were grown to 70% confluence. Following serum deprivation for 48 h, pretreatment for 30 min (media change only or 25 μM U0126) was followed by serum stimulation with or without U0126 (25 μM). Cell lysates were prepared at 9 h following serum stimulation.

DISCUSSION

vSMC derived from aortas from rats chronically treated with AAM are characterized by transformation to proliferative phenotypes [Cox and Ramos, 1990]. Phenotypic modulation of vSMC by chronic oxidative stress induced by AAM differentially modulates profiles of surface integrin expression [Parrish and Ramos, 1997]. Expression of α1 and α5 integrin subunits are substantially reduced on AAM cells, while expression of β3 integrin is increased compared to control cells. The proliferative advantage of AAM cells is maintained when cells are grown on plastic, pronectin, or fibronectin, but lost when grown on collagen, a ligand for the β_1 integrin subunit [Wilson et al., 2002]. Consistent with these observations, Thyberg's group has previously shown that the substrate is a crucial factor in modulating vSMC from contractile to synthetic/proliferative phenotypes [Thyberg et al., 1990].

To further explore the nature of integrin/ECM signaling in AAM-injured vSMC, we first evaluated the expression of the cdk inhibitors, p27 and p21, in randomly cycling AAM and control vSMC cultured on plastic or collagen substrates. p27 has been implicated in TGF-β and contact-induced inhibition of proliferation [Nigg, 1995], and inhibition of vSMC and fibroblast proliferation and migration [Diez-Juan and Andres, 2003]. In contrast, p21 has dual functions as a cell cycle inhibitor [Li and Brooks, 1999; Dotto, 2000] and as a permissive element for cell cycle progression in PDGF-stimulated vSMC [Weiss et al., 2000]. Differential expression of p27 and p21 has been observed in human atherosclerotic arteries [Tanner et al., 2000]. p27 was shown to be constitutively expressed in normal and atherosclerotic arteries, whereas p21 was elevated in human atherosclerotic arteries and undetectable in normal counterparts. It has been proposed that the cdk inhibitor p27 functions to inhibit cell proliferation during arterial repair, and p21 functions as a cofactor induced in the latter phases of arterial remodeling to cause G_1 arrest [Tanner et al.,

1998]. In our AAM injury model of atherogenesis, we show that p27 levels were not altered irrespective of the growth substrate, implying that p27 has little effect on the proliferative advantage of AAM cells. The increased expression of p21 observed in cycling AAM cells seeded on a permissive plastic substrate is consistent with p21 acting as a permissive element in vSMC cycle progression. Also, the normalization of p21 expression to control levels in AAM cells seeded on a collagen substrate establishes the matrix dependency of p21 expression in randomly cycling AAM injured cells.

In contrast to cycling cells, G_0-synchronized AAM cells express higher levels of both p27 and p21 compared to control cells when grown on plastic, whereas, no difference was seen with mitogen stimulation or growth on a collagen substrate. Cell cycle progression in both cell types resulted in decreased p27 levels on both substrates, with a more pronounced effect when cells were grown on collagen. These findings are consistent with the growth inhibitory function of p27. In contrast, following cell cycle progression, p21 protein expression increased in control and AAM cells when grown on either matrix, consistent with a positive role for p21 in cell cycle progression in this system. We found no difference in p21 or p27 expression following 9 h of serum stimulation between cell types irrespective of the growth matrix. This indicates that differences exist at the basal level in cdk inhibitors in AAM injured vSMC compared to control cells level, while mitogen stimulation results in similar responses for p27 and p21 in both cell types.

Mitogenic stimulation for both cell types on plastic or collagen results in decreased p27 expression and increased p21 expression. p27 levels are similar in both cell types during mitogen stimulation, and on both substrates, confirming that this cdk inhibitor is not involved in the proliferative advantage of oxidatively stressed vSMC. However, p21 expression shows less induction when cells are grown on a non-permissive substrate compared with growth on plastic providing further evidence of p21 as a positive regulator of growth in vSMC. The growth inhibitory effect of collagen in AAM cells may occur post-9 h of mitotic stimulation and can be explained through the differential effects of growth factors on phenotypic properties of vSMC [Yamamoto and Yamamoto, 1994]. For example, PDGF can initiate DNA synthesis. However, other growth factors, such as EGF are required for completion of the cell cycle.

Evidence is presented that p21 expression is upregulated in oxidatively injured vSMC seeded on a permissive substrate, and that upregulation, relative to control cells, is lost when cells are grown on a non-permissive substrate. Interestingly, overexpression of p27 and p21 overexpression following serum stimulation led to G_1 growth arrest [Tanner et al., 2000]. Thus, the effect of p21 and p27 expression on growth regulation in SMCs may depend on the cellular context in which they are expressed. It has been suggested that p21 can play dual functions in cell cycle regulation depending on stoichiometric ratios of p21 to cyclin/cdk complexes. Permissive effects of p21 include modulation of the assembly and nuclear localization of cyclin D1/cdk4/6 complexes [Dotto, 2000]. Also, the expression levels of p21 are dependent on the growth substrate, suggesting that the functions of the cell cycle regulatory proteins are subject to matrix regulation, which may be at the level of cellular localization of the cdk inhibitors, a process regulated by phosphorylation-dependent signaling pathways [Ritt et al., 2000; Blagosklonny, 2002]. Several recent reports suggest that both p21 and p27 may associate with the cytoplasmic tails of specific integrins and regulate smooth muscle migration and cytoskeletal organization. [Coqueret, 2003; Diez-Juan and Andres, 2003] p21 interacts with a myriad of proteins including the transcription factors C/EBP and NF-κB [Dotto, 2000], which have been implicated in oxidative stress signaling in vSMC [Allen and Tresini, 2000]. Thus, although we have not directly tested these possibilities, altered p21 expression in AAM cells may contribute to the proliferative phenoytpe through mechanisms that are not directly involved in cell cycle regulation.

To further evaluate the effect of chronic oxidative injury on vSMC, we examined the influence of the NF-κB inhibitor PDTC in this cell system. PDTC can have a variety of effects on cultured cells including pro-oxidant and antioxidant effects [Moellering et al., 1999; Meisner et al., 2000; Shi et al., 2000; Porcile et al., 2003]. These effects appear to be concentration dependent as, e.g., low PDTC concentrations increase AP-1 binding activity while higher PDTC concentrations inhibit NF-κB activity [Porcile et al., 2003]. In our cell system of chronically AAM injured vSMC, 100 nM

PDTC pretreatment had a greater effect on p27 and p21 levels in G_0 synchronized control cells grown on plastic than in any other treatment group. Additionally, following mitogen stimulation, p27 was further suppressed by the addition of PDTC, while PDTC had no effect on p21 levels when cells were grown on plastic. A greater difference in p21expresssion in AAM cells at 0 h versus 9 h PDTC treatment on collagen versus plastic compared with p27 was also observed. Thus, PDTC suppresses the collagen/mitogen induction of p21 in AAM cells but not the plastic/mitogen response; whereas, p27 shows enhanced suppression in AAM cells compared to control cells on both substrates. This provides evidence that differential p21 expression in oxidatively injured vSMC can be influenced by PDTC and the growth substrate.

The role of ERK1/2 in mediating extracellular matrix modulation of growth properties in oxidatively stressed vSMC was also investigated in these studies. MAP kinases are intricately involved in modulation of growth, proliferation, and differentiation by growth factors and integrins [Schwartz et al., 1995; Shattil and Ginsberg, 1997; Giancotti and Ruoslahti, 1999; Danen and Yamada, 2001]. Thus, MAP kinases may serve as points of integration for multiple signaling pathways, including those involved in matrix-regulated cell cycle activity and proliferation. As seen for p21, peak ERK1/2 activation in vSMC was influenced by the extracellular matrix, thus implicating interactions between ERK1/2 signaling and p21 in the regulation of vSMC proliferation. Thus, altered integrin/matrix regulation of ERK1/2 and downstream signaling events may contribute to oxidative injury induced proliferative phenotypes. This interpretation is consistent with our studies showing that basal levels of cyclin D1 are increased in AAM cells compared to control cells when cells are grown on plastic, but not collagen (Jones et al., unpublished). As cyclin D1 is an important cell cycle regulatory protein involved in transition from G_1 to S phase of the cell cycle, alterations in cell cycle regulatory proteins may contribute to proliferative dysregulation seen in oxidatively stressed vSMC.

Our results show that activation of ERK1/2 is of greater magnitude and duration in AAM cells compared to controls when seeded on a plastic substrate. ERK1/2 activity plays an integral part in cell cycle regulation, specifically the passage from G_1 to S phase of the cell cycle [Meloche et al., 1992; Weber et al., 1997]. MAP kinase activation can occur through matrix/integrin signaling in adherent cells [Morino et al., 1995]. Morino et al. [1995] (25) showed that fibroblast adherence to fibronectin as well as β_1 integrin crosslinking resulted in activation of MAP kinases. Assoian et al. [Assoian and Marcantonio, 1996; Assoian and Schwartz, 2001] suggested that integrin binding with fibronectin or vitronectin in vSMC leads to sustained ERK1/2 activity resulting in translocation of the active enzyme to the nucleus where it is required for synthesis of cyclin D1. Thus, interactions of integrins, MAP kinase, and cell cycle control proteins may mediate dysregulated growth and differentiation of AAM cells leading to induction of highly proliferative phenotypes. The early peak in ERK1/2 activation is likely a combined effect of growth factor and integrin/extracellular matrix signaling. These data are suggestive that ERK1/2 activation is an important contributing component of the proliferative advantage of oxidatively injured vSMC, but not the site of collagen modulation of the phenotype.

Following inhibition of ERK1/2 activity; however, differential expression of p21, but not p27, was observed between AAM and control cells. As cell cycle progression is blocked by MEK1/2 inhibition, increases in p27 levels are expected. In this model, no differences were observed in induction of p27 between the two cell types grown on either substrate. Thus, the alterations in p27 expression in AAM injured cells are not dependent on ERK activation. On the other hand, decreases in p21 expression following MEK1/2 inhibition were both cell type and matrix dependent with AAM cells and with the restrictive substrate collagen showing the most depressed p21 levels. It can be deduced, therefore, that ERK1/2 activation and p21 induction are related and contribute to the matrix dependent, proliferative advantage observed in oxidatively injured AAM vSMC.

In summary, evidence is presented here that chronic AAM injury alters expression of p21 and p27 in a matrix dependent manner. Antioxidant administration (PDTC) enhances the matrix dependent suppression of cdk inhibitors in vSMC that have undergone repeated cycles of oxidative injury by AAM. Differential peak MAP kinase activation and expression of cdk inhibitors, p27 and p21, in AAM cells grown on

plastic versus collagen indicate discrete alterations of integrin/ECM signaling in AAM injured vSMC. Thus, altered integrin/matrix regulation of cdk inhibitors, p27 and p21, ERK1/2 and further downstream signaling events may contribute to acquisition of proliferative phenotypes following oxidative injury by AAM and may represent common mechanisms for increased proliferation in other models of vascular oxidative injury during the atherogenic process.

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REFERENCES

Allen RG, Tresini M. 2000. Oxidative stress and gene regulation. Free Radic Biol Med 28:463–499.

Assoian RK, Marcantonio EE. 1996. The extracellular matrix as a cell cycle control element in atherosclerosis and restenosis. J Clin Invest 98:2436–2439.

Assoian RK, Schwartz MA. 2001. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. Curr Opin Genet Dev 11:48–53.

Blagosklonny MV. 2002. Are p27 and p21 Cytoplasmic Oncoproteins? Cell Cycle 1:391–393.

Coqueret O. 2003. New roles for p21 and p27 cell cycle inhibitors: A function for each cell compartment. Trends Cell Biol 13:65–70.

Cox LR, Ramos K. 1990. Allylamine-induced phenotypic modulation of aortic smooth muscle cells. J Exp Pathol (Oxford) 71:11–18.

Danen EH, Yamada KM. 2001. Fibronectin, integrins, and growth control. J Cell Physiol 189:1–13.

Diez-Juan A, Andres V. 2003. Coordinate control of proliferation and migration by the p27Kip1/cyclin-dependent kinase/retinoblastoma pathway in vascular smooth muscle cells and fibroblasts. Circ Res 92:402–410.

Dotto GP. 2000. p21(WAF1/Cip1): More than a break to the cell cycle? Biochim Biophys Acta 1471:M43–M56.

Giancotti FG, Ruoslahti E. 1999. Integrin signaling. Science 285:1028–1032.

Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. 2000. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 20:2175–2183.

Li JM, Brooks G. 1999. Cell cycle regulatory molecules (cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors) and the cardiovascular system; potential targets for therapy? Eur Heart J 20:406–420.

Meisner M, Schmidt J, Schywalsky M, Tschaikowsky K. 2000. Influence of pyrrolidine dithiocarbamate on the inflammatory response in macrophages and mouse endotoxin shock. Int J Immunopharmacol 22:83–90.

Meloche S, Seuwen K, Pages G, Pouyssegur J. 1992. Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: Correlation between late phase activation and mitogenicity. Mol Endocrinol 6:845–854.

Moellering D, McAndrew J, Jo H, Darley-Usmar VM. 1999. Effects of pyrrolidine dithiocarbamate on endothelial cells: Protection against oxidative stress. Free Radic Biol Med 26:1138–1145.

Morino N, Mimura T, Hamasaki K, Tobe K, Ueki K, Kikuchi K, Takehara K, Kadowaki T, Yazaki Y, Nojima Y. 1995. Matrix/integrin interaction activates the mitogen-activated protein kinase, p44erk-1 and p42erk-2. J Biol Chem 270:269–273.

Nigg EA. 1995. Cyclin-dependent protein kinases: Key regulators of the eukaryotic cell cycle. Bioessays 17:471–480.

Parrish AR, Ramos KS. 1997. Differential processing of osteopontin characterizes the proliferative vascular smooth muscle cell phenotype induced by allylamine. J Cell Biochem 65:267–275.

Porcile C, Stanzione S, Piccioli P, Bajetto A, Barbero S, Bisaglia M, Bonavia R, Florio T, Schettini G. 2003. Pyrrolidinedithiocarbamate induces apoptosis in cerebellar granule cells: Involvement of AP-1 and MAP kinases. Neurochem Int 43:31–38.

Ramos KS. 1999. Redox regulation of c-Ha-ras and osteopontin signaling in vascular smooth muscle cells: Implications in chemical atherogenesis. Annu Rev Pharmacol Toxicol 39:243–265.

Ramos KS, Parrish AR. 1995. Growth-related signaling as a target of toxic insult in vascular smooth muscle cells: Implications in atherogenesis. Life Sci 57:627–635.

Ritt MG, Mayor J, Wojcieszyn J, Smith R, Barton CL, Modiano JF. 2000. Sustained nuclear localization of p21/WAF-1 upon growth arrest induced by contact inhibition. Cancer Lett 158:73–84.

Rodgers L. 1998. Flow cytometry. In: Spector DL, Goldman RD, Leinwand LA, editors. Cells a laboratory manual. Culture and biochemical analysis of cells. New York: Cold Spring Harbor Laboratory Press. pp 16.1–16.12.

Schwartz MA, Schaller MD, Ginsberg MH. 1995. Integrins: Emerging paradigms of signal transduction. Annu Rev Cell Dev Biol 11:549–599.

Shattil SJ, Ginsberg MH. 1997. Integrin signaling in vascular biology. J Clin Invest 100:S91–S95.

Shi X, Leonard SS, Wang S, Ding M. 2000. Antioxidant properties of pyrrolidine dithiocarbamate and its protection against Cr(VI)-induced DNA strand breakage. Ann Clin Lab Sci 30:209–216.

Tanner FC, Yang ZY, Duckers D, Gordon G, Nabel EG. 1998. Expression of cyclin-dependent kinase inhibitors in vascular disease. Circ Res 82:1998.

Tanner FC, Boehm M, Akyurek LM, San H, Yang ZY, Tashiro J, Nabel GJ, Nabel EG. 2000. Differential effects of the cyclin-dependent kinase inhibitors p27(Kip1), p21(Cip1), and p16(Ink4) on vascular smooth muscle cell proliferation. Circulation 101:2022–2025.

Thyberg J, Hedin U, Sjolund M, Palmberg L, Bottger BA. 1990. Regulation of differentiated properties and proliferation of arterial smooth muscle cells. Arteriosclerosis 10:966–990.

Weber JD, Raben DM, Phillips PJ, Baldassare JJ. 1997. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. Biochem J 326(Pt. 1): 61–68.

Weiss RH, Joo A, Randour C. 2000. p21(Waf1/Cip1) is an assembly factor required for platelet-derived growth factor-induced vascular smooth muscle cell proliferation. J Biol Chem 275:10285–10290.

Wilson E, Parrish AR, Bral CM, Williams ES, Ramos KS. 2002. Collagen suppresses the proliferative phenotype of allylamine-injured vascular smooth muscle cells. Atherosclerosis 162:289–297.

Yamamoto M, Yamamoto K. 1994. Growth regualtion in primary culture of rabbit arterial smooth muscle cells by platelet-derived growth factor, insulin-like growth factor-I, and epidermal growth factor. Exp Cell Res 212: 62–68.