Journal of Cellular Biochemistry 65:267–275 (1997)

Differential Processing of Osteopontin Characterizes the Proliferative Vascular Smooth Muscle Cell Phenotype Induced by Allylamine

Alan R. Parrish and Kenneth S. Ramos*

Department of Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, Texas

Abstract Repeated cycles of vascular injury by allylamine induce vascular lesions similar to those seen in atherosclerotic vessels, or following balloon catheterization. Vascular (aortic) smooth muscle cells harvested from allylamine-treated animals (i.e., allylamine cells) acquire a proliferative advantage relative to control counterparts that is associated with differential secretion and extracellular matrix sequestration of several proteins. In the present study, we have characterized two of these proteins (M_r 52 and 36 kDa; pI 5.6 and 5.2, respectively) and their putative role in the expression of a proliferative phenotype. Because the physical properties of these proteins were comparable to those of osteopontin (OPN) and its thrombin-generated fragment(s), initial experiments were conducted to examine the expression and processing of OPN in this cell system. OPN mRNA expression was enhanced during early G_1 cell cycle progression in allylamine cells relative to control counterparts. However, comparable amounts of OPN (M_r 56, 52, and 50 kDa) were detected by Western analysis in media conditioned by both cell types using the OP-199 or B77-Rat1 antibodies to OPN. Allylamine cells, however, produced increased amounts of a 36 kDa protein recognized by the OP-199 antibody. Incubation of conditioned media from [^{35}S]methionine-labeled allylamine cells with thrombin decreased the intensity of the 52 kDa protein, while increasing the intensity of a 36 kDa protein. RT-PCR analysis demonstrated expression of a 1.2 kb OPN band in both cell types consistent with the predicted size of OPN mRNA, suggesting that the 36 kDa fragment recognized by OP-199 in allylamine cells was likely not due to altered splicing of the OPN transcript. To determine if OPN and/or the 36 kDa fragment played a central role in the proliferative capacity of allylamine cells, the effect of an antibody to an α_v integin subunit was examined. An antibody to the α_v subunit, but not α_4, nullified the proliferative advantage of allylamine cells relative to control counterparts, suggesting that integrin-mediated signaling is a key feature of the proliferative phenotype of allylamine cells. We conclude that enhanced proteolytic cleavage of OPN may characterize the modulation of vascular SMCs to a more proliferative phenotype following chemical injury by allylamine. J. Cell. Biochem. 65:267–275.

Key words: allylamine; osteopontin; vascular smooth muscle cells; vascular injury; atherosclerosis

Aberrant smooth muscle cell (SMC) proliferation is a pivotal point in the early events leading to atherosclerosis and re-stenosis [Ross, 1993; Schwartz et al., 1995]. With this in mind, studies in our laboratory have focused on the molecular mechanisms that mediate the deregulation of vascular SMC growth and differentiation following repeated cycles of chemical injury [reviewed in Ramos and Parrish, 1995]. Of particular interest has been the finding that allylamine, an atherogenic amine in vivo [Lalich et al., 1972; Boor et al., 1980], induces acquisition of a proliferative phenotype in vascular (aortic) SMCs that is sustained as a function of serial passage in vitro [Cox and Ramos, 1990]. The proliferative advantage of cells from allylamine-treated animals (henceforth referred to as allylamine cells) is associated with increased synthesis of proteins that become associated with the extracellular matrix (ECM) [Ramos et al., 1993]. Seeding of control cells on a matrix synthesized by allylamine cells affords control cells enhanced proliferative potential, thus implicating an ECM component(s) in the

Contract grant sponsor: National Institute of Environmental Health Sciences, contract grant numbers ES04849, ES00213; Contract grant sponsor: NIEHS, contract grant number 07273.

*Correspondence to: Dr. Kenneth S. Ramos, Department of Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4466. Email: kramos@vetmed.tamu

Received 2 July 1996; accepted 22 January 1997

enhanced mitogenic responsiveness of allylamine cells [Ramos et al., 1993].

Osteopontin (OPN) is a secreted acidic phosphoprotein (M_r 42–66 kDa) with effects on gene expression [Sauk et al., 1990], Ca^{++} regulation [Chen et al., 1993], and nitric oxide production [Hwang et al., 1994]. These effects are presumably mediated by the activation of the $\alpha_v\beta_3$ integrin following interaction with the conserved GRDGS sequence of OPN [reviewed in Denhardt and Guo, 1993]. OPN is overexpressed in transformed cell lines relative to non-tumorigenic counterparts [Senger et al., 1989; Su et al., 1993], and several reports have implicated OPN in the tumorigenic capacity of several cell lines [Behrend et al., 1994; Gardner et al., 1994]. Giachelli et al. [1991] first associated increased expression of OPN in SMCs with vascular injury by cloning an mRNA (2B7) which was increased 5-fold following balloon angioplasty of the rat carotid artery. OPN is elevated during neointimal formation and is a component of human atherosclerotic plaques [Giachelli et al., 1993], evidence which supports the view that OPN plays an important role in pathological processes associated with atherogenesis.

More recent work has focused on the role of OPN in SMC attachment and migration. OPN promotes adhesion and spreading of both vascular endothelial and SMCs, and is a potent chemotactic agent for SMCs in culture [Liaw et al., 1994]. While the migratory effect of OPN is mediated by the $\alpha_v\beta_3$ integrin [Yue et al., 1994; Liaw et al., 1995], interaction of OPN with the $\alpha_v\beta_1$ and $\alpha_v\beta_5$ integrin receptors may support SMC adhesion [Yue et al., 1994; Liaw et al., 1995]. These data suggest that interaction with distinct receptors is a mechanism by which OPN can regulate multiple cellular functions. OPN itself does not induce DNA synthesis, or enhance the effects of platelet-derived growth factor (PDGF) in SMCs, suggesting that OPN selectively influences SMC migration and attachment [Yue et al., 1994].

OPN contains a thrombin-sensitive dipeptide sequence (RS) in close proximity to the integrin-recognition domain, but controversy exists regarding the biological consequences of thrombin cleavage to generate a 35 kDa fragment. Two groups have described opposite effects on the adhesive properties of OPN following thrombin cleavage. Xuan et al. [1994] reported decreased attachment of PAP2 (ras-transformed, metastatic murine NIH 3T3 cells) and MDA-MB-435 human mammary carcinoma cells on dishes coated with thrombin-cleaved recombinant human OPN. Senger et al. [1994], however, demonstrated increased cell attachment and spreading of various human cell lines including HT1080 (fibrosarcoma), T24 (bladder carcinoma), HISM (intestinal smooth muscle), and HFL-1 (fetal lung fibroblast) on thrombin-cleaved rat OPN coated culture dishes. In addition, thrombin-cleaved OPN stimulated vascular endothelial cell migration in vitro to a greater extent than full-length OPN [Senger et al., 1996].

The present studies have been conducted to characterize two abundant proteins (M_r 52 and 36 kDa) which are differentially secreted and sequestered in the ECM of allylamine cells [Ramos et al., 1993]. Evidence is presented that OPN is overexpressed at the mRNA level in allylamine cells and that enhanced proteolytic cleavage of OPN generates a 36 kDa fragment that is enhanced in allylamine cells relative to control SMCs. The findings that blockade of α_v-mediated signaling nullifies the proliferative advantage of allylamine cells relative to control SMCs suggest that OPN and/or proteolytic fragments of the protein may participate in proliferative regulation in SMCs. These data provide strength to the emerging view that OPN is involved in disorders of growth and differentiation, such as cancer and atherosclerosis.

MATERIALS AND METHODS

Materials

Allylamine (99% purity) was purchased from Aldrich (Milwaukee, WI). Medium 199, L-glutamine, and the monoclonal anti-human α_4 (clone P4DC2) and α_v (clone VNR147) integrin subunit antibodies were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Intergen (Purchase, NY). Trypsin, epidermal growth factor, antibiotic/antimycotic solution, anti-mouse IgG, anti-rabbit IgG, and BCIP-NBT tablets were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Thymidine (55 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). [^{32}P]dCTP was purchased from NEN Radiochemicals (Grand Island, NY). OPN and β-tubulin plasmids were purchased from American Type Culture Collection (Rockville, MD). Tri-Reagent was purchased from Molecular Re-

search Center, Inc. (Cincinnati, OH). The OP-199 [Liaw et al., 1994] and B77-Rat1 [Senger et al., 1994] antibodies to OPN were kind gifts of Dr. Cecilia M. Giachelli (University of Washington, Seattle, WA) and Dr. Donald Senger (Beth Israel Hospital, Harvard Medical School, Boston, MA), respectively. The MPIIIB10_1 mouse monoclonal antibody to rat bone OPN developed by M. Solursh and A. Franzen was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-2-3144 from the NICHD. All other chemicals were purchased from Sigma Chemical Co.

Cell Culture

SMCs were isolated by successive enzymatic digestion of the aortae from adult male Sprague-Dawley rats gavaged with allylamine-HCl (70 mg/kg) or tap water daily for 20 days as previously described [Cox and Ramos, 1990]. This dosing regimen is associated with modulation of SMCs toward a highly mitogen-responsive state and has been proposed as a model of atherogenesis [Ramos, 1990]. Subcultures were maintained in Medium 199 supplemented with 10% FBS and 2 mM glutamine in 5% CO_2:95% air at 37°C. Subcultures were prepared by trypsinization of subconfluent cultures at a 1:4 ratio twice weekly. In these studies, cultures between passage level 15–22 were used and cells were seeded at an initial density of 100 cells/mm^2 unless otherwise noted.

RNA Extraction and Northern Analysis

Total RNA was extracted using a modified version of the procedure published by Chomczynski and Sacchi [1987]. Cells were seeded in 100-mm tissue culture dishes and growth-arrested by serum deprivation for 72 h in 0.1% FBS. Cultures were stimulated with 10% FBS and processed at the desired time points following synchronous cell cycle entry. RNA was extracted with chloroform and precipitated with isopropyl alcohol overnight at −20°C. RNA concentrations were determined spectrophotometrically and 15 µg was loaded onto a 1.2% agarose/1 M formaldehyde denaturing gel and electrophoresed in a 1 × SPC buffer (20 mM Na_2HPO_4, pH 6.8, and 2 mM CDTA) at 50 V for approximately 3 h. Samples were transferred from the gel to a nylon membrane by capillary action for at least 24 h before cross-linking using a UV cross-linker (Stratagene, La Jolla, CA). The membrane was prehybridized at 60°C for 24 h in hybridization buffer containing 5 × SSPE (0.75 M NaCl, 0.05 M Na_2HPO_4, 5 mM EDTA, pH 7.4), 1% SDS, 10% dextran sulfate, 0.1% polyvinyl pyrrolidine, 0.1% Ficoll, and 0.1% bovine serum albumin. The membrane was hybridized to an OPN cDNA (1.4 kb) excised from *Escherichia coli* DNA by Pst I/Xba I endonucleases or human β-tubulin cDNA (1.6 kb) cloned into the EcoRI site of pBluescript vector. The membrane was washed with 1 × SSPE twice at room temperature (15 min) and 1 × SSPE/2% SDS at 65°C for 45 min (2×) before exposure to X-ray film.

SDS-Page/Western Analysis

Allylamine and control cultures were plated on 150-mm plastic culture dishes and, following a 24-h attachment period, serum-free medium containing 25 µg/ml of bovine serum albumin was added for 24 h. Total secreted proteins precipitated with trichloroacetic acid (15%) to give a final protease inhibitor concentration of 0.9 mM phenylmethanesulphonyl fluoride, 1 mM N-ethylmaleimide, 23 mM EDTA, and 45 µg/ml pepstatin A. Following 24 h at 4°C, proteins were pelleted by centrifugation at 6,000*g*. Pellets were washed 2× with ethanol before re-suspending in 300 µl of 40% (v/v) glycerol, 25% (w/v) β-mercaptoethanol, 12% (w/v) SDS, 0.31 M Tris-HCl (pH 6.8), 25 mM EDTA, and 0.1% (w/v) pyronin Y. Protein concentration was determined using a modified Bradford assay [Bradford, 1976]. Samples were boiled for 5 min and 50 µg of protein was electrophoresed on a 12.5% (w/v) polyacrylamide gel according to published methods [Laemmli, 1970]. Proteins were transferred to a membrane overnight (15 V) and blocked for 2 h with 2% Tween/10% nonfat drymilk prior to the addition of the primary antibody (OP-199 1:500; MPIIIB10_1 1:10) for 16 h at 4°C. Rabbit anti-IgG (OP-199, B77-Rat1) or mouse anti-IgG (MPIIIB10_1) was used as the secondary antibody (1:400, 4 h) and proteins visualized using BCIP-NBT tablets. For studies involving the thrombin sensitivity of proteins secreted by allylamine and control SMCs, conditioned media from [^{35}S]methionine labeled allylamine cells was harvested and 5 U/ml of thrombin was added for 1 h at 37°C. Proteins were precipitated as previously described and 50,000 CPM separated on a 12.5%

polyacrylamide gel. Following gel drying, proteins were visualized by autoradiography.

RT-PCR Analysis

OPN gene expression was assessed using Reverse Transcription-Polymerase Chain Reaction (GeneAMP, Perkin Elmer, Oak Brook, IL). Specific primers for rat OPN were designed using Oligo 4.0 (National Biosciences, Plymouth, MN) according to the published sequence of the gene [Olberg et al., 1986], and generated by the Gene Technologies Laboratory at Texas A&M University. The downstream [5′-GCACAGAAAGAACAGAAGCGAAAT-3′, position 1228–1251, rat OPN mRNA] and upstream OPN primers [5′-CAGCCAAGGACCAACTACAACCAT-3′, position 58–81 of rat OPN mRNA] were designed to amplify a 1,194 bp fragment. Total RNA was extracted from randomly cycling control and allylamine SMCs. RNA was extracted with chloroform, precipitated with isopropyl alcohol overnight at −20°C, and then resuspended in nuclease-free H_2O. RNA (0.2 µg) was placed into a reaction mixture containing 5 mM MgCl, 1 × PCR buffer, 1 mM each dNTP, 1 U/µl RNase inhibitor, 2.5 U/µl reverse transcriptase, and 2.5 µM oligo d(T) in a total volume of 20 µl and incubated at room temperature for 10 min. Following 30 min at 42°C, samples were heated for 5 min at 99°C and cooled 5 min at 4°C. For PCR amplification, 2 µM of forward and reverse primers were added to sample with 2 mM MgCl, 0.25 U/µl of Taq polymerase in a total volume of 100 µl. Samples were then amplified by 30 cycles at 57°C as the annealing temperature. Amplified bands were visualized by ethidium bromide staining following separation using a 7.5% nondenaturing polyacrylamide gel as described by Young et al. [1990] to detect OPN splice variants. To determine the specificity of the amplication, reactions were performed without reverse transcriptase, Taq polymerase, or template.

Antibody Studies

Allylamine and control cultures were seeded at a density of 150 cells/mm^2 on 35-mm plastic culture dishes. Following a 3-h attachment period in complete medium (10% FBS), fresh media (10% FBS) containing 1 µCi [^{3}H]-thymidine/dish in the presence or absence of an antibody to OPN or the α_v integrin subunit were added. The antibody to the human α_v integrin subunit has previously been used in studies assessing the role of OPN in SMC attachment and migration [Liaw et al., 1995]. Thymidine incorporation was measured over a 24-h period. As controls, an antibody to the human integrin subunit α_4, as well as heat-inactivated α_v and α_4 (100°C, 10 min) were employed.

Statistics

Analysis of variance (ANOVA) in conjunction with Fisher's post-hoc test was used to determine the statistical significance of differences between control and treated cultures ($P < 0.05$). Values represent the mean ± SEM (n = 4) and experiments repeated in duplicate or triplicate.

RESULTS

Based on the physico-chemical properties of the 52 and 36 kDa proteins secreted by allylamine cells [Ramos et al., 1993], and the role of OPN in atherogenesis [Giachelli et al., 1993], we hypothesized that OPN was overexpressed and differentially processed in allylamine cells to generate a biologically active fragment(s). To test this hypothesis, initial measurements of OPN mRNA levels were conducted in growth-arrested and serum-stimulated cultures of allylamine and control SMCs to monitor gene expression as a function of cell cycle transit (Fig. 1). OPN mRNA levels were elevated in G_o-synchronized cultures of allylamine cells relative to control counterparts, as well as during early cell cycle transit following serum stimulation. Although OPN gene expression is often regarded as mitogen-dependent, expression in allylamine cells was upregulated under growth-arrested conditions.

We next examined OPN secretion into the culture medium of allylamine and control cells. Comparable levels of 56 and 50 kDa proteins recognized by both the B77-Rat1 (data not shown) and OP-199 antibodies to OPN were secreted over a 24-h period by control and allylamine cells (Fig. 2). Control cells also secreted a 52 kDa protein recognized by OP-199 that was also seen, to a lesser extent, in allylamine cells. Allylamine cells, however, exhibited increased amounts of a 36 kDa protein recognized by the OP-199 antibody.

Thrombin has previously been demonstrated to cleave OPN into two fragments in the 35 kDa range [Senger et al., 1994] and, therefore, we hypothesized that the 36 kDa protein expressed by allylamine cells represented a proteolytic

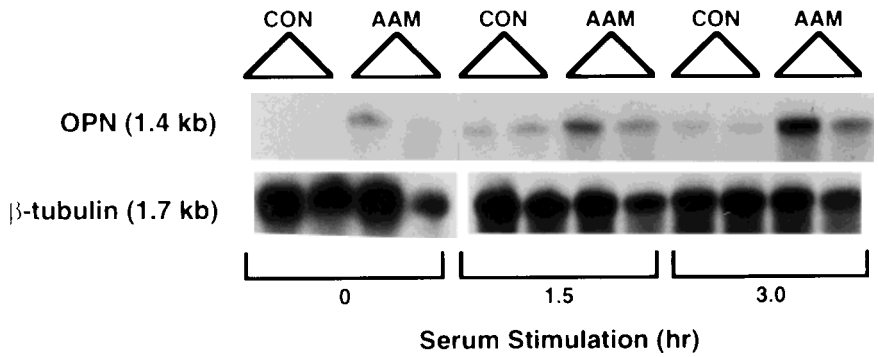

Fig. 1. Time course of OPN mRNA expression in serum-stimulated cultures of allylamine (AAM) and control (Con) SMCs. SMCs were synchronized in G_o by serum deprivation in 0.1% FBS for 72 h prior to mitogenic stimulation with 10% FBS for 1.5 or 3 h. Cells were harvested and total RNA extracted, separated and hybridized to a ^{32}P-labeled OPN or β-tubulin probe as described in Materials and Methods. Two different AAM and CON cell strains were used and similar results were seen in two independent experiments.

fragment of OPN. The ability of thrombin to cleave the 52 kDa protein was investigated in separate experiments by the addition of thrombin to the conditioned media of metabolically labeled allylamine SMCs. The addition of thrombin results in a marked decrease in the intensity of the 52 kDa band concomitant with the appearance of a 36 kDa protein in allylamine cells (Fig. 3). These data implicate proteolytic cleavage of OPN in the formation of the fragment present in the conditioned media of allylamine cells.

To determine if OPN processing occurred at the mRNA level, RT-PCR analysis of OPN gene expression was conducted. Amplification of OPN mRNA by RT-PCR revealed a 1.2 kb band in both control and allylamine cells, consistent with the predicted size of the full-length wild-type OPN mRNA (Fig. 4). Taken together, these results suggest that OPN protein is processed differentially in allylamine cells resulting in increased levels of the 36 kDa fragment.

To assess the role of integrin-related signaling in the proliferative capacity of allylamine cells, an antibody to the OPN receptor was employed to measure DNA synthetic rates in response to mitogenic stimulation as a measure of proliferative capacity. The α_v integrin antibody decreased DNA synthesis in allylamine cells, but was without effect in control cells (Fig. 5). The specificity of this response was examined using antibodies either to the α_4 integrin subunit, an integrin not implicated in OPN function, or heat-inactivated antibodies. Addition of these antibodies did not influence the mitogenic behavior of allylamine cells or control counterparts.

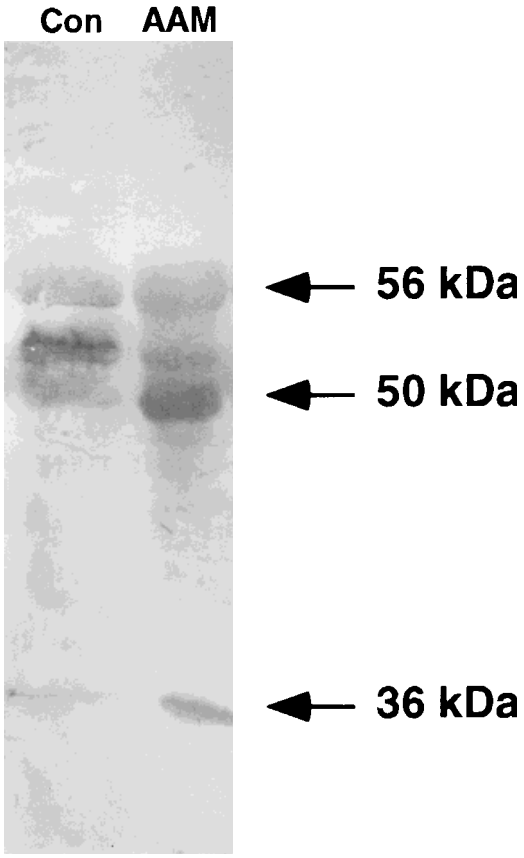

Fig. 2. Western blot analysis of osteopontin secreted into the media by allylamine (AAM) and control (Con) cells. Fifty micrograms of protein collected from serum-free cultures over a 24-h period were separated on a 12.5% polyacrylamide gel. Proteins were transfered to a membrane blocked for 2 h with 2% Tween/5% dry milk prior to addition of the primary antibody (OP-199 1:500) for 16 h. Rabbit anti-IgG conjugated to alkaline phosphatase was used as the secondary antibody (1:400) and proteins visualized using the alkaline phosphatase method. Similar results were seen in two independent experiments using different strains of control and allylamine cells.

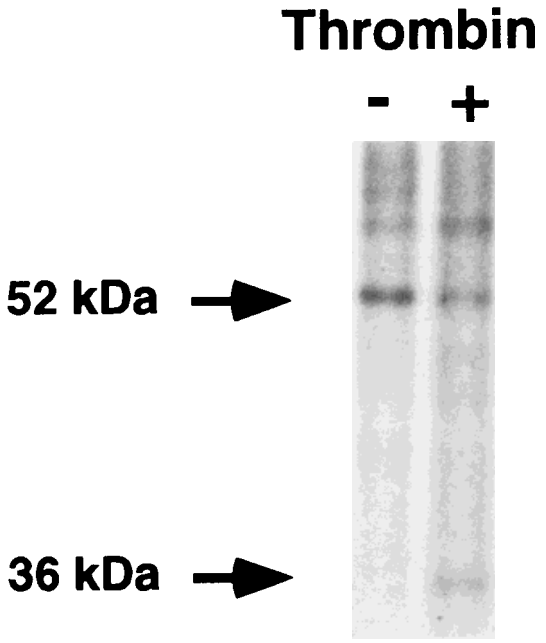

Fig. 3. Effect of exogenous thrombin on the relative abundance of the 52 kDa protein secreted by allylamine SMCs. Exogenous thrombin (5 U/ml) was added to conditioned media harvested from cultures of [^{35}S]methionine-labeled allylamine SMCs for 1 h. Fifty thousand CPM was separated on a 12.5% polyacrylamide gel and visualized by autoradiography. Similar results were seen in two independent experiments using different strains of control and allylamine cells.

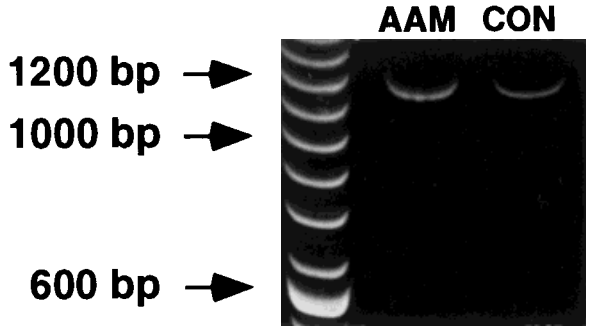

Fig. 4. OPN mRNA expression in randomly cycling cultures of allylamine (AAM) and control (Con) SMCs as assessed by RT-PCR. OPN gene expression was determined using specific primers as described in Materials and Methods. Similar results were seen in two independent experiments using different strains of control and allylamine cells.

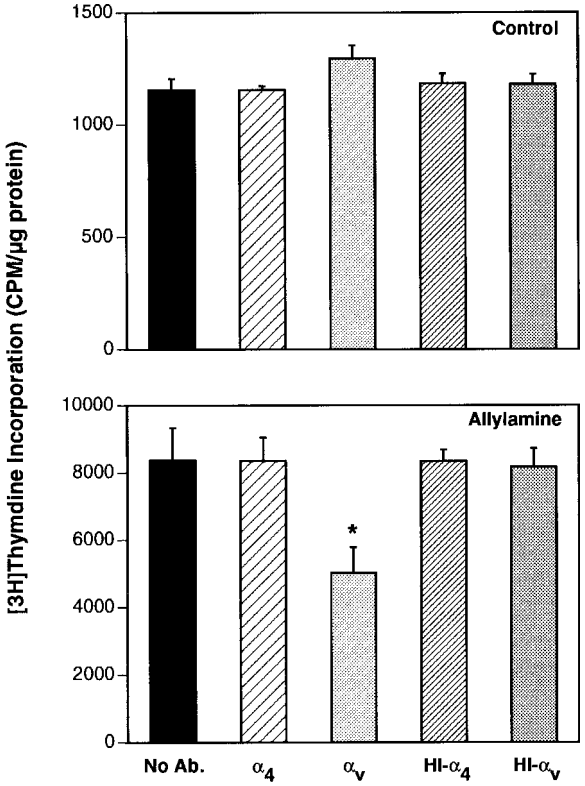

Fig. 5. [^{3}H]thymidine incorporation in cycling cultures of control and allylamine SMCs upon incubation for 24 h in the presence of an antibody to the human α_v integrin subunit. Following a 3-h attachment period, thymidine incorporation was measured upon addition of 1 µCi of [^{3}H]thymidine per dish over a 24-h period in the presence or absence of an antibody to the human α_4 integrin subunit, α_v integrin subunit, or heat-inactived (HI) antibodies. Data points represent the mean ± SEM of three dishes. *Significant difference from the respective control (no antibody) at the $P < .05$ level. Different scales are used for the y-axes. Similar results were seen in two independent experiments using different strains of control and allylamine cells.

DISCUSSION

The evidence presented here suggest that the proliferative SMC phenotype induced by allylamine is associated with expression of a biologically active proteolytic fragment of OPN. As in other forms of cellular injury [reviewed in Denhardt and Guo, 1993], upregulation of OPN gene expression in allylamine cells may be secondary to injury of SMCs following oxidative deamination of allylamine [Ramos et al., 1988]. However, in our cell system alterations in OPN expression and processing are sustained during serial propagation, suggesting that permanent deregulation of this pathway has occurred. A role for OPN and/or biologically active fragments of OPN in the proliferative phenotype induced by allylamine is consistent with our previous finding that control SMCs acquire a proliferative advantage comparable to that of allylamine cells when seeded on a matrix deposited by allylamine cells [Ramos et al., 1993]. In addition, expression of the enhanced proliferative capacity of allylamine cells as compared to controls is dependent on cellular interaction

with GRGDS-containing substrates (Parrish et al., manuscript submitted).

OPN gene expression is upregulated in allylamine cells relative to controls during G_o synchronization, as well as during early cell cycle progression. The time course of serum inducibility was similar to that reported by Gadeau et al. [1993] in which maximal induction of OPN gene expression occurred within 5 h of mitogen stimulation. OPN is minimally expressed under basal conditions and peaks during the early G_1 phase following serum stimulation of quiescent cells [Gadeau et al., 1993]. Because allylamine cells arrest in the G_o phase of the cycle following serum deprivation and density arrest [Bowes and Ramos, 1993], overexpression of OPN is likely not due to increased cell cycle–related activity, but rather, regulated by factors generated following mitogenic challenge. The serum inducibility of OPN in allylamine cells suggests that enhanced expression is not related to loss of serum-dependence.

Assessment of OPN expression in control and allylamine cells by Western blot analysis revealed comparable levels of 52 and 50 kDa proteins, respectively. This finding is not consistent with the overexpression of OPN mRNA levels in allylamine cells. Senger et al. [1996] also failed to detect increased soluble OPN in cells overexpressing OPN mRNA and suggested that this may be due to retention of OPN at the cell surface/receptors, or within the extracellular matrix. In our studies, this represents an attractive possibility since the proliferative advantage of allylamine cells involves secreted products that are sequestered in the matrix [Ramos et al., 1993]. Alternatively, the lack of correlation between mRNA and protein levels may be accounted for by differences in the degree of mitogenic stimulation since Northern analysis was conducted in mitogen-stimulated cultures, while OPN protein secretion was assessed under serum-free conditions. This possibility, however, was ruled out in experiments showing that OPN protein levels are comparable in mitogenically stimulated cultures of control and allylamine cells (data not shown). Furthermore, similar levels of parental OPN protein in allylamine and control SMCs can be accounted for by increased proteolytic cleavage of the parent protein in allylamine cells, leading to overexpression of the immunoreactive 36 kDa protein. This, in fact, may be the case since the total amount of immunoreactive proteins was increased in allylamine cells. Collectively, our results suggest that the salient difference between allylamine and control SMCs involves the appearance of the 36 kDa OPN fragment.

While the mechanism responsible for generation of the 36 kDa fragment is not known, the thrombin sensitivity of the 52 kDa protein secreted into the media of allylamine cells, coincident with the expression of unspliced OPN mRNA in allylamine cells, suggests that overexpression of the small OPN fragment results from enhanced proteolytic cleavage of OPN. It is of interest to note that the expression of a 32 kDa protein increases as a function of serial passage in control SMCs (Parrish and Ramos, unpublished results). Given that SMCs spontaneously modulate to more proliferative phenotypes as a function of serial passage, the appearance of the 32 kDa protein may be linked to proliferative status in these cells.

The finding that thrombin catalyzes the proteolytic cleavage of OPN to generate biologically active fragment(s) raises the possibility that the allylamine phenotype involves alterations in thrombin receptor and/or thrombin-related signaling. A role for the thrombin receptor in the upregulation of proliferative capacity in SMCs has been proposed on the basis that α-thrombin stimulates SMC proliferation by proteolytic activation of thrombin receptor [McNamara et al., 1993; Fager, 1995]. The hypothesis that an OPN and/or its proteolytic fragment(s) participate in the enhanced mitogenic responsiveness of allylamine cells is supported by the finding that antibodies which recognize the α_v integrin subunit, nullify the proliferative advantage of allylamine cells relative to control SMCs. Evidence that an OPN fragment retains biologic function has been demonstrated by others in studies showing that thrombin cleavage of OPN modulates the adhesive and chemotactic properties of the protein [Senger et al., 1994; Xuan et al., 1994].

A key role for OPN has been postulated in the regulation of migration [Liaw et al., 1994, 1995; Yue et al., 1994], and possibly proliferation [Giachelli et al., 1991; Gadeau et al., 1993], during the pathologic progression of several disorders. In the case of atherosclerosis and re-stenosis, this view is based on the findings that OPN is sequestered within the matrix of the vascular wall and is present in human atherosclerotic plaques [Giachelli et al., 1993]. In addition, blockade of $\alpha_v\beta_3$ functions reduces

vascular lesion formation following injury in vivo [Choi et al., 1994; Matsuno et al., 1994]. The data shown demonstrate that alterations in the expression and processing of OPN characterize the proliferative SMC phenotype induced following allylamine injury in vivo. In addition, the findings that blockade of $\alpha_v\beta_3$-mediated signaling nullifies the proliferative advantage of allylamine cells relative to control SMCS suggest that OPN and/or a fragment of OPN participates in proliferative regulation of SMCs.

ACKNOWLEDGMENTS

The authors thank Ms. Edna Sanchez for her technical assistance, Nyla Harper for her assistance with Western blotting, and Cody L. Wilson for assistance with primer design. The authors acknowledge Dr. Cecilia M. Giachelli (University of Washington, Seattle, WA) and Dr. Donald Senger (Beth Israel Hospital, Harvard Medical School, Boston, MA) for kindly providing the OP-199 and B77-Rat1 antibodies, respectively. This work was supported in part by grant ES04849 to K.S.R. from the National Institute of Environmental Health Sciences. K.S.R. is the recipient of Research Career Development Award ES 00213. A.R.P. is a pre-doctoral fellow in NIEHS Toxicology Training (grant ES 07273).

REFERENCES

Behrend EI, Craig AM, Wilson SM, Denhardt DT, Chambers AF (1994): Reduced malignancy of ras-transformed NIH3T3 cells expressing antisense osteopontin mRNA. Cancer Res 54:832–837.

Boor PJ, Nelson TJ, Chieco P (1980): Allylamine cardiotoxicity. II. Histopathology and histochemistry. Am J Pathol 100:739–751.

Bowes III RC, Ramos KS (1993): Allylamine enhances c-Ha-ras protooncogene expression in rat aortic smooth muscle cells. Toxicol Lett 66:263–272.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Chen J, Singh K, Mukherjee BB, Sodek J (1993): Developmental expression of osteopontin (OPN) mRNA in rat tissues: Evidence for a role of OPN in bone formation and resorption. Matrix 13:113–123.

Choi ET, Engel L, Callow AD, Sun S, Trachtenberg J, Santoro S, Ryan US (1994): Inhibition of neointimal hyperplasia by blocking alpha v beta 3 integrin with a small peptide antagonist gpenGRGDSPCA. J Vasc Surg 19:125–134.

Chomczynski P, Sacchi N (1987): Single step method RNA isolation by acid guanidium thiocyanate phenol-chloroform extraction. Anal Biochem 162:156–159.

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

Denhardt DT, Guo X (1993): Osteopontin: A protein with diverse functions. FASEB J 7:1475–1482.

Fager G (1995): Thrombin and proliferation of vascular smooth muscle cells. Circ Res 77:645–650.

Gadeau AP, Campan M, Millet D, Candresse T, Desgranges C (1993): Osteopontin overexpression is associated with arterial smooth muscle cell proliferation in vitro. Arter Thromb 13:120–125.

Gardner HA, Berse B, Senger DR (1994): Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed Rat1 fibroblasts. Oncogene 9:2321–2326.

Giachelli C, Bae N, Lombardi D, Majesky M, Schwartz S (1991): Molecular cloning and characterization of 2B7, a rat mRNA which distinguishes smooth muscle cell phenotypes in vitro and is identical to osteopontin (secreted phosphoprotein 1, 2aR). Biochem Biophys Res Commun 177:867–873.

Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM (1993): Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J Clin Invest 92:1686–1696.

Hwang S-M, Lopez CA, Heck DE, Gardner CR, Laskin DL, Laskin JD, Denhardt DT (1994): Osteopontin inhibits induction of nitric oxide synthase activity by inflammatory mediators in mouse kidney epithelial cells. J Biol Chem 269:711–715.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lalich JJ, Allen JR, Paik WCW (1972): Myocardial fibrosis and smooth muscle cell hyperplasia in coronary arteries of allylamine-fed rats. Am J Pathol 66:225–233.

Liaw L, Almeida M, Hart CE, Schwartz SM, Giachelli CM (1994): Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. Circ Res 74:214–224.

Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, Giachelli CM (1995): The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. J Clin Invest 95:713–724.

Matsuno H, Stassen JM, Vermylen J, Deckmyn H (1994): Inhibition of integrin function by a cyclic RGD-containing peptide prevents neointima formation. Circulation 90:2203–2206.

McNamara CA, Sarembock IJ, Gimple LW, Fenton JW, Coughlin SR, Owens GK (1993): Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by proteolytically activated receptor. J Clin Invest 91:94–98.

Olberg A, Franzen A, Heinegard D (1986): Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an arg-gly-asp cell-binding sequence. Proc Natl Acad Sci USA 83:8819–8823.

Ramos K (1990): Comparative angiotoxic responses of avian and rodent species: Implications in atherogenesis. J Toxicol Environ Health 29:357–376.

Ramos K, Cox LR (1987): Primary cultures of aortic smooth muscle cells: I. An in vitro model to study xenobiotic-induced vascular cytotoxicity. In Vitro Cell Dev Biol 23: 288–296.

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.

Ramos K, Grossman SL, Cox LR (1988): Allylamine-induced vascular toxicity in vitro: Prevention by semicarbazide-sensitive amine oxidase inhibitors. Toxicol Appl Pharmacol 96:61–71.

Ramos KS, Weber TJ, Liau G (1993): Altered protein secretion and extracellular matrix deposition is associated with the proliferative phenotype induced by allylamine in aortic smooth muscle cells. Biochem J 289:57–63.

Ross R (1993): The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature 362:801–809.

Sauk JJ, Van Kampen CL, Norris K, Foster R, Somerman MJ (1990): Expression of constitutive and inducible HAS70 and HSP47 is enhanced in cells persistently spread on OPN or collagen. Biochem Biophys Res Commun 172:135–142.

Schwartz SM, deBlois D, O'Brien ERM (1995): The intima: Soil for atherosclerosis and restenosis. Circ Res 77:445–465.

Senger DR, Perruzzi CA, Papadopoulos A (1989): Elevated expression of secreted phosphoprotein 1 (osteopontin, 2ar) as a consequence of neoplastic transformation. Anticancer Res 9:1291–1300.

Senger DR, Perruzzi CA, Papdopoulos-Sergiou A, Van de Water L (1994): Adhesive properties of osteopontin: Regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. Mol Biol Cell 5:565–574.

Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Perruzzi CA, Detmar M (1996): Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the $\alpha_v\beta_3$ integrin, osteopontin and thrombin. Am J Pathol 149:293–305.

Su ZZ, Austin VN, Zimmer SG, Fischer PB (1993): Defining the critical gene expression changes associated with expression and suppression of the tumorigenic and metastatic phenotype in Ha-ras-transformed cloned rat embryo fibroblast cells. Oncogene 8:1211–1219.

Xuan J-W, Hota C, Chambers AF (1994): Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. J Cell Biochem 54:247–255.

Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, Fisher LW (1990): cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics 7:491–502.

Yue T-L, McKenna PJ, Ohlstein EH, Farach-Carson MC, Butler WT, Johanson K, McDevitt P, Feuerstein GZ, Stadel JM (1994): Osteopontin-stimulated vascular smooth muscle cell migration is mediated by β3 integrin. Exp Cell Res 214:459–464.