Altered protein secretion and extracellular matrix deposition is associated with the proliferative phenotype induced by allylamine in aortic smooth muscle cells

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Repeated cycles of allylamine-induced aortic injury *in vivo* modulate the proliferative potential of smooth muscle cells (SMCs) during serial propagation *in vitro*. This modulation may be partly mediated by disturbances in polyphosphoinositide metabolism which afford allylamine-treated cells a growth advantage over control cells [Cox, Murphy and Ramos (1990) *Exp. Mol. Pathol.* **53**, 52–63]. The present studies were conducted to further evaluate the mechanisms which mediate the enhanced proliferative potential of allylamine cells. Cellular growth and/or [³H]thymidine incorporation into DNA were evaluated in control and allylamine cells seeded on plastic culture dishes or glass coverslips in the presence of 0.1, 1 or 10 % fetal bovine serum (FBS). On either substrate, incubation in 0.1 % FBS for 48 h inhibited DNA synthesis in cultures of both cell types, but the inhibitory response was more pronounced in allylamine cells. Subsequent challenge with 10 % FBS increased thymidine incorporation to a greater extent in allylamine cells. Interestingly, enhanced DNA synthesis of allylamine cells was associated with increased cell numbers only when seeded on a glass surface. The

enhanced growth rate on glass was not due to increased plating efficiency since comparable attachment rates were observed for both cell types. Reseeding of control cells on glass substrates pre-coated by allylamine cells afforded control cells a growth advantage comparable with that observed for allylamine cultures. Conditioned media from growth-arrested, as well as cycling cultures, of allylamine cells stimulated DNA synthesis in cultures of either cell type to a greater extent than conditioned media from control counterparts. In addition, the responsiveness of allylamine cells to secreted products was enhanced relative to that of control cells. Metabolic labelling studies revealed that the synthesis and/or secretion of 52, 46, 33 and 28 kDa proteins was enhanced in allylamine cultures relative to controls, and that the expression of two proteins of 30 and 31 kDa only occurred in allylamine cultures. We conclude that the enhanced growth response of allylamine cells is associated with both altered protein secretion and differential extracellular matrix deposition.

INTRODUCTION

Vascular injury induced by biological or chemical agents is often associated with smooth muscle cell (SMC) proliferation [1–5]. These lesions are morphologically similar to those induced by experimental manipulations such as balloon embolectomy [6,7] and may mimic the process of spontaneous atherogenesis [8,9]. Recent efforts in this laboratory have focused on the study of allylamine-induced vascular injury as a tool to examine the cellular and molecular mechanisms involved in vascular SMC growth and differentiation. Allylamine (3-aminopropene) is a toxic aliphatic amine which exhibits an unusual degree of vascular selectivity when administered by a variety of routes in several animal species [10]. The toxic effects of this chemical are associated with the development of vascular lesions which resemble those found in atherosclerotic vessels [11]. As in other experimental models of atherogenesis [12,13], repeated cycles of vascular injury upon exposure of rats to allylamine *in vivo* modulates the proliferative behaviour of aortic SMCs in culture [14]. This modulation is associated with morphological and biochemical alterations which are preserved after serial propagation in culture [14].

At the ultrastructural level, SMCs isolated from allylamine-

treated rats (henceforth referred to as 'allylamine cells') and established in primary culture display a rounded morphology, an extensive network of rough endoplasmic reticulum, and reduced myofilament density [14]. Such features are consistent with the loss of contractile responsiveness to noradrenaline and increased cellular proliferation in allylamine cells relative to cells from control animals. The proliferative advantage of allylamine cells may be mediated by enhanced polyphosphoinositide metabolism [15]. Measurement of ³²P incorporation into components of the phosphoinositide cycle revealed that allylamine affects, either directly or indirectly, both precursors and products of phosphoinositide metabolism. Thus the ability of polyphosphoinositide-derived second messengers to initiate the cascade of cellular events which culminates in mitosis is enhanced by allylamine treatment.

The present studies were conducted (1) to evaluate the influence of growth conditions *in vitro* on the expression and maintenance of the proliferative state induced by allylamine and (2) to determine if alterations in extracellular matrix (ECM) synthesis and/or deposition mediate the proliferative advantage of SMCs associated with allylamine treatment *in vivo*. Our results show that allylamine-induced modulation of aortic SMC proliferation is mediated by alterations in the synthesis and/or deposition of

ECM components, which in turn regulate proliferation programmes in cultured aortic SMCs.

MATERIALS AND METHODS

Materials

Allylamine (99% purity) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Fetal bovine serum (FBS) was purchased from Intergen (New York, NY, U.S.A.). L-[2,3-³H]Proline was obtained from New England Nuclear (Boston, MA, U.S.A.) and [³⁵S]methionine was obtained from Amersham (Arlington Heights, IL, U.S.A.). Fluorescein isothiocyanate-conjugated anti-mouse IgG was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Type I collagen was from Upstate Biotechnology (Lake Placid, NY, U.S.A.), and vitronectin was from Telios Pharmaceuticals (San Diego, CA, U.S.A.).

Cell culture

SMCs were isolated by successive enzymic digestion of aortae from Sprague–Dawley rats (Sasco, Houston, TX, U.S.A.) (175–200 g) gavaged with allylamine hydrochloride (70 mg/kg) or tap water daily for 20 days [16]. This dosing regimen is associated with the development of aortic lesions [17]. Cells were grown in Medium 199 supplemented with 10% FBS and 2 mM glutamine in 5% CO₂/95% air at 37 °C. Secondary cultures were prepared by trypsinization of subconfluent cultures. Cells were harvested weekly and split at a 1:4 ratio. All experiments were conducted in secondary cultures at passage levels 3–8.

Cell growth assays

Subcultured cells were seeded on plastic culture dishes or glass coverslips at a density of 600 cells/mm² and grown to confluence. Cultures of both cell types were growth-arrested by incubation in 0.1% (v/v) FBS for 48 h and subsequently challenged with 1% or 10% (v/v) serum for various times. Cells were trypsinized and then counted using a haemocytometer. For measurements of DNA synthesis, cultures were labelled with 0.5–5 µCi/ml [³H]thymidine triphosphate for 1 h and processed as described previously [14].

RNA isolation and Northern or slot blot analysis

Total RNA was isolated as described by Liao and Chan [18] with minor modifications. Cells were washed with PBS, and subsequently 4.5 ml of 4 M guanidine isothiocyanate containing 30 mM sodium acetate and 150 mM β-mercaptoethanol, pH 7.0, was added. The solution was passed 20 times through a 19-gauge needle and layered on to a 5.7 M caesium chloride cushion containing 30 mM sodium acetate, pH 5.0. After centrifugation, the RNA pellet was resuspended in 10 mM Tris/HCl/1 mM EDTA, pH 8.0, and extracted by phenol/chloroform (1:1, v/v) and chloroform/2-methylpropan-1-ol (4:1, v/v) respectively. The RNA was ethanol-precipitated and the concentration and purity determined by measurement of absorbance at 260, 280 and 320 nm. The purified RNA was electrophoresed on a 1% (w/v) denaturing formaldehyde agarose gel, transferred to nitrocellulose and hybridized under stringent conditions [18]. Slot blots were performed using a Schleicher & Schuell apparatus as previously described [19]. A mouse 1.1 kb α-smooth muscle actin cDNA probe was a gift from Drs. B. H. Min and A. R. Strauch (The Ohio State University) [20]. DNA probes used for

the detection of α₁(III) and α₁(IV) collagen, fibronectin and thrombospondin mRNA levels have been described previously [18]. Quantification of autoradiograms was done using a Hoefer GS 300 scanning densitometer.

Collagen synthesis

Subconfluent cultures were labelled with 5 µCi/ml [2,3-³H]-proline in serum-free medium containing 50 µg/ml ascorbate. After 24 h the cells were rinsed and processed to determine cellular collagen synthesis as described previously [14]. Cells were suspended in Hepes buffer (pH 7.4) and sonicated at 50 W for 20 s. Trichloroacetic acid (10%, w/v) was added to the cell suspensions and proteins were pelleted by centrifugation. Pelleted proteins were then suspended in 0.1 M NaOH and placed at 37 °C for 5 min with occasional shaking to facilitate protein dissolution. Aliquots of samples (200 µl) were added to a reaction mixture containing 0.08 M HCl, 60 µM Hepes (pH 7.2), 2.5 mM *N*-ethylmaleimide, 0.25 µmol of CaCl₂ and 5.2 µg of collagenase (Type III). Samples were incubated at 37 °C with shaking for 1.5 h. Trichloroacetic acid was added to each sample to terminate the collagenase digestion. Samples were then centrifuged to pellet undigested protein, and the supernatant was counted for radioactivity by liquid scintillation. Blanks were subtracted from each collagenase-treated sample.

Conditioned media

Subconfluent cultures of SMC were incubated for 65 h in Medium 199 containing 0.1% or 10% (v/v) FBS. Cultures were then incubated in serum-free medium for 24 h, after which the medium was collected and centrifuged for 10 min at 500 g. Conditioned medium from both growth-arrested and cycling cultures of allylamine and control cells was used to grow freshly seeded cells. The extent of [³H]thymidine incorporation after cross-treatment with conditioned media was measured 12, 24 and 48 h after initial seeding.

³⁵S labelling of ECM proteins

Cycling SMC cultures were washed with methionine-free medium for 30 min and then incubated for 6 h in methionine- and serum-free medium containing 50 µCi/ml of L-[³⁵S]methionine (specific radioactivity 1000 Ci/mmol) and 100 µg/ml BSA. Conditioned medium containing 0.9 mM phenylmethanesulphonyl fluoride, 1 mM *N*-ethylmaleimide, 23 mM EDTA and 45 µg/ml pepstatin A was harvested, and total secreted proteins were precipitated with trichloroacetic acid (15%, w/v) and incubated at 4 °C for 1 h. Proteins were pelleted by centrifugation at 6000 g. Pellets were dried and resuspended 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. Samples were boiled for 5 min and a 5 µl aliquot was counted for radioactivity in a scintillation counter. Approx. 50000 c.p.m. of sample was electrophoresed on a 7% or 10% (w/v) polyacrylamide gel according to the method of Laemmli [20a]. Gels were dried and processed for quantification of individual bands using a Betascope 603 Blot Analyzer or exposed to X-Omat X-ray film for autoradiography.

Cell adhesion assay

Appropriate ECM substrates were diluted in coating buffer (26 mM Na₂CO₃, 74 mM NaHCO₃, pH 9.5), incubated in micro-

Figure 1 Growth of aortic SMCs from control (■) and allylamine-treated (●) animals in response to 1 or 10% FBS on plastic (a) or glass (b)

Subcultured cells at passage level three were seeded at a density of 600 cells/mm² and grown to confluence. Results shown represent the average number of cells in three replicate dishes for respective cell types. Similar patterns were obtained in four separate experiments. Cultures were trypsinized and counted in a haemocytometer. The S.E.M. for individual points never exceeded 5% of respective means.

titre dishes overnight at 4 °C and subsequently blocked with 3 mg/ml BSA for 2 h at room temperature. Cells were harvested and equilibrated for 30 min and a total of 1×10^6 cells were incubated per well for 1 h at 37 °C. After incubation, cells were washed three times with PBS and 50 μ l of 8% (w/v) glutaraldehyde was added for 20 min. The cell layer was washed with PBS and stained with 0.2% (w/v) Crystal Violet for 10 min. Attached cells were evaluated by microscopy and quantification was performed on an e.l.i.s.a. reader at 570 nm.

Statistics

Where appropriate, Student's *t* test or analysis of variance were used to assess the statistical significance of the differences observed. Results always represent the mean $\pm$ S.E.M. Asterisks denote statistically significant differences at the $P < 0.05$ level.

RESULTS

Enhanced growth of allylamine cells is substrate-dependent

The DNA synthetic rate of control and allylamine SMCs seeded on plastic dishes before and after serum challenge was examined. The mitogenic response of allylamine cells was significantly higher than that of controls. Incubation of SMCs on plastic with 10% FBS for 24 h increased thymidine incorporation by $124.1 \pm 21.7\%$ in controls and $223.6 \pm 48.8\%$ in allylamine cells ($n = 5$, $P < 0.05$). The enhanced response was not substrate-dependent, since increased DNA synthesis was also observed

when cells were seeded on a glass surface (results not shown). These results suggest that SMCs derived from allylamine-treated animals maintained an enhanced DNA synthetic ability in serial culture. We next examined whether a difference in cell proliferation between control and allylamine cells is also present. Rat SMCs were seeded on plastic or glass and counted at various times following serum challenge. No difference in the proliferative capacity of control and allylamine cells was observed when cells were seeded on plastic dishes (Figure 1a). However, allylamine cells grew more rapidly and maintained a higher cell density when seeded on a glass surface (Figure 1b).

This response was independent of the serum concentration in the medium and was observed at all times tested. However, it should be noted that the growth of allylamine cells on either substrate was serum-dependent. Because differences in growth potential on glass may be due to differential attachment of cells after plating, studies were conducted to assess the plating efficiency of control and allylamine cells on a glass surface. The data presented in Table 1 show that control and allylamine cells attached readily to glass, reaching similar maximal attachment within 120 min. Collectively, these results demonstrate that SMCs isolated from allylamine-treated animals exhibit an enhanced proliferative capacity when seeded on a glass substrate and that this pattern is not due to differential plating efficiency.

Altered ECM synthesis and deposition may account for the growth advantage of allylamine cells

Since allylamine cells seeded on glass were able to proliferate more rapidly and grow to a higher cell density than control cells,

Table 1 Plating efficiency of subcultured aortic SMCs isolated from control and allylamine-treated animals seeded on a glass substrate

SMCs were allowed to attach for 5, 10, 30, 60, 120 or 180 min and subsequently trypsinized and counted. Cells were seeded at a density of 300 cells/mm² in growth medium containing 10% FBS to a final density of 1×10^5 cells/ml. Similar results were observed in three separate experiments using three different cell strains. Results shown represent the means $\pm$ S.E.M. of three replicate dishes.

Time (min)	$10^{-4} \times$ Cell number	
	Control	Allylamine-treated
5	4.9 ± 1.2	6.8 ± 0.8
10	18.7 ± 1.0	18.3 ± 3.5
30	59.3 ± 8.9	53.8 ± 6.4
60	63.9 ± 7.5	74.7 ± 14.8
120	85.7 ± 9.9	88.0 ± 6.2

Figure 2 DNA synthesis in cross-plated aortic SMCs from control and allylamine-treated animals in secondary culture

Subcultures of control (C) or allylamine (AAM) cells were grown to confluence for 5 days. Cultures were then trypsinized and immediately cross-plated into original control (C) or allylamine (AAM) dishes at a density of 150 cells/mm² under standard growth conditions. Results shown represent the means $\pm$ S.E.M. of three replicate cultures. Similar results were observed in two additional experiments. * denotes significance at the $P < 0.05$ level.

Table 2 Cross-plating of subcultured aortic SMCs from control (C) and allylamine (AAM)-treated animals seeded on a glass substrate

Cells were seeded at a density of 200 cells/mm². Numbers represent the mean cell counts for duplicate dishes. Similar results were observed in two separate experiments using different cell strains. NA, not available.

Cross-plating profile	Days after plating ...	$10^{-4} \times$ Cell number		
		4	5	7
C-C		10.2	14.2	14.0
C-AAM		21.2	24.9	30.8
AAM-AAM		25.0	23.3	31.4
AAM-C		26.3	28.1	NA

we next examined whether the ECM deposited by these cells can influence the proliferation of control cells. The results shown in Figure 2 indicate that control cells seeded on a substrate synthesized by allylamine cells exhibited an almost 3-fold increase in DNA synthetic ability when compared with control cells seeded on a substrate synthesized by control cells. In addition,

Figure 3 Cellular collagen synthesis in subconfluent cultures of control (□) and allylamine (▨) SMCs

Five-day-old cultures were labelled with 5 μ Ci/ml [³H]proline for 24 h. Proline incorporation was assayed after a collagenase (Type III) digestion of cellular protein. Results are expressed as c.p.m. per μ g of cellular protein. Values represent the means $\pm$ S.E.M. of five replicate dishes. * denotes significance at the $P < 0.05$ level for individual comparisons as shown by the brackets. Enhanced proline was also observed in the medium.

Figure 4 Expression of smooth muscle α -actin, fibronectin, thrombospondin, $\alpha_1(\text{III})$ collagen and $\alpha_1(\text{IV})$ collagen mRNA levels in control and allylamine SMCs

SMCs were seeded at 8×10^3 cells/cm² and cultured for 4 days. Fresh medium containing 10% FBS was added to the dishes 10 h prior to harvesting for RNA. Total RNA isolated from six independent cell strains were analysed for expression of specific mRNAs by Northern or slot-blot analysis. 1A, 5A and 6A are allylamine cells, while 1C, 9C and 11C are control cells. (a) Northern blot analysis of smooth muscle α -actin. Arrows mark the β - and α -actin species. (b) Slot-blot analysis of ECM mRNA levels; 1 and 5 μ g portions of RNA were analysed to ensure linearity and consistency of detection, but only the 1 μ g slots are shown. Lane 1, fibronectin; lane 2, thrombospondin; lane 3, type III collagen; lane 4, type IV collagen.

allylamine cells seeded on a control substrate continued to exhibit enhanced DNA synthetic capacity.

We next examined whether the enhanced DNA synthesis observed in allylamine cells actually reflected an increase in cell number. Similar cross-seeding experiments were performed and cell numbers were determined after 4, 5 and 7 days (Table 2). We found that by day 4 control cells seeded on the substrate synthesized by allylamine cells exhibited a 100% increase in cell number when compared with control cells re-seeded on control substrates. This difference in cell number was maintained after 7 days, when control cells have reached a saturating density.

These observations suggest that the increased proliferative capacity of allylamine cells may be due to a difference in the deposition of ECM components. This possibility is consistent

Figure 5 DNA synthesis in control (□) and allylamine (▨) aortic SMCs challenged with conditioned media from growth-arrested and cycling cultures of either cell type for 24 h

Values represent the means $\pm$ S.E.M. of five replicate dishes. *denotes significance at the $P < 0.05$ level for individual comparisons as shown by the brackets.

Figure 6 SDS/PAGE of medium proteins secreted by control and allylamine SMCs seeded on plastic

Subcultured smooth muscle cells between the passages 5 and 7 were grown for 5 days in Medium 199 containing 10% FBS and then pulsed for 6 h with [35 S]methionine in serum- and methionine-free medium. Radiolabelled secreted proteins were harvested into protease inhibitors and visualized by SDS/PAGE (7% or 10% gels) and autoradiography. Several protein bands and their apparent molecular masses (kDa) as indicated by arrows were consistently enhanced in allylamine cultures relative to controls. Lanes 1–4 show four control cell strains and lanes 5–8 show four allylamine cell strains. Similar results were obtained in three separate experiments.

with our finding that allylamine cells were able to synthesize more proline-containing protein than control cells (Figure 3), suggesting that they may produce a larger amount of collagen than SMCs obtained from the aortae of control animals.

The expression of smooth muscle α -actin was assayed to determine whether decreased expression of this contractile protein gene was associated with the increased proliferative capacity

exhibited by allylamine cells. Six independent strains of control and allylamine SMCs were examined. We found that both control and allylamine cells expressed 2.1 and 1.6 kb mRNAs that were recognized by the smooth muscle α -actin probe (Figure 4a). The 2.1 kb message represents cross-hybridization to the β - and γ -actin species, while the 1.6 kb mRNA is the smooth muscle α -actin [18]. Clearly, the expression of smooth muscle α -actin was undiminished in allylamine cells, indicating that the increased proliferative capacity of these cells was not associated with decreased expression of this gene. Based on these data and on the analysis of four additional allylamine cell strains and two additional control cell strains, higher expression of α -actin in allylamine cells was consistently observed.

Additional studies were conducted to determine whether expression of certain ECM genes was enhanced in allylamine cells. We examined the levels of fibronectin and thrombospondin, since their expression is associated with proliferation [19,20], and the mRNA levels of two major collagen moieties, the α_1 (III) and α_1 (IV) collagens. RNA slot blot analysis of the expression of the ECM genes fibronectin, thrombospondin, type III and type IV collagen revealed that the mRNA levels of these genes were not appreciably altered in six independent control and allylamine cell strains (Figure 4b). Since a much larger difference in the synthesis of proline-containing polypeptides between growth-arrested allylamine and control SMCs was observed (Figure 3), we also examined mRNA levels for these genes after culturing in the presence of 1% FBS. However, once again we found no difference in the mRNA expression of these major ECM proteins (results not shown). The ability of these cells to adhere to different substrates was also analysed. Both control and allylamine cells adhered best to type I collagen (results not shown). In contrast, adhesion of these cells to vitronectin was poor and usually well below 50% of that found for type I collagen, while adhesion to fibronectin was intermediate. Overall, there was not a significant difference in the ability of control and allylamine cells to adhere to these substrates.

Conditioned media from allylamine cells enhance DNA synthesis

A second possibility considered was that allylamine cells synthesize and secrete mitogenic factors into the media which are incorporated into the ECM. Therefore conditioned media of allylamine cells were examined for mitogenic activity. When conditioned media from allylamine cells were compared with media from control cells, we found that DNA synthesis of allylamine cells was enhanced greater than 2-fold (Figure 5). Enhanced DNA synthesis was also observed in control cells, but the increase in DNA synthesis was not as pronounced as that observed in allylamine cells. The appearance of mitogenic material was independent of the growth state of these cells, since the conditioned media of growth-arrested allylamine cells also exhibited the same ability to elevate DNA synthesis.

We next examined the proteins secreted by these cells into the conditioned media. Metabolic labelling studies revealed that the secretion of proteins of 52, 46, 33 and 28 kDa was enhanced in allylamine cells relative to controls, while two proteins of 31 and 30 kDa were only detected in allylamine cells (Figure 6). It is possible that one or more of these proteins is responsible for the enhanced DNA synthesis in allylamine cells.

DISCUSSION

Role of the ECM in the regulation of allylamine SMC proliferation

The enhanced mitogenic responsiveness of allylamine cells is consistent with our previous report that increased turnover of

membrane polyphosphoinositides in response to FBS is observed in allylamine cells [15]. Increased polyphosphoinositide turnover has been found in highly proliferative vascular SMCs from spontaneously hypertensive rats [23], and in SMCs isolated from blood vessels following balloon injury [6]. The enhanced serum responsiveness of allylamine cells may involve up-regulation of signal transduction pathways involved in mitogenesis. This interpretation is consistent with recent studies in our laboratory showing that the entry of allylamine cells into the S phase of the cell cycle is enhanced (R. C. Bowes and K. S. Ramos, unpublished work). Because these alterations are preserved after serial passage *in vitro*, we conclude that toxic insult causes permanent modulation of the cellular programming involved in the regulation of SMC growth. Our initial studies of vascular SMC growth following allylamine-induced vascular injury *in vivo* were conducted using cells seeded on a glass surface. This approach facilitated the assessment of morphological, ultrastructural and cytochemical profiles under similar experimental conditions. In those studies, phenotypic differences involving alterations in the proliferative and synthetic behaviour of control and allylamine cells were documented [14]. Interestingly, the enhanced DNA synthesis in allylamine cells is associated with increased cell numbers only when cells are seeded on a glass surface. Because glass seeding would, of necessity, require synthesis and deposition of proteins involved in cellular attachment and proliferation, differences in proliferative potential between control and allylamine cells may be related to alterations in the synthesis and/or deposition of ECM components. Our cross-plating studies support this possibility, since the growth of control cells was comparable with that of allylamine cells when seeded on a substrate pre-coated by allylamine cells.

The ECM of SMCs consists of a complex assembly of various collagens, elastin, fibronectin, thrombospondin and glycoconjugates that participate in the regulation of cellular morphology, growth and differentiation [18,21,22,24,25]. Our results suggest that the proliferative advantage of allylamine cells may be mediated in part by differential synthesis and/or deposition of ECM components. Alternatively, because the ECM is also known to sequester growth factors [26], differential deposition of growth factors in the matrix may also contribute to the proliferative advantage of allylamine cells. We also found that allylamine cells synthesized a larger amount of proline-containing proteins than control cells, suggesting that allylamine cells may produce a larger amount of collagen proteins than controls. The difference in collagen synthesis between control and allylamine cells is particularly interesting in view of studies by Stavenov [27] which show that injury to arterial SMCs by particles from tobacco smoke is associated with release of a protein which stimulates the secretion of collagen. Whether the enhanced proliferation of cells seeded on ECM deposited by allylamine cells is due to altered collagen synthesis is presently unclear.

Altered protein secretion in allylamine cells

Conditioned media from allylamine cells were able to stimulate DNA synthesis to a greater extent than conditioned media from control cells. These results indicate that allylamine cells may secrete products that enhance mitogenesis. We have identified a number of polypeptides that are more abundant in conditioned media from allylamine cells. In addition, two proteins of 30 and 31 kDa appear to be uniquely expressed in these cells. These proteins may be involved in increasing DNA synthesis in recipient

cells. It is presently unclear whether the molecule(s) responsible for the increased DNA synthesis are also deposited in the ECM and are responsible for the enhanced mitogenic activity in the matrix of allylamine cells.

In conclusion, we have found that SMCs isolated from allylamine-treated animals continue to maintain enhanced growth characteristics. The enhanced growth response is associated with both altered protein secretion and increased ECM deposition. The possibility of increased deposition of growth factors in the ECM provides an attractive explanation for our finding that both conditioned media and ECM of allylamine cells enhance DNA synthesis. To date, we have not detected altered expression of the B chain of platelet-derived growth factor, acidic or basic fibroblast growth factor, insulin-like growth factor II or transforming growth factor- β I in these cells (G. Liao, unpublished work). However, further studies are clearly warranted to test this hypothesis. A second possibility is that altered ECM deposition may act to enhance DNA synthesis directly. For example, expression of an alternatively spliced fibronectin and increased synthesis of thrombospondin are both associated with SMC proliferation, and fibronectin is able to directly activate the Na/H antiporter [22,28,29]. Identification of the protein(s) responsible for the increase in DNA synthesis may provide important clues toward understanding SMC proliferation after vascular injury.

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