Subendothelial Cells From Normal Bovine Arteries Exhibit Autonomous Growth and Constitutively Activated Intracellular Signaling

Maria G. Frid, Almaz A. Aldashev, Raphael A. Nemenoff, Ryuji Higashito, Jay Y. Westcott, Kurt R. Stenmark

Abstract—The arterial media is comprised of heterogeneous smooth muscle cell (SMC) subpopulations with markedly different growth responses to pathophysiological stimuli. Little information exists regarding the intracellular signaling pathways that contribute to these differences. Therefore, we investigated the growth-related signaling pathways in a unique subset of subendothelial SMCs (L1 cells) from normal, mature, bovine arteries and compared them with those in “traditional” SMCs derived from the middle media (L2 SMCs). Subendothelial L1 cells exhibited serum-independent autonomous growth, not observed in L2 SMCs. Autonomous growth of L1 cells was driven largely by the constitutively activated extracellular signal–regulated kinase (ERK-1/2) cascade. Inhibition of upstream activators of ERKs (MAP kinase kinase-1, p21ras, receptor tyrosine kinases, and Gi protein–coupled receptors) led to suppression of autonomous growth in these cells. L1 cells also exhibited constitutive activation of important downstream targets of ERKs (cytosolic phospholipase A2, cyclooxygenase-2) and secreted large amounts of prostaglandins. Importantly, L1 cells secreted potent mitogenic factor(s), which could potentially contribute in an autocrine fashion to the constitutive activation of these cells. Our data suggest that unique arterial cells with autonomous growth potential and constitutively activated signaling pathways exist in normal arteries and may contribute selectively to the pathogenesis of vascular diseases.

Key Words: smooth muscle cell proliferation ■ heterogeneity ■ mitogen-activated protein kinase ■ G proteins ■ prostaglandins

The pathogenesis of many cardiovascular diseases such as arteriosclerosis, postangioplasty restenosis, pulmonary hypertension, and diabetic vasculopathy is characterized by abnormal proliferation of arterial smooth muscle cells (SMCs), resulting in intimal and/or medial thickening. Numerous studies in animal models and in humans have demonstrated that cells in the neointima differ markedly from SMCs in the tunica media and that many of the biochemical and proliferative characteristics of neointimal cells are similar to those displayed by embryonic, fetal, and/or neonatal vascular SMCs (reviewed in Reference 1). It has been suggested that the accumulation of nonmuscle-like cells in the neointima may not simply be the result of a “dedifferentiation” of mature SMCs migrating from the tunica media but is potentially the result of expansion of a phenotypically unique subset of vascular cells.2–5 This hypothesis is supported by both in vivo and in vitro studies demonstrating that relatively undifferentiated SMCs with enhanced growth potential exist in the uninjured vascular media.6–10 We have previously reported that a unique subset of SMCs could be isolated from the subendothelial aspect of normal, mature, bovine arterial media.11 These phenotypically unique subendothelial cells had the ability for serum-independent growth and exhibited remarkably augmented growth in response to various growth-modulating stimuli, including hypoxia, compared with SMCs derived from adjacent middle media. The mechanisms responsible for the augmented growth potential of subendothelial cells are presently unknown; however, a better understanding of these mechanisms could facilitate the design of more specific treatment strategies.

Proliferation in most cell types is driven by activation of the mitogen-activated protein kinases (MAPks) p44^Mapk and p42^Mapk, also known as extracellular signal–regulated kinases (ERKs), ERK-1 and ERK-2.12 Activation of the ERK pathway is initiated by ligand binding to cell-surface receptors and is mediated largely through 2 major receptor-mediated signaling pathways: receptor tyrosine kinases (RTks) and G protein–coupled receptors (GPCRs).13,14 One pathway in-
volves the activation of RTKs by traditional polypeptide growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF). A second signaling pathway acts through 7 membrane-spanning receptors coupled to heterotrimeric GTP-binding proteins (G proteins). Several ligands that signal via distinct G-protein subtypes have been shown to elicit mitogenic responses. Thrombin and lysophosphatidic acid have been shown to stimulate growth via pertussis toxin–sensitive GiPCRs. In some but not all cells, vasoactive agents such as angiotensin II and endothelin, which act through GqPCRs, have also been shown to initiate proliferation. There is accumulating evidence that activation of 1 particular receptor-mediated signaling pathway can amplify intracellular signaling within a parallel but separate pathway. Additionally, continuous or “constitutive” activation of the MAPK pathway, as might occur in a cell secreting autocrine growth factors acting via either GPCRs or RTKs, could lead to a “priming” effect on the cell, rendering it more susceptible to activation by other mitogenic stimuli and resulting in subsequent proliferation.

Studies in established cell lines and transformed cells from tumors have dissected in great detail the intracellular signaling mechanisms regulating enhanced growth of these cells. However, the signaling pathways contributing to the enhanced proliferative capacity of unique subsets of SMCs in the arterial wall remain obscure. The purpose of the present study, therefore, was to investigate the growth-related ERK pathway(s) in a unique subset of subendothelial SMCs (termed L1 cells) derived from normal, mature, bovine arteries, which exhibit serum-independent, self-driven growth. We also assessed the members of the eicosanoid production pathway, previously shown to be important in controlling SMC proliferation, viz, cytosolic phospholipase A2 (cPLA2), the inducible form of cyclooxygenase (COX-2), and prostaglandins (PGE2 and PGI2). For comparison purposes, we used SMCs isolated from the middle media (termed L2 SMCs), which exhibit a relatively slow, serum-dependent growth pattern traditionally described for vascular SMCs from the conducting, large, elastic arteries of large mammalian species.

Methods

Isolation of Cell Subpopulations
Main pulmonary arteries and aortic arches were obtained from adult (2-year-old) cows at a local slaughterhouse. Vessels were cut open, and the endothelium was removed by gentle scraping of the luminal surface of the vessel with a scalpel blade. The tunica media was then mechanically dissected into a very thin subendothelial layer and a middle layer, and tissue explants from each medial layer were incubated in complete Dulbecco’s modified Eagle’s medium (DMEM, Sigma Chemical Co) or 10% plasma-derived serum (Cocalico Biologicals, Inc). Because previous in vivo studies had demonstrated the presence of nonmuscle-like cells in the subendothelial compartment of the mature, bovine arterial media, our objective was to selectively isolate these cells in culture. In primary cultures of subendothelial tissue explants incubated in 10% calf serum, 2 morphologically distinct cell subpopulations were observed. Cells of 1 subpopulation (hereafter termed L1 cells), comprising <10% of all cell colonies, appeared small and rhomboidal and at confluence, formed a dense, multilayered network. Cells of the other subpopulation appeared bipolar or spindle-shaped and at confluence, formed the “hill-and-valley” pattern traditionally described for SMCs. To selectively isolate cell colonies with a rhomboidal appearance (L1 cell colonies), we used “ring-based” isolation techniques. In brief, to isolate individual colonies of cells with a distinct rhomboidal appearance, plastic or Teflon rings (5 to 10 mm in diameter; greased on the bottom) were placed over each cell colony of interest. Cells within the ring were trypsinized and transferred to a 24-multiwell plate for expansion. Because small rhomboidal cells represented only a minority of cell colonies in primary cultures from subendothelial media grown in 10% serum, attempts were made to selectively increase the number of this cell type in primary culture. Because use of plasma instead of serum had previously been shown to allow selective growth of unique SMC subpopulations, we incubated subendothelial tissue explants in medium supplemented with 10% plasma-containing DMEM. Under these conditions, rhomboidal cell colonies were observed to predominate (>90%). When rhomboidal–appearing L1 cells initially grown in 10% plasma were subsequently subcultured in 10% serum-containing medium, their characteristic rhomboidal appearance did not change. Thus, utilizing selective isolation techniques and/or culture medium supplemented with 10% plasma, we were able to isolate and expand in culture a subpopulation of nonmuscle-like cells. Virtually all cell colonies isolated from the middle media (L2) explants grown in 10% serum exhibited uniform morphological characteristics. These cells (hereafter termed L2 SMCs) exhibited a spindle-shaped, bipolar appearance and at confluence, showed the hill-and-valley pattern traditionally described for vascular SMCs. No cell growth was observed when middle-media explants were incubated in 10% plasma.

All experiments were carried out on cells at passages 3 to 7. Cell cultures were tested for Mycoplasma contamination by using a Gen-Probe Mycoplasma TC rapid detection system (Gen-Probe Inc) and were negative for such contamination.

Cell Growth Assays
Cells were plated onto 24-multiwell plates at a density of 10 or 20×10^3 cells per well in DMEM supplemented with 10% calf serum. On day 1, cells were rinsed with PBS and DMEM supplemented with 0.1% calf serum, 10% calf serum, 10% plasma, or 10% medium conditioned by L1 cells for 72 hours (termed L1-conditioned medium). Cells (4 wells for each condition) were trypsinized and counted at different time points in a standard SPortlite hemacytometer (Baxter). Data were expressed as cell number x1000 per well.

DNA Synthesis Assay
DNA synthesis in distinct cell subpopulations in response to various mitogens, inhibitors, serum, or L1-conditioned medium (see description above) was determined by measuring [3H]thymidine incorporation described elsewhere. The following purified peptide mitogens were used in this study: PDGF-BB (10 ng/mL, Bachem Fine Chemicals), bFGF (30 ng/mL, Bachem), EGF (100 nmol/L, UBI), thrombin (0.01 U/mL, Sigma), endothelin-1 (500 nmol/L, Sigma), and lysophosphatidic acid (10 µmol/L, Sigma). The mitogen doses used in this study had been previously shown to elicit maximal responses in bovine SMCs (data not shown). The following inhibitors were used in the study: pertussis toxin (potent inhibitor of the GiGPCR pathway; 100 ng/mL; 3 hours before the addition of mitogens; List Biologicals), genistein (inhibitor of RTKs; 100 µmol/L; 15 minutes before the addition of mitogens; UBI), PD98059 (a specific MAP kinase kinase-1 [MEK-1] inhibitor; 10 µmol/L; Biomol Research), BZA-5B (a farnesyl transferase inhibitor that blocks activation of p21WAF, 100 µmol/L; Genentech), indomethacin and sulindac sulfide (both nonselective COX inhibitors; both at 10 µmol/L; Sigma), and nordihydroguaiaretic acid (a nonselective lipoygenase inhibitor; 5 µmol/L; Sigma).

Conditioned Medium and Coculture Experiments
To determine whether L1 cells secrete growth-modulating activity, both live coculture experiments as well as conditioned medium experiments were performed. Coculture experiments with L1 cells as the source cells and growth-arrested L2 SMCs as the target cells were performed as previously described by Majack and colleagues (Cook et al). For conditioned medium experiments, source L1 cells at 90% confluence were rinsed with PBS and maintained in serum-
deprived (0.1% calf serum) medium for 72 hours, and then this conditioned medium was collected and either added fresh to growth-arrested target L2 SMCs, along with \([^3]H\)thymidine (0.5 µCi/mL) for 24 hours to assess DNA synthesis, or divided into aliquots and snap-frozen in LN\(_2\). Freezing/thawing of the conditioned medium did not change its growth-promoting effect. Both live coculture and conditioned medium experiments yielded similar results.

**Measurement of MAPK Activity**

MAPK activity was analyzed by an immunocomplex kinase assay as described previously.\(^{23}\) Subconfluent cell cultures in 6-well plates were serum deprived (0.1% calf serum) for 72 hours and then stimulated for 5 minutes with 10% calf serum or individual mitogens. Cells were then washed with ice-cold PBS and lysed in 0.3 mL of lysis buffer (25 mmol/L Tris-HCl, pH 7.2; 50 mmol/L \(\beta\)-glycerophosphate; 0.5% Triton X-100; 1 mmol/L EDTA; 100 µmol/L Na\(_2\)VO\(_4\); 1 mmol/L DTT; 2 mmol/L MgCl\(_2\); 0.06 U/mL aprotinin; 0.1 mmol/L PMSF; and 20 µmol/L leupeptin). Insoluble material was pelleted by centrifugation (14 000 g for 10 minutes), and supernatants were quantitated by the Bio-Rad protein assay according to Bradford (Bio-Rad Laboratories). ERK-1 and ERK-2 were immunoprecipitated by incubation for 2 hours with anti-ERK-1 and anti-ERK-2 antibodies (Santa Cruz Biotechnology) conjugated to protein A–Sepharose beads. The beads were washed in lysis buffer, and kinase activity was determined by using an EGF-receptor peptide as previously described.\(^{24}\) Results were expressed as pico moles of \(^{32}\)P phosphate incorporated into substrate per minute per milligram of extracted protein and are representative of at least 3 independent experiments performed in duplicate.

**Measurement of PLA\(_2\) Activity and PG Production**

Measurement of cPLA\(_2\) activity was performed as previously described.\(^{25}\) In brief, cells were minced with a Dounce homogenizer in homogenization buffer, pH 7.4 (250 mmol/L sucrose, 50 mmol/L HEPES, 1 mmol/L EDTA, and 1 mmol/L EGTA) with protease inhibitors (20 µmol/L pepstatin, 20 µmol/L leupeptin, 0.1 mmol/L PMSF, and 1000 kU/mL aprotinin). Extracts were centrifuged at 100 000 g for 1 hour. Supernatants were incubated with 15 µmol/L \[^{14}\]C\)-arachidonylphosphatidylcholine (Amersham Corp) in the presence of 4 µmol/L CaCl\(_2\) for 30 minutes at 37°C. The reaction was quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture. Plates were quenched with the addition of 40 mL of ethanol:acetic acid (98:2) mixture.

Immunoblotting of cPLA\(_2\) and COX Isoforms

Extracts matched for protein content were analyzed by 8% SDS-polyacrylamide gel electrophoresis and Western blotting with specific antibodies (monoclonal anti-cPLA\(_2\) antibody and goat polyclonal anti-COX-2 antibodies, both purchased from Santa Cruz, Inc, and both used at a dilution of 1:200). Secondary antibodies were conjugated with horseradish peroxidase and used according to the manufacturer’s (Santa Cruz) protocol. Detection was performed with an ECL-Plus (Amersham Corp) according to the manufacturer’s directions.

**Results**

**Phenotypically Unique Subendothelial L1 Cells Exhibit Augmented Growth Potential**

Cells isolated from the subendothelial aspect of the vessel wall (L1 cells) were small and rhomboidal with long, lamellar extensions and formed a dense, multilayered network at confluence. In contrast, cells isolated from the middle media (L2 SMCs) were larger and spindle-shaped and at confluence, exhibited a hill-and-valley appearance traditionally described for vascular SMCs (Figure 1). As previously described,\(^{31}\) subendothelial L1 cells expressed little \(\alpha\)-SM actin and no SM myosin, whereas middle-media L2 SMCs expressed high levels of both \(\alpha\)-SM actin and SM myosin. These 2 phenotypically distinct cell subpopulations exhibited markedly different growth capabilities when tested under similar conditions. After a prolonged period of serum deprivation (72 hours in 0.1% calf serum), basal levels of DNA synthesis (as measured by \([^3]H\)thymidine incorporation) in subendothelial L1 cells was 6- to 9-fold higher than that in middle-media L2 SMCs (Figure 2A). In response to serum stimulation (10% calf serum, 24 hours), subendothelial L1 cells exhibited much higher rates of \([^3]H\)thymidine incorporation than did middle-media L2 SMCs (Figure 2A). Similarly, L1 cells exhibited a higher rate of proliferation (as defined by an increase in cell number) and a higher saturation density (number of cells/cm\(^2\) at plateau of the growth curve) in 10% calf serum compared with L2 SMCs (Figure 2B). Most interestingly, L1 cells demonstrated an ability for autonomous or self-driven growth, as defined by significant increases in cell number when grown under serum-deprived (0.1% calf serum) conditions (Figure 2C). L2 SMCs remained quiescent in 0.1% calf serum (Figure 2C).

**L1 Cells Exhibit Constitutively Activated MAPK and Eicosanoid Production Pathways Under Serum-Deprived Conditions**

Cell proliferation is known to be regulated mainly through 2 major receptor-mediated signaling pathways: the RTK-mediated and the GPCR pathways. Therefore, we first tested whether activation of either pathway was contributing to the autonomous, self-driven growth of subendothelial L1 cells. We utilized antagonists of both pathways: pertussis toxin, a potent inhibitor of the Gi/G0 pathway, and genistein, an inhibitor of RTKs. We found that treatment with either pertussis toxin or genistein significantly decreased basal (0.1% calf serum, 72 hours) levels of \([^3]H\)thymidine incorporation in subendothelial L1 cells (Figure 3A), suggesting that both RTK and GPCR pathways were constitutively activated in L1 cells and contributed to their autonomous growth. In contrast, pertussis toxin and genistein had no effect on basal levels of DNA synthesis in middle-media L2 SMCs (Figure 3B).

Because proliferation in most cell types has been shown to be correlated with activation of MAPK (p44\(^{\text{MAPK}}\) and p42\(^{\text{MAPK}}\), also known as ERK-1 and ERK-2, respectively) and because both RTK-mediated and GPCR pathways have been shown to converge on activation of ERKs, we evaluated basal (after 72 hours in 0.1% calf serum) ERK-1/ERK-2 activity in both cell types and the effects of the inhibitors of GPCR and RTK pathways (pertussis toxin and genistein) on basal ERK activity. As shown in Figures 3C and 3D, basal ERK activity (open bars) in L1 cells was 2.5-fold higher than that in middle-media L2 SMCs. Both pertussis toxin and genistein dramatically suppressed basal ERK activity in L1 cells but had no effect on basal ERK activity in L2 SMCs.

High basal levels of ERK activity suggested that constitutively activated ERKs were playing an essential role in the
self-driven growth of subendothelial L1 cells. To test this hypothesis, we employed PD98059, a highly selective inhibitor of MEK-1 activation that has been shown to effectively inhibit activation of ERKs. Treatment of L1 cells with 10 μmol/L PD98059 markedly (by 80%) inhibited autonomous, self-driven growth (assessed by an increase in cell number in serum-deprived medium; Figure 4). In contrast, PD98059 exerted minimal effect on the low, basal levels of DNA synthesis in L2 SMCs (data not shown). We also assessed the role of the small G protein Ras (p21ras), an upstream activator of MAPK, in mediating MAPK-dependent autonomous growth of subendothelial L1 cells. We used the farnesyl transferase inhibitor BZA-5B, which had been previously demonstrated to effectively block activation of Ras. BZA-5B (100 μmol/L) only partially (43%) inhibited growth of L1 cells under serum-deprived conditions (Figure 4), suggesting that the self-driven growth of L1 cells is mediated through both Ras-dependent and Ras-independent signaling pathways.

It has recently been shown that differential growth responses of vascular SMCs may be the result of differences in the availability of downstream targets for MAPK, including cPLA2. cPLA2 is known to be phosphorylated and activated by MAPK, and cPLA2 expression is at least partially driven by activation of Ras. PLA2 is a key enzyme in the eicosanoid production pathway, a pathway with many important biological actions, including regulation of vascular SMC growth. We therefore sought to determine whether the elevated levels of MAPK activity in autonomously growing L1 cells under basal conditions would be associated with increased expression of cPLA2 and other members of eicosanoid production pathways. Under basal (serum-free) conditions, cPLA2 protein expression and activity were markedly elevated in subendothelial L1 cells compared with middle-media L2 SMCs (Figures 5A and 5B). Arachidonic acid, liberated by cPLA2, is metabolized by constitutive (COX-1) or inducible (COX-2) COXs to PGs. We observed high levels of expression of inducible COX-2 in L1 cells under serum-deprived conditions (Figure 6A). COX-2 was not detectable in L2 SMCs under identical conditions (Figure 6A). L1 cells also demonstrated remarkably high basal levels of PGE2 and PGI2 (the latter measured by its stable analog 6-keto-PGF1α).
released into the culture medium compared with L2 SMCs (Figure 6B).

To assess the contribution of eicosanoids to the autonomous growth of L1 cells, we examined the effect of 2 types of inhibitors of eicosanoid production: nonselective COX inhibitors, which suppress PG production (indomethacin and sulindac sulfide), and a nonselective lipooxygenase inhibitor, nordihydroguaiaretic acid, which suppresses hydroxyeicosatetraenoic acid production. Sulindac sulfide and indomethacin inhibited PG production at the concentrations used but did not have any effect on basal DNA synthesis of L1 cells, whereas nordihydroguaiaretic acid significantly (by 50%) inhibited basal DNA synthesis in L1 cells grown in serum-deprived medium (data not shown).

L1 Cells Are More Sensitive to Mitogenic Signals via the GiPCR Pathway Than Are L2 SMCs (Agonist and Antagonist Studies)

As shown above, subendothelial L1 cells grow faster than do middle-media L2 SMCs not only under conditions of serum deprivation but also under serum-stimulated conditions (see Figure 2B). Experiments in other cell systems have shown that constitutive activation of a particular signaling pathway can significantly affect the magnitude and/or responsiveness of a cell to other mitogenic stimuli.18 These observations suggested that the augmented growth of L1 cells in response to serum could be due to an enhanced ability of L1 cells, compared with L2 SMCs, to amplify exogenous growth signals acting through multiple receptor-mediated signaling pathways. Therefore, we assessed the contribution of both GiPCR and RTK-mediated signaling pathways to the enhanced serum-stimulated growth of L1 cells by utilizing agonists and antagonists of both pathways. The data presented in Table 1 show that specific agonists of RTK, viz, PDGF-BB and bFGF, increased DNA synthesis in both L1 cells and L2 SMCs. Interestingly, EGF, another well-known RTK agonist, did not significantly stimulate growth in either cell type (data not shown). On the other hand, thrombin, an agonist of GiPCRs,15 was a potent stimulator of DNA synthesis in L1 cells, whereas it had almost no effect on L2 SMCs (Table 1). Similarly, lysophosphatidic acid, which has
been shown to signal through a Gi-coupled pathway in certain cell types, stimulated DNA synthesis in L1 cells but not in L2 SMCs (Table 1). Endothelin-1 and angiotensin II, which have been shown to signal primarily through activation of Gq rather than Gi, did not stimulate DNA synthesis in either L1 or L2 SMCs under the conditions tested (0.1% calf serum for 72 hours before addition of these agonists; data not shown).

To further evaluate the contribution of RTK-mediated and GiPCR signaling pathways to the augmented serum-stimulated growth of subendothelial L1 cells, we examined the effects of antagonists of these pathways on serum- and peptide mitogen–stimulated growth of L1 cells compared with middle-media L2 SMCs. Data presented in Table 2 demonstrate that pretreatment with pertussis toxin reduced the stimulatory effect of 10% serum on the growth of L1 cells by 80% and reduced the thrombin-stimulated increase in [3H]thymidine uptake by 87.7%. In middle-media L2 SMCs, however, pertussis toxin pretreatment reduced the mitogenic effect of 10% serum by 28.8%. (As shown above, there was no stimulatory effect of thrombin on DNA synthesis in L2 SMCs.) As expected, pertussis toxin did not have any effect on stimulation by PDGF in either cell type (data not shown).

Genistein markedly attenuated the mitogenic effect of 10% serum and PDGF in both L1 and L2 SMCs and the mitogenic effect of thrombin in L1 cells (data not shown). The latter findings are compatible with previous observations demonstrating that genistein not only inhibits RTK signaling but can also inhibit signaling via Gi-coupled pathway(s) in some cell types.

Some but Not All Colonies of Subendothelial L1 Cells Secrete a Paracrine Mitogenic Factor(s)

The observations that subendothelial L1 cells exhibited autonomous, serum-independent growth and demonstrated a constitutively activated MAPK cascade, which was effectively blocked by RTK and GiPCR inhibitors, raised the possibility that these cells could be producing mitogenic factor(s). To test this hypothesis, we assessed the effects of serum-free medium conditioned by subendothelial L1 cells for 72 hours (L1-conditioned medium) on DNA synthesis in quiescent (72 hours of serum deprivation) L2 SMCs. We performed both live coculture and conditioned medium experiments (see Methods) and found that both methods yielded similar data with regard to stimulation of DNA synthesis in stimulated growth of subendothelial L1 cells, we examined the effects of antagonists of these pathways on serum- and peptide mitogen–stimulated growth of L1 cells compared with middle-media L2 SMCs. Data presented in Table 2 demonstrate that pretreatment with pertussis toxin reduced the stimulatory effect of 10% serum on the growth of L1 cells by 80% and reduced the thrombin-stimulated increase in [3H]thymidine uptake by 87.7%. In middle-media L2 SMCs, however, pertussis toxin pretreatment reduced the mitogenic effect of 10% serum by 28.8%. (As shown above, there was no stimulatory effect of thrombin on DNA synthesis in L2 SMCs.) As expected, pertussis toxin did not have any effect on stimulation by PDGF in either cell type (data not shown).

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![Figure 4](image1.png)

**Figure 4.** Autonomous growth of subendothelial L1 cells, as defined by an increase in cell number on day 8 under serum-deprived (0.1% calf serum) conditions was markedly inhibited by treatment with the MEK-1 inhibitor PD 098059 (10 μmol/L, black bar) and attenuated by treatment with the p21 inhibitor BZA-5B (100 μmol/L, hatched bar). *P < 0.01 compared with untreated day 8 controls. Values are mean ± SE (n = 3 replicate wells). Similar results were obtained in 3 other independent experiments. CS indicates calf serum.

![Figure 5](image2.png)

**Figure 5.** cPLA2 protein expression (A) and activity (B) were markedly elevated in subendothelial L1 cells compared with middle-media L2 SMCs. A, Western blot analysis of cPLA2 expression in protein extracts from L1 cells and L2 SMCs electrophoresed in an 8% SDS gel. A peptide with electrophoretic mobility corresponding to a molecular mass of 85 kDa was recognized with specific anti-cPLA2 antibodies (Santa Cruz). B, cPLA2 activity was measured in extracts prepared from unstimulated L1 cells and L2 SMCs after 72 hours under serum-deprived (0.1% calf serum) conditions. *P < 0.001 compared with L2 SMCs.

![Figure 6](image3.png)

**Figure 6.** Subendothelial L1 cells expressed the inducible form of COX, COX-2, and demonstrated remarkably high levels of PG secretion under unstimulated, serum-deprived conditions (72 hours in 0.1% calf serum) compared with middle-media L2 SMCs. A, L1 cells express COX-2 under basal, unstimulated conditions, whereas COX-2 is undetectable in L2 SMCs under identical conditions. B, L1 cells released remarkably high amounts of PGE2 and PGL2 (as measured by its stable analogue 6-keto-PGF1α) released into the culture medium under unstimulated conditions compared with L2 SMCs. *P < 0.001 compared with L2 SMCs.
growth-arrested L2 SMCs. (Therefore, only the data on conditioned medium experiments are presented here.) We found that in some colonies of L1 cells, serum-free medium conditioned by these cells for 72 hours dramatically (11-fold) increased \[^{3}H\]thymidine uptake in quiescent medial L2 SMCs (Figure 7). Conditioned medium from other colonies of L1 cells, which exhibited all of the characteristics described above (autonomous growth, constitutively activated ERKs, markedly high levels of cPLA \(_{2}\) and COX-2 expression, and PG production), did not exert any mitogenic effect on either L2 SMCs or NIH 3T3 fibroblasts (data not shown). We also evaluated the effect of serum-deprived medium conditioned by L2 SMCs on 3T3 fibroblasts and observed no detectable paracrine mitogenic activity (data not shown). Preliminary data from our laboratory demonstrate that the paracrine mitogenic factors present in L1-conditioned medium from certain L1 cell colonies belong to the heparin-binding family of growth factors. When L1-conditioned medium was passed over a heparin-Sepharose column, all mitogenic activity in the run-through fraction was abolished (data not shown). Elution of the fraction bound to the heparin-Sepharose column by various concentrations of NaCl demonstrated that the peak of mitogenic activity was present in the fraction eluting with 0.75 mol/L NaCl (data not shown). High-performance liquid chromatography purification of the fraction with peak mitogenic activity and further analysis by SDS-polyacrylamide gel electrophoresis as well as amino acid sequencing of the proteins in this fraction demonstrated that there are at least 2 unique members of the heparin-binding family of growth factors (manuscript in preparation).

**Effect of L1-Conditioned Medium, Containing Paracrine Mitogenic Factors, on MAPK and Eicosanoid Production Pathways in L2 SMCs**

Because we observed high levels of intrinsic ERK activity as well as high levels of cPLA \(_{2}\) and COX-2 expression in L1 but not in L2 SMCs, we wanted to assess whether the conditioned medium from those L1 cell colonies that secreted paracrine mitogenic factor(s) would increase ERK-1/ERK-2 activity and the levels of cPLA \(_{2}\) and COX-2 expression in middle-media L2 SMCs to the levels observed in L1 cells. A 5-minute exposure of quiescent L2 SMCs to 10% L1-conditioned medium increased ERK-1/ERK-2 activity by 4.6-fold (Table 3), as might have been expected from the L1-conditioned medium effect on L2 SMC proliferation (as shown in Figure 7). Interestingly, however, addition of L1-conditioned medium to quiescent L2 SMCs for 24, 48, or 72 hours did not significantly affect the low level of cPLA \(_{2}\) or COX-2 expression as determined by Western blotting assay (data not shown). The effect of L1-conditioned medium on PG production in L2 SMCs could not be tested because L1-conditioned medium itself contains very large amounts of PGE\(_{2}\) and PGI\(_{2}\) (see data above). However, we tested the ability of L2 SMCs to secrete these prostanooids in response to stimulation with arachidonic acid (10 \(\mu\)mol/L, added for 30 minutes). PG production in L2 SMCs remained low even under stimulation with arachidonic acid (Table 3). Compared with control (30 minutes in serum-free medium), there was a moderate increase in PGE\(_{2}\) and PGI\(_{2}\) (the latter measured by 6-keto-PGF\(_{2\alpha}\)) production by L2 SMCs (Table 3); however, these levels were still dramatically (80%) lower than those in unstimulated L1 cells (cf Figure 5D).

**Discussion**

In the present study, we began to evaluate the pro-proliferative intracellular signaling pathways in bovine arterial subendothelial SMCs (L1 cells) that, in contrast to traditional vascular SMCs, exhibit the capacity for autonomous, self-driven growth. Under conditions of prolonged serum deprivation, subendothelial L1 cells were found to exhibit constitutively activated MAPK (ERK-1/ERK-2) and eicosanoid production (cPLA\(_{2}\)/COX-2) pathways. In contrast, SMCs isolated from the adjacent middle media (L2 SMCs), which exhibited slow growth under serum-stimulated conditions and remained quiescent under serum deprivation, had low basal levels of ERK-1/ERK-2 and very low levels of cPLA\(_{2}\) and COX-2 expression. Both agonist and antagonist studies performed on L1 cells indicated the critical role of the

### Table 1. Subendothelial L1 Cells and Middle-Media L2 SMCs Differ in Their Mitogenic Responses to Growth Factors Acting via GPCR Pathways

<table>
<thead>
<tr>
<th>0.1% CS</th>
<th>PDGF-BB</th>
<th>bFGF</th>
<th>Thrombin</th>
<th>LPA</th>
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<td>1510±121*</td>
<td>1750±197*</td>
<td>2025±85*</td>
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<tr>
<td>L2 SMCs</td>
<td>45.4±9.1</td>
<td>1125±240*</td>
<td>995±83*</td>
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</tbody>
</table>

\(\text{dpm} \) indicates disintegrations per minute; CS, calf serum; and LPA, lysophosphatidic acid. Values are mean±SE (n=4 replicate wells).

*\(P<0.01\) compared with 0.1% CS control.

### Table 2. Subendothelial L1 Cells Are More Sensitive to Pertussis Toxin–Induced Inhibition of Growth Than Are Middle-Media L2 SMCs

<table>
<thead>
<tr>
<th>0.1% CS</th>
<th>10% CS</th>
<th>PTx+10% CS</th>
<th>Thrombin</th>
<th>PTx+Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 cells</td>
<td>615±267.7</td>
<td>2124±267.7</td>
<td>917±81.2 (80%)</td>
<td>2190±83.4</td>
</tr>
<tr>
<td>L2 SMCs</td>
<td>45.4±166.3</td>
<td>1260±166.3</td>
<td>910±83.5 (28.8%)</td>
<td>51.7±3.4</td>
</tr>
</tbody>
</table>

\(\text{dpm} \) indicates disintegrations per minute; CS, calf serum; PTx, pertussis toxin; and NS, not significant. In parentheses is the percent inhibition compared with the stimulatory effect of 10% CS or thrombin.
GiPCR pathway in their augmented growth capabilities. In contrast, the middle-media L2 SMCs appeared to mediate mitogenic signals predominantly via RTK-mediated pathways. We found that some colonies of L1 cells secreted paracrine mitogenic factors, whereas other L1 cell colonies did not secrete any detectable paracrine mitogenic activity. Both secreting and nonsecreting L1 cells were indistinguishable on the basis of all other characteristics described in the present study (autonomous growth, constitutively activated MAPK, and eicosanoid production pathways), which suggested that secretion of the paracrine mitogenic factors is not a prerequisite for the autonomous growth capability of L1 cells. Moreover, when conditioned medium from L1 cells secreting paracrine mitogenic factor(s) was added to L2 SMCs, it did not affect cPLA2 and/or COX-2 expression in these latter SMCs. In other words, the conditioned medium from L1 cells did not convert the L2 SMCs into those of an L1 phenotype. Taken together, these data suggest that there are intrinsic functional differences between distinct subsets of vascular cells residing in close proximity in the arterial wall and specifically, that the growth of a unique subset of subendothelial cells is controlled by mechanisms distinct from those mediating growth in a population of more traditionally differentiated SMCs in the middle media.

Autonomous growth capability has been described for mesenchymal cells derived from patients with diseases such as atherosclerosis as well as pulmonary and renal interstitial fibrosis. The possibility has been raised that the presence of a cell type with an enhanced proliferative phenotype in the diseased organ could explain the progression of the fibroproliferative response without further exogenous signaling. Studies in experimental animals have described autonomous growth capabilities in SMCs derived from embryonic, normal adult, and injured arteries. Based on these previous observations, the question was raised as to whether expression of an autonomous growth phenotype is a potential of all medial SMCs in response to injury or whether a unique subset of cells with autonomous growth potential normally exists in the uninjured arterial wall and in response to injury, is selectively recruited into the vascular repair process. Recent studies in several species have demonstrated that the arterial media is comprised of heterogeneous populations of SMCs that exhibit distinctly different proliferative capabilities. These data, together with our finding of a unique subset of cells (L1 cells) with autonomous growth potential and derived from the subendothelial compartment of normal tunica media, support the hypothesis that specific subsets of vascular cells can contribute in unique ways to the process of neointimal thickening after vascular injury. However, the concept of selective contribution of such cells to the disease process does not rule out the possibility that other more differentiated SMCs in the arterial media modulate their phenotype in response to injury and are also recruited, in different ways, into the vascular remodeling process.

Little is known of the signaling pathways utilized by nontransformed mesenchymal cells with autonomous growth potential. In the present study, we began to evaluate the intracellular mechanisms regulating the autonomous growth of subendothelial L1 cells isolated from normal, mature, arterial media presuming that this information might provide insight into understanding neointimal thickening after vascular injury. We found that under conditions of prolonged

![Figure 7](image_url)

**Figure 7.** Certain colonies of subendothelial L1 cells secrete mitogenic factor(s) that can stimulate DNA synthesis (A) and increase cell number (B) in middle-media L2 SMCs. A, For DNA synthesis assay, serum-free medium was conditioned by L1 cells for 72 hours and then added to cultures of growth-arrested (72 hours in 0.1% calf serum) L2 SMCs for 24 hours, along with [3H]thymidine (see Methods). Growth-arrested L2 SMCs (0.1% calf serum, open bar) had a low level of [3H]thymidine incorporation, whereas after exposure to L1-conditioned medium (hatched bar), L2 SMCs exhibited an 11-fold increase in DNA synthesis. *P<0.001 compared with unstimulated controls. Values are mean±SE (n=4 replicate wells). Similar results were obtained in 6 other independent experiments. B, For cell growth assay, growth-arrested L2 SMCs (72 hours in 0.1% calf serum) were incubated in either 0.1% calf serum (dotted bar), 10% calf serum (black bar), or 10% L1-conditioned medium (hatched bar) for 5 days. Cells were trypsinized and counted in a standard hemacytometer at days 1 and 5. Values are mean±SE (n=4 replicate wells). *P<0.001 compared with day 1 controls. CS indicates calf serum.

**TABLE 3. Effect of L1-Conditioned Medium on ERK-1/ERK-2 Activity and PG Production in Quiescent L2 SMCs**

<table>
<thead>
<tr>
<th></th>
<th>0.1% CS</th>
<th>L1-Conditioned Medium</th>
<th>Arachidonic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK-1/ERK-2 activity, pmol 32P · mg -1 · min -1</td>
<td>7.9±1.4</td>
<td>36.3±3.7</td>
<td>ND</td>
</tr>
<tr>
<td>PGE2, pg · cell×10 -3 · h -1</td>
<td>3.5±0.6</td>
<td>ND</td>
<td>6.1±1.4</td>
</tr>
<tr>
<td>6-Keto-PGF2α, pg · cell×10 -3 · h -1</td>
<td>0.4±0.1</td>
<td>ND</td>
<td>3.2±1.2</td>
</tr>
</tbody>
</table>

CS indicates calf serum; ND, not determined. Values are mean±SE.
serum deprivation, L1 cells exhibited constitutively activated ERK-1/ERK-2. Based on experiments with inhibitors of upstream activators of ERKs (such as inhibitors of MEK-1, Ras, RTK, and GiPCR pathways), the constitutively activated MAPK pathway was found to be critical for autonomous growth of L1 cells. The finding that self-driven growth of L1 cells is markedly inhibited by pertussis toxin, a potent inhibitor of the GiPCR pathway, emphasized a crucial role of Gi-coupled events in contributing to autonomous growth potential. This finding, together with the observation that DNA synthesis in L1 cells can be readily activated by agonists of the GiPCR pathway, such as thrombin and lysophosphatidic acid, suggested a heightened activity and/or sensitivity of the GiPCR pathway in L1 cells. Several possibilities may be considered in explaining these findings. First is the production by L1 cells of an autocrine factor that acts through a GiPCR. This factor(s) could be acting in a juxtacrine fashion or could be secreted in very low quantity, not sufficient to stimulate mitogenesis in other cell types. Importantly, such a hypothetical GiPCR agonist could also contribute to the enhanced growth capabilities of L1 cells by interacting with other growth-promoting pathways. There is accumulating experimental evidence that activation of the GiPCR signaling pathway can markedly amplify the responses produced by a separate coincident activation of other receptors. A second possibility is that of mutation-independent constitutive activation of a heptaspanning receptor in the absence of a ligand, as has been recently demonstrated in the nonmalignant human colon epithelial cell line NCM460. The third most unlikely possibility is that of a constitutively active Gi, as has been described for many tumor cells with autonomous growth potential and bearing activating mutations in G-protein subunits.

In addition to constitutive activation of ERKs, remarkably high levels of cPLA2 and COX-2 protein expression and activity were observed in L1 cells. A critical role for cPLA2 in proliferation of human vascular SMCs has recently been proposed. Constitutively elevated MAPK activity in subendothelial L1 cells could potentially serve as an intracellular “motor” in maintaining elevated levels of cPLA2 activity in these cells, since cPLA2 is known to be phosphorylated and activated by MAPK. Interestingly, previous experiments showed that overexpression of H-Ras in rat vascular SMCs induced elevated levels of cPLA2 expression and activity. Concomitantly, these SMCs exhibited marked downregulation of α-SM actin compared with wild-type SMCs, a characteristic similar to that observed in L1 cells. It is possible, therefore, that elevated cPLA2 activity contributes to both the proliferative phenotype and the differentiation status of L1 cells.

Increased activity of cPLA2 is associated with increases in the levels of free arachidonic acid, which are then metabolized to a variety of bioactive eicosanoids, including PGs, through the action of COXs. We found that L1 cells express markedly elevated levels of the inducible form of COXs, COX-2, and they secrete large amounts of PGs. Several studies have demonstrated a pronounced inhibitory effect of prostanooids on vascular SMC growth. In contrast, we found that inhibition of PG production had no effect on autonomous growth of L1 cells. These data are consistent with a previous report by Morisaki et al demonstrating a lack of inhibition of DNA synthesis by endogenous and exogenous PGs in cultured neointimal compared with medial SMCs from injured rabbit aortas. Available data from clinical trials, though conflicting, in general also do not support the concept that PGs can be successfully used to suppress excessive SMC proliferation in restenosis after percutaneous transluminal coronary angioplasty. Moreover, elevated production of PGs by a number of rapidly growing cancer and transformed cells has been suggested as an important contributor to enhanced cell proliferation. Taken together, these data suggest that activation of prostanooid production pathways can have divergent effects in different subsets of vascular SMCs and support the idea that growth may be controlled differently in distinct subsets of vascular SMCs.

In contrast to our observations that inhibition of COX activity in L1 cells did not affect their growth rate, we found that inhibition of lipoygenase production by the nonselective lipoygenase inhibitor nordihydroguaiaretic acid markedly suppressed basal DNA synthesis. These observations are in agreement with studies by Rao et al on rat aortic SMCs, which showed that 15-hydroxyeicosatetraenoic acid, a 15-lipoxygenase product of arachidonic acid, accounts for some of the arachidonic acid–induced activation of ERKs. Future studies are essential to elucidate the role of the lipoygenase system in the autonomous, self-driven growth of subendothelial L1 cells.

Our data with observations by other investigators suggest that phenotypically and functionally unique vascular cells with high proliferative potential (similar to L1 cells described herein) normally exist in the subendothelial region of large, elastic arteries, raising the possibility of their selective contribution to the pathogenesis of vascular diseases. The basis for the differences in growth capabilities of distinct subsets of vascular cells might lie in our finding that different SMC subpopulations use distinctly different intracellular signaling mechanisms to control growth. These observations suggest that pharmacological therapies aimed at inhibiting vascular remodeling should consider the specific growth control pathways utilized by the cells involved.

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