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GROWTH AND PHENOTYPIC CHARACTERIZATION OF PORCINE CORONARY ARTERY SMOOTH MUSCLE CELLS

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SUMMARY

Vascular smooth muscle cell (VSMC) proliferation significantly contributes to atherosclerotic plaque formation and limits the success rate of percutaneous transluminal coronary angioplasty. We derived a population of porcine coronary artery SMCs to characterize VSMC proliferation and phenotype in preparation to study the molecular actions of VSMC mitogens and antiproliferative agents. Growth assays were designed to minimize the estrogen content in the culture medium, since this steroid hormone significantly influences VSMC growth and the expression of VSMC mitogens and their receptors. Culture conditions were identified such that this criterion was achieved while maintaining a significant VSMC growth rate. Cells cultured in serum-free medium, regardless of growth factor supplements, did not remain adherent to a plastic culture substrate, nor did they proliferate. Dextran-coated charcoal (DCC)-treated sera, including fetal bovine, calf, and porcine, supported VSMC adhesion, but not growth. Whole fetal bovine serum (FBS) produced the best proliferative response. A type-I collagen-coated culture surface significantly enhanced VSMC growth, but only in culture medium containing non-DCC-treated FBS. Flow cytometry analyses confirmed the mitogenic effects of this substrate. The VSMCs exhibited a morphological change on type-I collagen, but this was not accompanied by a change in VSMC phenotype. Our data indicate that culture of these porcine coronary artery SMCs in 2.5% FBS plus 10 ng platelet-derived growth factor-BB per ml in phenol red-free medium on type-I collagen may be the optimal conditions for studying the molecular aspects of VSMC mitogens and antiproliferative agents.

Key words: serum; estrogen; mitogens; type-I collagen.

INTRODUCTION

Segments of the canine pulmonary artery (6) and porcine coronary artery (21) cultured ex vivo have provided models used to study the proliferation of vascular smooth muscle cells (VSMCs). A clear advantage of these models is that the VSMC remains in its nearly natural environment. However, VSMC proliferation studies in ex vivo models are confounded by the ability of other cells in the vessel wall, such as endothelial cells or fibroblasts, to proliferate. Investigators can isolate VSMCs by separating them from the vessel wall to avoid this problem. The disadvantage is that the VSMCs are further removed from their natural surroundings. Nevertheless, the decrease in experimental variables and the ability to address different issues regarding VSMC function make the isolation and characterization of cellular models an important goal.

The pivotal role that VSMC proliferation plays in atherosclerosis and restenosis following percutaneous transluminal coronary angioplasty serves as a major impetus for investigators to establish VSMC experimental models from which a variety of questions regarding VSMC growth can be addressed. We chose to derive a population of SMCs from porcine coronary artery explants as a model for VSMC growth and phenotypic characterization. The derivation method specifically selected for VSMCs capable of migrating and proliferating. These cells are so-called synthetic cells because VSMCs with these characteristics also secrete extracellular matrix proteins including collagen and elastin (15). These properties contribute to arterial occlusion and therefore endow this VSMC subtype with the potential to act in a pathogenic manner. Consequently, we considered characterization of this VSMC subtype before examining the molecular actions of VSMC mitogens and antiproliferative agents. The current investigation was aimed at observing the characteristics of VSMC behavior under several conditions including variations in sera, VSMC mitogens, and cell culture substrates. Further studies of the molecular actions of these agents and of antiproliferative factors on these cells are in progress. These investigations may facilitate improved design of pharmacological therapies that can effectively inhibit the VSMC hyperplasia component of atherosclerosis and restenosis.

Estradiol-17β is a recognized inhibitor of VSMC growth (21), yet the mechanism responsible for this phenomenon remains unclear. One goal for the current report was to identify in vitro conditions that would permit significant growth of these porcine coronary SMCs while eliminating or minimizing the levels of estrogen in the culture medium. The reasons for this goal are twofold: 1) estrogen can con-

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found the characterization of VSMC growth, since it can regulate the expression of the VSMC mitogen, insulin-like growth factor-1 (IGF-1) (4), its receptor (18), and another VSMC mitogen, epidermal growth factor (EGF) (8); and 2) estrogen can significantly reduce the expression of its receptor (16), the protein through which it elicits some of its biological effects (19).

Female Yorkshire pig left-anterior descending (LAD) coronary arteries were harvested to provide explant tissue from which a population of VSMCs could be derived. There are several advantages to choosing this species: 1) pig hearts are relatively easy to obtain; 2) pig hearts are large and permit comparatively easy LAD artery dissection; and 3) pig tissue morphology is similar to that of humans (20). Indeed, porcine heart valves have been used for xenograft transplantation in humans (12). The SMCs derived from the LAD artery also make this a desirable model choice, since coronary artery disease is the leading cause of death in industrialized nations and the LAD artery is the second most common site of atherosclerotic plaque formation (25).

Materials and Methods

Derivation of porcine coronary artery smooth muscle cells. Hearts were obtained 2–3 min following sacrifice of three 5-mo.-old, sexually mature female Yorkshire pigs housed at the Horst Meats Company slaughterhouse in Hagerstown, MD. The VSMCs used in this study were pooled from outgrowths of three separate LAD arteries derived from each of the three porcine hearts. For cell isolation, sterile, ice-cold 1 × phosphate-buffered saline (PBS) (Biofluids, Inc., Rockville, MD) was flushed through the aorta, followed by brief massage to remove blood clots. The hearts were placed in a sterile bag containing ice-cold PBS. Hearts were transported to Georgetown University on ice in a styrofoam cooler (approximately 2 h). The proximal, epicardial portion of the LAD artery was removed from each heart and placed into sterile ice-cold Dulbecco's minimum essential medium (DMEM) (Biofluids). Next, the vessels were transferred to petri dishes containing enough PBS to cover the dish surface. While partially bathed in PBS, the remaining connective tissue was removed from the vessel wall. The vessels were cut into 3-mm-long rings and denuded of their endothelial lining by gentle rubbing of the endothelial layer with sterile, closed forceps.

To open each arterial ring, a single cut was made with sterile scissors. The tissues (explants) were placed, one per well, intimal side down, onto the plastic surface of sterile, 24-well culture plates. Explants were incubated in 300 μl each of prewarmed (37° C) DMEM containing phenol red, 10% fetal bovine serum (FBS) (GIBCO BRL, Gaithersburg, MD), 1% glutamine (Biofluids), and 1% penicillin/streptomycin (Biofluids). After reaching confluence, the cell monolayers were removed from the plastic surface by incubation in trypsin-versene (Biofluids) for 5 min at room temperature. An equal volume of serum-containing medium was added to the trypsinized cells before they were passed to new dishes/flasks. Aliquots of cells (Passage 2) were centrifuged, resuspended in dimethylsulfoxide-containing freezing medium (Cell Freezing Medium B, Biofluids) containing FBS (20%) and distributed into vials (NUNC™ Brand Products, Denmark) for long-term storage in liquid nitrogen.

On the basis of morphology of the cultured cells, fibroblasts were the most likely contaminating cells. The porcine cells, but not normal human fibroblasts, stained positively for smooth muscle myosin presence by immunofluorescence. The antibody used is capable of detecting this protein in human and porcine species. This antigen is the most definitive antigenic marker used to identify SMCs (15). The cells were found to be free of mycoplasma contamination.

Dextran-coated charcoal treatment of serum. Whole FBS, porcine serum (PS) (Lampire Biological Laboratories, Ottsville, PA), and calf serum (GIBCO BRL) were treated with dextran-coated charcoal (DCC) to remove steroid hormones. On Day one, concentrated DCC was prepared as follows: 25 g of charcoal (Sigma) were mixed with 1 L of water in a 4-L polypropylene beaker and stirred for 10 min. Oils and fines were removed, stirring was repeated, and the oils and fines were again aspirated. Dextran (M_r = 500 000, 2.5 g, Sigma) was added to the charcoal solution and the volume adjusted to 1 L.

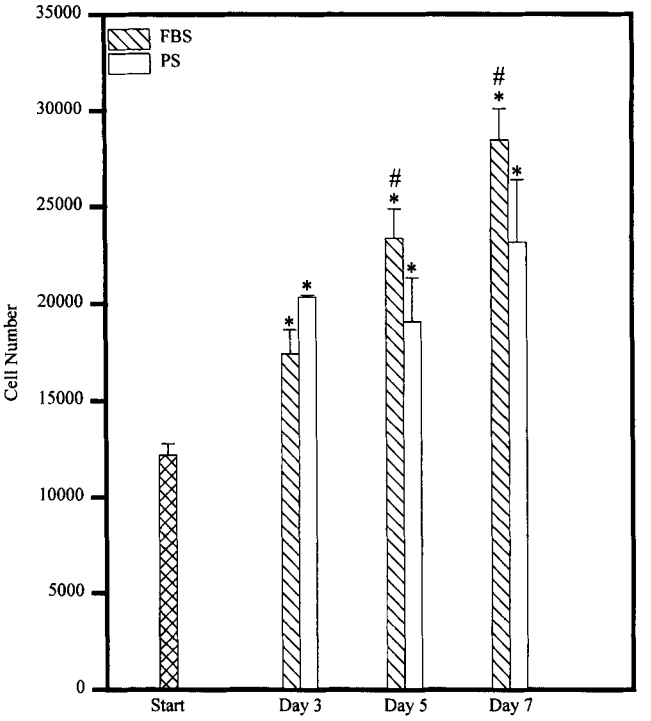

FIG. 1. Growth effects of various sera on porcine coronary artery smooth muscle cells. Porcine coronary artery SMCs were seeded in 5% FBS in IMEM containing phenol red into plastic 24-well plates. After 24 h, the cells were treated with the indicated sera in phenol red-free IMEM, and the average number of cells per well was determined (Start). Whole fetal bovine serum (FBS), whole porcine serum (PS) and a variety of dextran-coated charcoal-treated sera (FBS, calf serum, and porcine serum; data not shown) were included at final concentrations of 10% in the culture media. The culture media were changed on each indicated cell harvest d. Cells were harvested for counting in a Coulter counter. The Student-Newman-Keuls multiple comparison test was used to detect significant differences among means. Each column represents the mean and SEM of six replicates (* denotes significant growth increase vs. start and # denotes significant growth increase vs. previous harvest d).

Ten milliliters of 1 *M* Tris base were added and mixed, and the solution was covered with plastic wrap and placed at 4° C for equilibration overnight.

Sulfatase (1000 units; Sigma) was added to sera, mixed well, and incubated at 37° C for 2 h. The sulfatase-treated sera were mixed with the charcoal solvent and stirred at 56° C for 40 min. The charcoal/sera solutions were centrifuged at 27 000 × g for 10 min and the sera supernatants were removed and serially filtered by vacuum through a prefilter, a 0.45-μm filter, and a 0.2-μm filter into sterile tubes for storage at −20° C.

Growth curve analysis of porcine coronary artery smooth muscle cells. Passage 4–7 VSMCs were seeded at 5000 cells/cm² into plastic (Sarstedt, Newton, NC) and into type-I collagen-coated (Becton Dickinson Labware, Bedford, MA) 24-well dishes for culture at 37° C, 95% O_2:5% CO_2 for various time periods. In Fig. 1, VSMCs were seeded in 5% FBS in improved minimum essential medium (IMEM) (Biofluids) containing phenol red. Twenty-four h after their seeding, the VSMCs were treated with FBS, PS, DCC-treated FBS (SFBS), DCC-treated calf serum (CCS), or DCC-treated porcine serum, all at final concentrations of 10%, in phenol red-free IMEM. Regarding Figs. 2, 4, and 5, the VSMCs were seeded as described for Fig. 1. All of the cells were washed with 0.1% BSA in phenol red-free IMEM twice daily for 3 d to strip steroid hormones from the cell culture environment. The cells were maintained in 5% CCS in phenol red-free IMEM throughout the stripping period. Four d following their seeding, the cells were treated as described.

In Fig. 3, the VSMCs were seeded, as described above, into plastic or type-I collagen-coated 24-well plates. Cells examined under estrogen-depleted

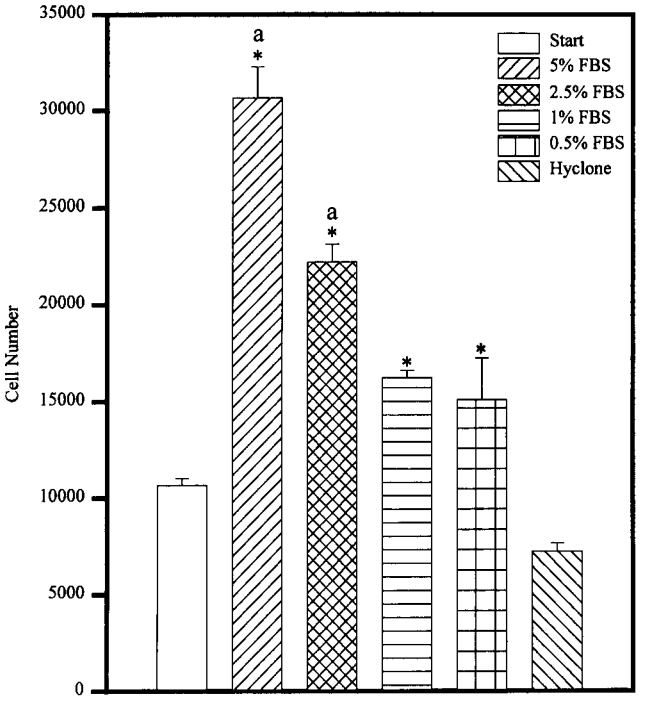

FIG. 2. Growth effects of various fetal bovine serum concentrations on porcine coronary artery smooth muscle cells. Porcine coronary artery SMCs were seeded in 5% FBS in IMEM containing phenol red into plastic 24-well plates. For the next 3 d, the cell environments were stripped of steroid hormones with washes in 0.1% BSA twice daily. The cells were maintained in 5% CCS in phenol red-free IMEM during this period. On the fourth d following their seeding, the cells were treated with the indicated FBS doses or the HyClone serum substitute in phenol red-free IMEM and the average number of cells per well was determined (Start). All cells were harvested for counting 48 h after treatment applications. The Student-Newman-Keuls multiple comparison test was used to detect significant differences among means. Each column represents the mean and SEM of six replicates (* denotes significant growth increase vs. control and [a] denotes significant difference vs. incubation in 1% and 0.5% FBS).

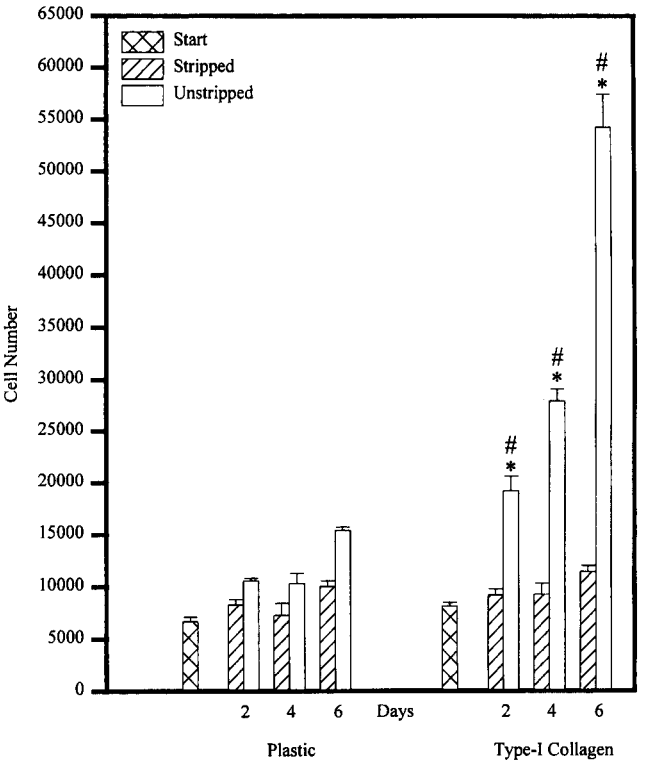

FIG. 3. Substrate effects on porcine coronary artery smooth muscle cell growth: plastic versus type-I collagen. Porcine coronary artery SMCs were seeded into plastic and into type-I collagen-coated 24-well plates in 5% FBS in IMEM containing phenol red. Designated stripped cells were depleted of steroid hormones by washes in 0.1% BSA and were cultured in 5% CCS in phenol red-free IMEM for three consecutive d before treatment. Nonstripped cells were treated 24 h following their seeding. Treatments included 5% SFBS in phenol red-free IMEM plus growth factor supplements (VSMCs that were previously cultured under steroid hormone-stripped conditions) or 5% FBS in IMEM containing phenol red plus growth factor supplements (VSMCs that were not stripped of steroid hormones). The combination of growth factor supplements was EGF (0.5 ng/ml) plus INS (5 ug/ml) and FGF (2 ng/ml). Three wells of cells from each type of plate were harvested and counted on treatment d to determine the Start counts. The cells were incubated for the indicated periods, then counted by Coulter counter. The remaining cells received fresh media at each time point. The Student-Newman-Keuls multiple comparison test was used to detect significant differences among means. Each column represents the mean and SEM of six replicates (* denotes significant growth increase vs. equivalent time and culture medium condition on plastic and [#] denotes, for equivalent times, significant difference vs. steroid hormone-stripped culture condition on collagen).

conditions were stripped of steroid hormones for 3 d as described above. Four d after seeding, these cells were treated with 5% SFBS plus growth factor supplements. Cells evaluated under nonestrogen-depleted conditions were treated 24 h following their seeding in 5% FBS in IMEM containing phenol red plus growth factor supplements. For all growth assays, on each treatment d and at designated termination d, cells were trypsinized and counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) to determine initial and final cell counts, respectively.

Growth factor preparation and application. Human recombinant platelet-derived growth factor (PDGF)-BB, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin (INS) (Sigma Chemical Co., St. Louis, MO), and IGF-1 (Upstate Biotechnology Inc., Lake Placid, NY) were prepared for cell culture use. Stock solutions of each growth factor were prepared as follows: PDGF-BB (20 ng/μl) was dissolved in 500 μl sterile 4 m*M* HCL containing 0.1% BSA (Sigma) and stored in 20-μl aliquots at −20° C until needed; EGF (10 ng/ml), IGF-1 (100 ng/ml), bFGF (25 ng/μl), and INS (5000 ng/μl) were dissolved in sterile 1× PBS containing 0.1% BSA and stored in 20-μl aliquots at −20° C until needed. It was unclear which PDGF receptors (αα, ββ, αβ) were expressed on the porcine VSMC surface. Therefore, PDGF-BB was used because unlike PDGF-AA or PDGF-AB, it binds to all PDGF receptor isotypes (7).

The mitogens were used at concentrations (5, 10, and 20 ng/ml) that were effective in former studies (9,24) to significantly stimulate VSMC proliferation. Although the cells used for this study were derived from a different vessel (coronary artery vs. aorta), we considered the growth factor doses appropriate.

Flow cytometry. Porcine VSMCs (Passages 5 and 6) were seeded at 5000 cells/cm^2 into two plastic (Sarstedt) and into two type-I collagen-coated (Becton Dickinson Labware) T-175 cm^2 flasks in 5% FBS in IMEM containing phenol red. After 24 h, the cell environments were stripped of steroid hormones with washes in 0.1% BSA twice daily for three consecutive d. The cells were maintained in 5% CCS in phenol-red free IMEM throughout the hormone-stripping period. The cells cultured on both substrates were treated 4 d following their seeding. Treatment medium included 2.5% FBS in phenol red-free IMEM. Following incubation for 24 h at 37° C, the cells were trypsinized, washed in 1× PBS, and stored in a citrate/dimethylsulfoxide buffer at −80° C until cell-cycle analysis was performed as described elsewhere (22). The cells were stained with propidium iodide before their analysis in a FAC Star^{PLUS} flow cytometer (Becton Dickinson, San Jose, CA).

Electron microscopy. To evaluate the ability for substrates to induce phenotypic changes, the VSMCs were cultured and subsequently prepared for ultrastructure observation by transmission electron microscopy (TEM). The cells were seeded at 5000 cells/cm² into five plastic and into five type-I collagen-coated T-175 cm² flasks with 5% FBS in IMEM containing phenol red. The cells were allowed to adhere to the flask surfaces in this medium for 24 h. For 3 d following, the cells were stripped of steroid hormones with washes in 0.1% BSA twice daily and were maintained in 5% CCS in phenol red-free IMEM during the stripping period. On the fourth d following cell seeding, the culture medium was changed to 2.5% FBS in phenol-red free IMEM.

Vascular smooth muscle cell processing for TEM analysis has been described previously (26). Briefly, after 48 h of incubation at 37° C, the cells from each flask were fixed in 15 ml of 3% glutaraldehyde–cacodylate buffer for 1 h at 4° C. Following a series of washes in 0.1 *M* cacodylate buffer, the cells were fixed in osmium tetroxide for 1 h at 4° C and subsequently stained with 2% uranyl acetate and lead citrate for 30 min at room temperature. The cell pellets were serially dehydrated in ethanol and embedded in Spurr's resin solution at room temperature for 15 h. A diamond sectioning knife was used to cut ultrathin (90–100 nm) sections of the cell pellets for viewing by TEM.

Ultrastructural analyses of VSMCs previously cultured on plastic and on type-I collagen were performed by TEM on a JEOL 1200 EX electron microscope (Peabody, MA). Cells were randomly chosen for observation. Upon viewing a cell, instrument focus and magnification were adjusted to permit clear, resolute images of the organelles and cytoskeletal features of interest: rough endoplasmic reticula (RER), mitochondria, golgi apparati, and actin filaments. Each randomly chosen field was photographed.

Four different reviewers, blinded to the experimental treatments administered, inspected the TEM photographs to identify the appropriate organelles and actin filaments. Thirteen photographs, representing porcine VSMCs cultured on plastic and on type-I collagen, respectively, were examined. Each reviewer counted and recorded the number of organelles in each photograph. The amount of actin staining was indicated by an ordinal scoring system with a score of (−) indicating no filaments present and scores of (+), (++), and (+++) designating relative increasing amounts of actin compared to a cell with no filaments. Additionally, reviewers described the orientation of actin filaments as either parallel, perpendicular, or a mix of both orientations to the longitudinal axis of the cell. For each photograph, the organelle and actin counts were summed and averaged. Average counts from each photograph (13 plastic and 13 collagen) were summed and averaged to calculate means and standard errors of the mean for each organelle and actin for each substrate condition.

Phase-contrast microscopy. Passage 6 porcine VSMCs were seeded at 5000 cells/cm² in 5% FBS in IMEM containing phenol red into each well of a plastic 6-well dish. Three wells contained one plastic coverslip each and three wells contained one type-I collagen-coated coverslip each. After permitting cell adhesion for 24 h, the cells were stripped of steroid hormones with washes in 0.1% BSA twice daily for three consecutive d. The culture medium during the stripping period was 5% CCS in phenol red-free IMEM. The cells were incubated in 2.5% FBS in phenol red-free IMEM for 48 h before each coverslip was removed and mounted with 80% glycerol onto a glass slide before examination by phase-contrast microscopy. Inspections were performed at 400× magnification with a Zeiss microscope (Germany).

Statistics. The Student-Newman-Keuls multiple comparison test was used to detect significant differences among mean values describing cell growth. Significant differences among means generated from flow cytometry and electron microscopy analyses were demonstrated by the paired Student's *t*-test. For both methods, a value of $P < 0.05$ was used to determine statistical significance.

Results

Growth characterization. Serum-free medium could not support VSMC adhesion or growth (data not shown). Subsequently, the VSMCs were cultured in a variety of sera at 10% final concentration in phenol red-free IMEM. Porcine VSMCs cultured in DCC-treated sera did not grow (data not shown), whereas FBS and PS were potent growth stimulants for these cells (Fig. 1, $P < 0.05$). We reasoned that FBS would have contained higher concentrations of growth factors compared to whole calf serum and therefore opted not to use the latter serum in this experiment, because only FBS stimulated exponential cell growth ($P < 0.05$).

Fig. 2 depicts VSMC growth in FBS concentrations of 0.5%, 1%, 2.5%, 5%, and in a steroid hormone-free serum supplement (HyClone Laboratories, Inc., Logan, UT) at 1× in phenol red-free IMEM. All concentrations of FBS promoted significant growth ($P < 0.05$), whereas the steroid hormone-free supplement was unable to support VSMC adhesion and growth. Five percent FBS elicited significantly better growth than all other FBS concentrations ($P < 0.05$). However, 2.5% FBS stimulated slightly more than one cell doubling in 48 h, yet it contains less endogenous estrogen than 5% FBS. Vascular smooth muscle cell growth in 2.5% FBS was significantly greater than in 1% FBS ($P < 0.05$). One percent FBS and 0.5% FBS were equally effective.

Fig. 3 shows VSMC growth responses following steroid hormone-stripping and non-steroid hormone-stripping before treatment with 5% SFBS or 5% FBS, respectively. Each of these serum preparations contained a combination of EGF (0.5 ng/ml), INS (5 μg/ml), and FGF (2 ng/ml) in phenol red-free IMEM. We hypothesized that multiple stimuli applied simultaneously would elicit significant VSMC growth. Subsequent experiments would be aimed at using mitogens individually to evaluate the mitogenic activity of each one. The latter approach reduced experimental variables and enabled more defined information pertaining to the factor(s) responsible for VSMC growth in these experiments. Vascular smooth muscle cells cultured on type-I collagen grew significantly better than those cultured on plastic ($P < 0.05$). Cells cultured on type-I collagen and stimulated under nonsteroid hormone-stripped conditions, grew significantly better than cells stimulated on this substrate under stripped conditions ($P < 0.05$).

Vascular smooth muscle cells were cultured in 2.5% FBS plus PDGF-BB, EGF, IGF-1, or INS at 5, 10, and 20 ng/ml on type-I collagen. The data indicate that all doses of PDGF-BB significantly stimulated VSMC proliferation ($P < 0.05$), even when compared with the other growth factors (Fig. 4). The VSMCs were not significantly affected by the latter agents. A dose of 10 ng PDGF-BB per ml stimulated significantly better growth than either the 5 or 20 ng/ml dose. Whereas VSMC growth in 2.5% FBS on plastic yielded an approximate cell doubling in 48 h (Fig. 2), cells grown on type-I collagen with PDGF-BB at 10 ng/ml more than doubled in number in 48 h (Fig. 4). All growth factor combinations (Fig. 5) produced significant increases in VSMC proliferation ($P < 0.05$). However, they were not significantly different from the VSMC growth response to 10 ng PDGF-BB per ml alone ($P < 0.05$).

Cell-cycle analysis: plastic vs. type-I collagen substrates. Flow cytometry analyses of VSMCs were performed to investigate the disparity in proliferative rates observed for cells cultured on plastic versus those cultured on type-I collagen. This technique had been useful to detect a VSMC phenotypic switch induced by type-I collagen (16). Our goal was to determine the percentage of cells included in the growth fraction ($S + G_2/M$ phases) within each population of cells that was cultured on its respective substrate. The data indicated that the percentage of cells in S-phase, when cultured on type-I collagen, was consistently twofold greater or more than the percentage of VSMCs in S-phase when cultured on plastic ($P < 0.05$) (Table 1). The percentage of cells in the growth fraction when cultured on collagen was roughly one-third more than that of cells cultured on plastic. These data reflect the significantly higher number of cells observed when VSMCs were cultured on type-I collagen in-

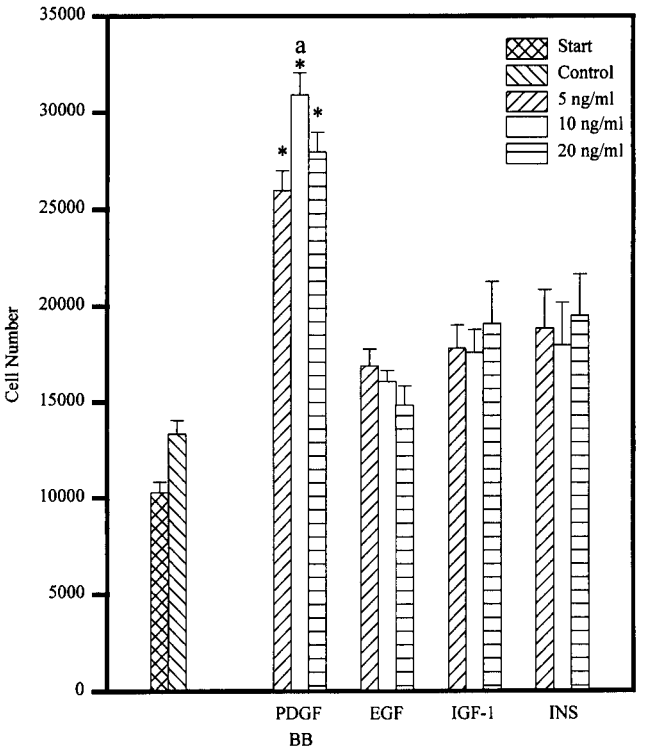

FIG. 4. Proliferative effects of growth factor supplements on porcine coronary artery smooth muscle cells. Cells were seeded in 5% FBS in IMEM containing phenol red into type-I collagen-coated 24-well plates. The cells were washed twice daily with 0.1% BSA and were cultured in 5% CCS in phenol red-free IMEM for the next 3 d. Triplicate sets of cell populations were treated with or without (control) the indicated growth factors (5, 10, or 20 ng/ml) in 2.5% FBS in phenol red-free IMEM for 48 h. Three wells of cells were harvested and counted on the treatment d to determine the average number of cells per well (Start). The Student-Newman-Keuls multiple comparison test was used to detect significant differences among means. Each column represents the mean and SEM of nine replicates (* denotes significant growth increase vs. control and [a] denotes significant difference vs. incubation in 5 and 20 ng PDGF-BB per ml).

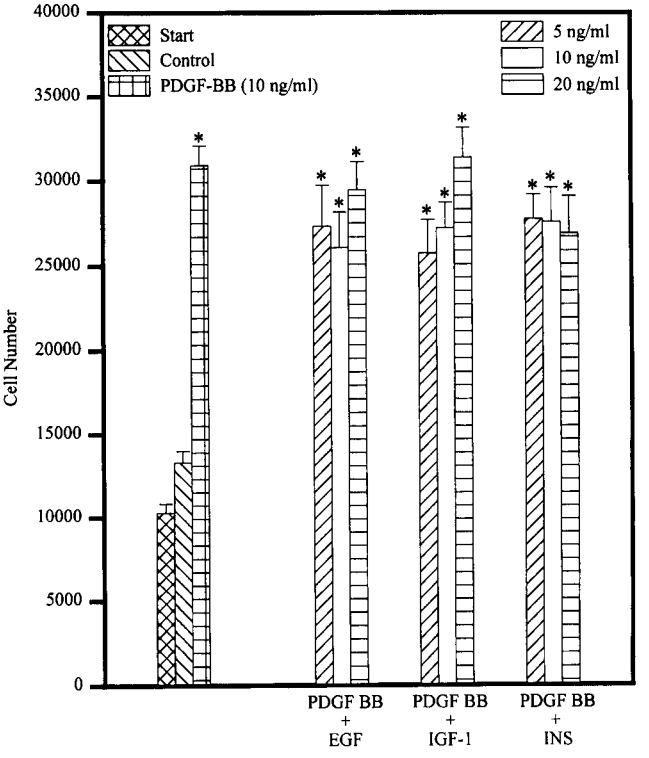

FIG. 5. Combinatorial proliferative effects of PDGF-BB and other growth factor supplements on porcine coronary artery smooth muscle cells. Cells were seeded in 5% FBS in IMEM containing phenol red into type-I collagen-coated 24-well plates. The cells were washed twice daily with 0.1% BSA and were cultured in 5% CCS in phenol red-free IMEM for the next 3 d. Triplicate sets of cells were treated with or without (control) the indicated growth factors (5, 10, or 20 ng/ml) in 2.5% FBS in phenol red-free IMEM for 48 h. Three wells of cells were harvested and counted on the treatment d to determine the average number of cells in each well (Start). The Student-Newman-Keuls multiple comparison test was used to detect significant differences among means. Each column represents the mean and SEM (* denotes significant growth increase vs. control).

stead of plastic (Fig. 3). Consistent with these results, was our finding that a larger percentage of cells cultured on plastic were in G_0/G_1 (mitogenically quiescent) than cells cultured on type-I collagen.

Electron microscopy: plastic versus type-I collagen substrates. The increases in VSMC growth and proportion in the growth fraction when cultured on type-I collagen suggested that the cells had switched to a more synthetic, less contractile phenotype. To investigate this hypothesis, an ultrastructural study of the VSMCs by TEM analysis was conducted to detect collective quantitative differences in organelles/cytoskeletal features that would indicate a change in VSMC phenotype. The organelle/cytoskeletal features of interest included RER, mitochondria, golgi apparati, actin filaments, and actin filament orientation (2).

TEM produced clear, artifact-free images of cellular ultrastructure of vascular smooth muscle cells after their culture on plastic and on type-I collagen substrates. Rough endoplasmic reticula appeared as vacuolated, ribosome-studded structures. Mitochondria were readily recognized by their cristae and double-layered membranes. Rough endoplasmic reticula and mitochondria varied in size, but this was uninformative. Golgi apparati clearly appeared as stacks of closely associated curved plates from which secretory granules were seen budding off. No defined polarity or pattern was evident with respect to the positions of these organelles in these cells. Nuclei, however, were centrally located and occupied at least 30–40% of the total cell

TABLE 1

CELL CYCLE ANALYSIS OF PORCINE VSMCs CULTURED ON PLASTIC AND ON TYPE=I COLLAGEN

Substratum	$\%G_0/G_1$ Mean	$\%G_0/G_1$ SEM	%S Mean	%S SEM	$\%G_2/M$ Mean	$\%G_2/M$ SEM	Significant Difference ($p < 0.05$)
Plastic	78.75	13.2	7.75	6	13	7	No
Type-I Collagen	71	8	17.5[a]	5	11.5	4.6	Yes

[a]The percentage of cells in the growth fraction (%S) when cultured on collagen was significantly different from that of cells cultured on plastic ($P < 0.05$).

TABLE 2

AVERAGE INDIVIDUAL ORGANELLE AND ACTIN FILAMENT CONTENT VALUES FOR PORCINE CORONARY ARTERY SMOOTH MUSCLE CELLS CULTURED ON PLASTIC AND ON TYPE-I COLLAGEN

Organelle and actin filaments	Plastic		Collagen		Significant difference ($P < 0.05$)
	Mean	SEM	Mean	SEM	
Rough endoplasmic reticula	4.9	1.96	6	2.78	No
Mitochondria	2.6	2.56	3.2	2.16	No
Golgi apparati	0.19	0.4	2	0.72	Yes
Actin filaments	1.23	0.94	0.85	0.94	No

area. Euchromatin, which was indicative of a noncondensed, gene transcription-ready state, was evident in all nuclei observed. Bundles of actin filaments were observed, primarily arranged parallel to the longitudinal axis of the cell.

On average, there was more of every organelle counted per cell in VSMCs cultured on type-I collagen than in VSMCs cultured on plastic. However, only the number of golgi apparati per cell was significantly greater ($P < 0.05$) in VSMCs cultured on type-I collagen than on plastic (Table 2). There were no significant differences observed in actin filament content or orientation per cell.

Phase-contrast microscopy: plastic versus type-I collagen substrates. Fig. 6 shows the VSMCs cultured on plastic (*Panel A*) and on type-I collagen (*Panel B*), respectively. Although both cell populations demonstrated characteristic VSMC spindle shapes, the cells cultured on type-I collagen appeared smaller and less regular morphologically than those cultured on plastic.

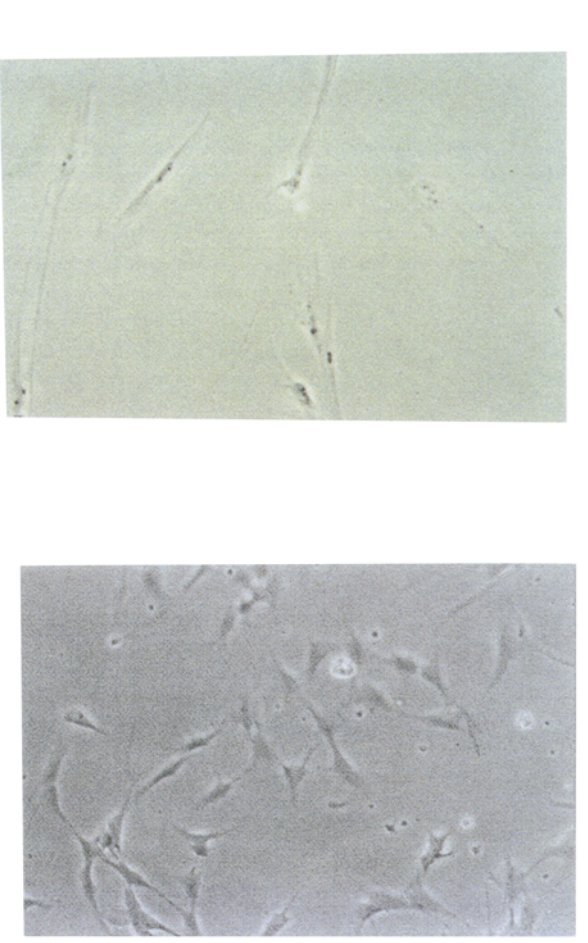

FIG. 6. Vascular smooth muscle cells cultured on plastic and on type-I collagen. Porcine VSMCs were seeded in 5% FBS in IMEM containing phenol red onto plastic or onto type-I collagen-coated coverslips. After 24 h, cells were stripped of steroid hormones for 3 d with washes in 0.1% BSA twice daily. The cell culture medium throughout the stripping period was 5% CCS in IMEM without phenol red. Next, the cells were cultured in 2.5% FBS in IMEM without phenol red for 2 d. Coverslips were mounted onto one glass slide each for viewing by phase-contrast microscopy. The images in this figure were produced at 400×. The characteristic VSMC spindle-shaped morphology is evident for cells cultured on plastic (*Panel A*). For cells cultured on type-I collagen, the characteristic VSMC spindle-shaped morphology is evident, yet these cells appear smaller and less regular morphologically than those cells cultured on plastic (*Panel B*).

DISCUSSION

We have studied porcine coronary artery SMC growth and ultrastructural characteristics. The porcine VSMCs did not remain adherent to the culture surface, nor did they grow in serum-free medium. Of the sera tested, FBS provided the best growth response. A final concentration of 2.5% FBS appeared optimal, considering our desire to minimize endogenous estrogen levels while maintaining some proliferation. In an attempt to enhance VSMC growth, we cultured cells on type-I collagen and discovered that this substrate significantly increased porcine VSMC growth beyond that observed on plastic, but only when the cells were cultured in whole FBS. A suspected concomitant change in VSMC phenotype was not observed. Platelet-derived growth factor-BB was the best mitogen for these cells on collagen. Combinations of this competence factor with a variety of progression factors failed to enhance VSMC growth relative to that with PDGF-BB alone. Taken together, these experiments indicate that porcine coronary SMCs cultured in 2.5% FBS plus 10 ng PDGF-BB per ml on type-I collagen may be optimal to study the molecular aspects of porcine coronary artery SMC proliferation and its inhibition by antiproliferative agents.

Serum-free medium was ineffective at supporting both VSMC adhesion and growth. Apparently, autocrine, paracrine, and growth factor response capabilities were compromised. Perhaps the lack of critical cellular adhesion factors such as fibronectin (10) and laminin (11) was limiting. Nontransformed cells require adhesion before they can proliferate in vitro (1). The current data suggest that serum contains adhesion factors that are critical for growth of these cells. Therefore, we introduced serum into the medium to support VSMC adhesion and proliferation.

The need to minimize the estrogen content in the cell culture medium prompted us to test the mitogenic capability of DCC-treated serum. Unlike serum-free medium, SFBS and CCS supported VSMC adhesion, but not growth. This implies that different factors are required for these functions in these cells. Even when adequately attached to plastic, the VSMCs did not proliferate. Whole FBS promoted continuous cell growth, suggesting that the DCC treatment removed or inactivated critical growth factors such as PDGF, EGF, IGF-1, and INS, or adhesion factors, examples of which were provided above. Specific identification of these serum-derived agents

and possible biological manifestations (i.e., apoptosis, hsp70 expression, etc.) was beyond the scope of this investigation. It was necessary to titrate the FBS concentration to reduce the endogenous estrogen level to a minimum, while maintaining adequate cell proliferation.

Whereas the VSMCs grew best in 5% FBS, the cells doubled in 48 h when grown in 2.5% FBS. The latter concentration appeared optimal, since it would contain less estrogen than 5% FBS. Neither 1% FBS nor 0.5% FBS provided sufficient mitogenic stimuli for these cells. We suspected that an artificial, steroid hormone-free, nutrient-enriched serum supplement would have been optimal for growth of these cells. However, these experiments did not yield such data.

The LAD artery SMCs were considered synthetic-like by virtue of their ability to migrate from the explants and to proliferate. Growth factor-induced proliferation would likely require the VSMCs to maintain this phenotype (5). Type-I collagen can change VSMC phenotype from contractile to synthetic in vitro (27). Additionally, this collagen subtype is the most abundant in the atherosclerotic plaque (13) and, as suggested in vitro (27), may be the substrate upon which synthetic VSMCs proliferate in vivo. These points support our use of type-I collagen as a substrate to promote VSMC growth.

Porcine coronary artery SMCs cultured on type-I collagen grew significantly faster than cells cultured on plastic. Flow cytometry analyses confirmed these observations by demonstrating that a greater percentage of cells cultured on type-I collagen were in the growth fraction. Data generated from TEM analyses, based upon established criteria (2), indicated that a phenotypic switch had not occurred when the VSMCs were cultured on type-I collagen. However, the significant increase in golgi apparati observed in cells cultured on type-I collagen indicated that these cells had become further synthetic-like within the proposed spectrum (17) of VSMC phenotypes. Vascular smooth muscle cells cultured on type-I collagen experienced significant mitogenesis relative to cells cultured on plastic, apparently without experiencing a completely defined transition to the synthetic phenotype.

Type-I collagen was a significantly better growth substrate than plastic, but only when cells were cultured in non-DCC-treated FBS. These observations suggest that serum components and type-I collagen acted cooperatively to stimulate VSMC growth. Evidently, the stripping process removed serum-derived growth factors that were necessary to complement the type-I collagen-induced contribution. Previous studies indicate that the latter phenomenon may include growth factor receptor expression (14) and/or growth factor receptor potentiation (3). In the current model, type-I collagen-induced growth factor expression and potentiation would have been fruitless without adequate levels of ligand (in stripped serum) to activate the receptors. Alternatively, the growth factors required unidentified serum components to elicit their full mitogenic potential. A similar requirement was observed for PDGF when it was found that only in the presence of plasma proteins could it stimulate significant 3T3 fibroblast growth (23).

The combination of applied growth factors, in the presumed presence of serum-derived PDGF (Fig. 3), promoted the maximum amount of VSMC growth observed in this study. A dissection of growth factor stimulation revealed that PDGF, in the presumed presence of serum-derived progression factors, yielded significant mitogenesis (Fig. 4). Exogenous progression factor addition to exogenous PDGF reduced VSMC mitogenesis compared to that observed with PDGF alone (Fig. 5). Further molecular studies are required to investigate these phenomena. These data suggest that exogenously added progression factors were not necessary to achieve maximum mitogenic rates in this experiment. In contrast, it may have been the concerted effects of endogenous EGF, IGF-1, and/or INS, plus exogenously added PDGF-BB, that promoted maximum VSMC growth. We hypothesize that the combinatorial addition of these progression factors to this particular culture medium would fail to elicit a growth response equivalent to that demonstrated with PDGF added by itself. Since the individual addition of PDGF, but not the progression factors, stimulated the greatest growth (Fig. 4), it is inferred that PDGF presence in the serum was most critical.

We have characterized porcine coronary artery SMC growth and phenotype in response to several growth factors and substrates. Variations in VSMC behavior (i.e., no cell adherence; cell adherence, but no growth; cell adherence and growth; and different rates of cell growth) were observed in response to a variety of cell culture conditions (serum-free medium, DCC-treated serum, whole serum, plastic and type-I collagen substrates, and growth factor supplementation of medium that contained a minimal amount of serum). These observations have generated several questions for further investigation. For example, what is the essential difference between serum-derived factors that permit VSMC adherence and those that permit both VSMC adherence and growth? The significant, but submaximal proliferative rate and the relative reduction in variables required to achieve this rate make the combination of PDGF-BB (10 ng/ml) in 2.5% FBS with type-I collagen as substrate a possible optimal culture model to study the molecular mechanisms of proliferation and growth inhibition in these coronary SMCs. Regarding investigation of the latter phenomenon, we have detected estrogen receptor mRNA and protein in these VSMCs cultured under the apparent optimal culture conditions. This information has afforded us the opportunity to investigate the mechanism through which estrogen and antiestrogens effect VSMC proliferation (Lavigne et al. Steroids, in press).

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