

Glucocorticoid Inhibition of Vascular Smooth Muscle Cell Proliferation: Influence of Homologous Extracellular Matrix and Serum Mitogens

JOHN P. LONGENECKER, LAURA A. KILTY, and LORIN K. JOHNSON

Department of Pathology, Stanford University School of Medicine and the Geriatric Research Education and Clinical Center, Palo Alto Veterans Administration Medical Center, Palo Alto, California 94304. Dr. Longenecker's and Dr. Johnson's present address is California Biotechnology, Inc., Mountain View, California 94043.

ABSTRACT We examined the influence of glucocorticoid hormones on the proliferation of cultured adult bovine aortic smooth muscle cells (BASM) using both primary mass cultures and a cloned strain. Cloned BASM cells maintained on plastic culture dishes were inhibited by ~40% by dexamethasone treatment but showed no inhibition when grown on homologous extracellular matrix (ECM) coated dishes. Dexamethasone inhibited growth of primary cultures by 73% on plastic and by 45% on ECM. The inhibitory effect was specific for the glucocorticoids, dexamethasone, corticosterone, and cortisol and was not observed with progesterone, aldosterone, estradiol or 17- α OH progesterone.

In cloned cells, the abolition of glucocorticoid inhibition by ECM was independent of seeding density and serum concentration. The inhibition on plastic was dependent on serum concentrations >1% and resulted in both a slow rate of proliferation and a lower saturation density. A specific subset of peptides detected on two-dimensional gels was induced by glucocorticoids under growth inhibitory conditions but was not induced when the cells were grown on ECM.

Primary cultures grown on ECM and exposed to Dulbecco's modified Eagle's Medium (DME) containing high density lipoprotein and transferrin grew at 40% of the rate observed for cultures exposed to DME with 10% serum. Both conditions showed growth inhibition of 70% in the presence of dexamethasone. The addition of epidermal and platelet-derived growth factors in DME containing high density lipoprotein and transferrin to cells grown on ECM resulted in growth rates comparable to that observed with cultures exposed to 10% serum and were inhibited 45% by dexamethasone. These results suggest that glucocorticoids inhibit smooth muscle proliferation by decreasing the sensitivity of the cells to mitogenic stimulation by high density lipoprotein when the cells are maintained on a homologous substrate.

Alterations in the control of vascular smooth muscle cell proliferation are major factors in the development of atherosclerotic vessel disease (1). An understanding of factors that either stimulate or inhibit vascular smooth muscle cell proliferation is therefore a prerequisite to understanding and possibly controlling the pathogenesis of vascular disease. A major contribution to our knowledge concerning the control of vascular smooth muscle cell proliferation has come from the use of cultured aortic smooth muscle cells. Two growth factors

found in serum, platelet-derived growth factor (PDGF)¹ (2), and epidermal growth factor (EGF) (3), as well as a noncirculating fibroblast growth factor (FGF) (3), have been identi-

¹ *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; DEX, dexamethasone; BASM, bovine aortic smooth muscle; HDL, high density lipoprotein.

fied as mitogens for these cells. A polypeptide secreted by endothelial cells has been observed to stimulate smooth muscle cell proliferation in culture (4). It also has been observed that aortic smooth muscle cells, when grown on extracellular matrix derived from corneal endothelial cells, can proliferate maximally in the absence of PDGF provided HDL, EGF, and insulin or somatomedin C are present (5). Because smooth muscle cells *in vivo* are in constant contact with a variety of extracellular components, these later experiments suggest that the study of cultured smooth muscle cells when grown on plastic surfaces *in vitro* may yield results that differ considerably from that which occurs *in vivo*.

Although a large body of information has accumulated concerning factors that stimulate vascular smooth muscle cell proliferation, little is known about factors which are inhibitory to smooth muscle cell growth. Recently, a heparin-like substance secreted by endothelial cells has been characterized that inhibits smooth muscle cell proliferation *in vitro* (6). We have also reported that glucocorticoids inhibit the growth of aortic smooth muscle but not aortic endothelial cells in culture (7). This inhibitory response was observed in both primary mass cultures and a cloned strain of bovine aortic smooth muscle (BASM) cells. In addition, the noncirculating mitogen, FGF, was found to totally abolish the glucocorticoid-mediated inhibition of smooth muscle cell proliferation (7).

These results suggest that glucocorticoids may selectively inhibit the responsiveness of smooth muscle cells to the mitogenic action of some component(s) in serum. Because of these observations, and the finding that the extracellular substrate can also modify the responsiveness of smooth muscle cells to serum growth factors, we examined the influence of glucocorticoids on the growth of BASM cells maintained on a homologous extracellular matrix and in serum-free culture media. The data show that glucocorticoids are growth inhibitory to BASM cells under all growth conditions examined and support the proposition that myointimal hyperplasia may be directly inhibited *in vivo* by these steroids.

MATERIALS AND METHODS

FGF and EGF were the generous gifts of Dr. D. Gospodarowicz. The FGF was prepared as described (8) from bovine brain and solubilized at 50 $\mu\text{g}/\text{ml}$ in Dulbecco's modified Eagle's medium (DME-H16) containing 0.1% bovine serum albumin. EGF was purified as described by Savage and Cohen (9). Human high density lipoprotein (HDL) was purified from freshly drawn blood by the method of Havell et al. (10). PDGF was purchased from Collaborative Research Inc. (Lexington, MA). DME-H16, Fungizone, glutamine, penicillin, streptomycin, trypsin-EDTA, and Hanks' balanced salt solution were obtained from Grand Island Biological Co. (Grand Island, NY). Collagenase was purchased from Worthington Biochemicals (Freehold, NJ). Calf serum (lot 2275) was obtained from Sterile Systems Inc. (Logan, UT). [^{35}S]methionine (>1,000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Acrylamide, bis-acrylamide, and ammonium persulfate were obtained from Bio-Rad Laboratories (Richmond, CA). SDS was a product of BDH Chemicals Ltd. (Poole, England), and carrier ampholines were obtained from LKB Instruments (Rockville, MD). All other chemical and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture: Primary cultures of BASM cells were prepared from an adult bovine aortic arch by collagenase digestion according to the method of Chambley-Campbell et al. (11). Following establishment of the primary culture, cells were harvested by trypsinization (0.01% trypsin and 0.02% EDTA) and cloned as described by Gospodarowicz et al. (12), in the presence of FGF. Primary cultures were used during the second to sixth passage (1:50 split ratio). Stock cultures of both primary and cloned BASM cells were maintained in DME-H16 supplemented with 10% calf serum, penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), and Fungizone (2.5 $\mu\text{g}/\text{ml}$) in a 5% CO_2 -humidified incubator at 37°C. Stocks of the cloned cells received 250 ng/ml FGF added every other day until they reached subconfluent densities.

For growth rate determinations, cells were seeded at an initial density of 2.0×10^4 cells per 35 mm well in 6-well plates. 24 h later, any additions, i.e., hormones or growth factors, were made and, at the end of the growth period, triplicate plates were trypsinized into single-cell suspensions, and the cells were counted with a hemocytometer.

Preparation of ECM: Cultures of cloned BASM cells were used for the preparation of ECM-coated tissue culture dishes as previously described (13). These cells were grown to confluence, in the absence of FGF, washed twice with phosphate-buffered saline, then exposed to 0.25 M NH_4OH in distilled water for 10 min at 25°C followed by several washes with distilled water. The resulting ECM-coated dishes were stored at 4°C in distilled water for up to 3 mo without loss of their growth-promoting activity.

Metabolic Labeling and Two-dimensional Gel Electrophoresis: Cells were seeded into 2-cm² culture wells, either previously coated with ECM or not, at a density of 5×10^3 cells/well. 6 h later, dexamethasone (1 μM) or FGF (250 ng/ml) was added and the cells were incubated for an additional 48 h. Cultures were then washed twice in DME containing 10 μM methionine and incubated in the same media for 1 h with 400 $\mu\text{Ci}/\text{ml}$ [^{35}S]methionine. Following the labeling period, the cells were washed twice with PBS at 4°C and solubilized in 75 μl of O'Farrell lysis buffer (14). 500,000 acid-precipitable cpm were then fractionated on equilibrium two-dimensional gels as previously described (14). The second dimension consisted of a 10–16% exponential gradient of acrylamide in SDS. Following fixation and staining (50% trichloroacetic, 0.1% Coomassie Blue), the gels were destained (7% acetic acid), dried under vacuum, and autoradiographed for 10 d on Kodak NS2T film.

RESULTS

Influence of Smooth Muscle ECM on Glucocorticoid Responsiveness of BASM Cells

The proliferation of a primary mass culture as well as one cloned strain of aortic smooth muscle cells was examined over a 5-d period in the presence and absence of the synthetic glucocorticoid DEX. As can be seen in Table I, neither the mass culture nor the cloned strain showed differences in plating efficiency on either the plastic substrate or ECM-coated dishes. In addition, the mass culture grew almost equally well on plastic culture dishes as on dishes covered with ECM when the cells were not exposed to DEX. However, mass cultures exposed to DEX and maintained on plastic reached a density which was only 28% of that observed for control cells. On matrix-coated dishes proliferation in the

TABLE I
Influence of Smooth Muscle ECM on the Growth Response of Smooth Muscle Cells to Glucocorticoids

| Conditions | Cells/dish $\times 10^{-4}$ * | | | |
|------------------------|-------------------------------|-------------------------|--------------------|-------------------------|
| | Plastic (% of control) | | ECM (% of control) | |
| | 6 h | 5 d | 6 h | 5 d |
| Mass culture | | | | |
| Control | 1.2 \pm 0.2 | 51.5 \pm 2.5 | 1.1 \pm 0.1 | 55.1 \pm 5.2 |
| Dex (1 μM) | 1.1 \pm 0.1 | 16.5 \pm 1.8 (28%) | 1.0 \pm 0.1 | 32.1 \pm 2.5 (58%) |
| Clone BASM | | | | |
| Control | 1.3 \pm 0.2 | 42.2 \pm 4.0 | 1.4 \pm 0.2 | 82.5 \pm 6.5 |
| Dex (1 μM) | 1.5 \pm 0.3 | 28.0 \pm 1.5 (56%) | 1.6 \pm 0.4 | 78.4 \pm 8.0 (95%) |

* Cells from either primary mass cultures or a cloned strain were seeded into 35-mm dishes either coated or not with smooth muscle ECM, as described in Materials and Methods, at a density of 2×10^4 cells/dish. Some plates were then counted to determine the plating efficiency at 6 h. DEX was added 24 h later and the remainder of the cells were trypsinized and counted on the fifth day. The mean and standard deviation of triplicate values for each condition are shown.

presence of the glucocorticoid was 58% of the control. With the cloned smooth muscle cells, the use of ECM-coated dishes allowed a twofold increase in the final cell density during the 5-day growth period under control conditions. In the presence of DEX, these cells grown on plastic were inhibited by 46% but showed no significant glucocorticoid inhibition when grown on ECM-coated dishes.

The steroid-induced inhibition of the mass culture shown on Table 1 was also observed to be glucocorticoid specific and was observed with the natural circulating bovine glucocorticoid, cortisol (62% inhibition). Corticosterone used at this same concentration (0.1 μM) produced a 41% inhibition while progesterone consistently produced a slight stimulation of growth (17%), possibly by antagonizing the effect of endogenous cortisol in the calf serum. Aldosterone, estradiol, and 17 α OH progesterone did not significantly inhibit smooth muscle cell growth. The inhibition of aortic smooth muscle cell growth appears to be glucocorticoid specific and is influenced by the extracellular substrate on which the cells are grown.

Effect of ECM, Serum, and Plating Density on the Glucocorticoid-induced Inhibition of Cloned BASM Cell Growth

The use of a cloned strain of BASM cells provided the advantage that all cells in the culture were responding to the growth conditions similarly. Because the results of Table 1 showed that the ECM could totally overcome the inhibitory influence of the glucocorticoid on the cloned strain, we used this cloned strain to examine the kinetics, seeding density and serum requirements of these growth responses.

The data of Fig. 1 show the kinetics of BASM cell growth in the presence and absence of 1 μM DEX over a 9-d period on either plastic or BASM-derived ECM-coated culture dishes. In this experiment the plating efficiency measured at 6 h was the same on plastic and matrix. It is also clear that there is no significant difference in the efficiency of plating as a result of glucocorticoid treatment since the number of cells at day 1 was the same in both presence and absence of DEX. There is, however, a significant difference in the growth of the cells on plastic and ECM, in terms of both the proliferation of the cells (i.e., doubling time), as shown by the slope of the growth curve, and the saturation density reached. Furthermore, DEX was found not to be inhibitory to the growth of these cells when they are plated on ECM-coated dishes, but did significantly inhibit the growth of the cells on plastic. This DEX-induced inhibition is characterized by a decreased period of log-phase growth and an apparent lower saturation density. The data of Fig. 1 are remarkably similar to our earlier observations using FGF in place of ECM-coated dishes (7). FGF stimulates the growth rate and saturation density of this BASM clone when grown on plastic almost identical to that shown here for cells grown on ECM-coated dishes. In addition, FGF overcomes the glucocorticoid inhibition of smooth muscle growth observed in its absence.

We next asked whether the glucocorticoid inhibition of BASM cell growth was dependent on factors in the serum. The data of Fig. 2A show the effect of reducing the serum concentration on the growth (5 d) of BASM cells in the presence and absence of DEX (1 μM) on plastic and ECM-coated dishes. Although the ECM-stimulation of growth occurs at all serum concentrations examined, the serum titration

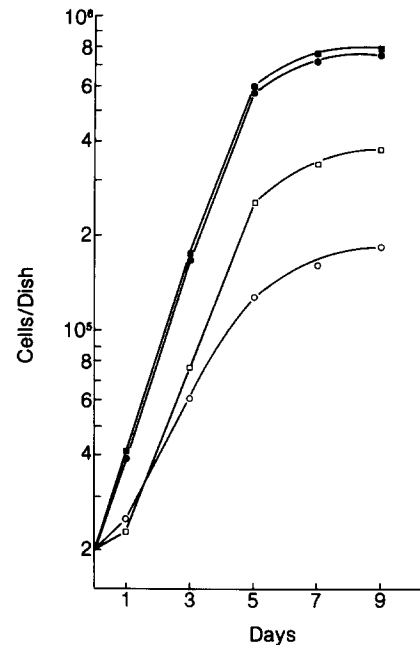


FIGURE 1 Growth of BASM cells in the presence and absence of DEX on plastic and ECM-coated dishes. Cloned BASM cells were plated at 2×10^4 cells/35-mm tissue culture dishes which were either coated or not with BASM-derived ECM. At the times shown, cells were harvested by trypsinization and counted. This figure shows the number of cells per 35-mm well over a 9-d growth period. Solid symbols represent growth on ECM, and the open symbols represent those grown on plastic. Cells grown in the presence of 10^{-6} M DEX are shown in circles.

curves are nearly parallel for ECM and plastic, both showing optimal growth at serum concentrations between 5 and 10%. DEX did not affect BASM cell growth on ECM-coated dishes at any serum concentration. For cells grown on plastic, however, DEX did inhibit growth at higher serum concentrations, but not at concentrations $<2.5\%$.

Because ECM-coated dishes appear to be "mitogenic" for BASM cell growth, and as the response to serum growth factors may be sensitive to the initial density at which the cells are plated, we also examined the influence of plating density on the growth of cloned BASM cells in the presence and absence of DEX on both plastic and ECM-coated dishes. The data of Fig. 2B show that cells grown on ECM-coated dishes are far less sensitive to the initial plating density and that DEX has no effect on their growth at any density. For cells grown on plastic, however, there is a significant inhibition of growth by DEX for cells plated at higher cell densities, but a much smaller inhibition at lower densities. It is of note that a common density limit appears in the data of Fig. 2A and B, for cells grown on plastic in the presence of DEX. Under these conditions, the growth of cells slows and begins to plateau at a cell density of approximately 10^5 cells/well. The reason or mechanism by which DEX results in a lower saturation density is unclear.

Since DEX inhibits the growth of BASM cells grown on plastic, and since ECM is mitogenic for these cells, it was of interest to determine whether DEX could influence the mitogenic activity of the matrix material. BASM cells were grown in the presence and absence of DEX for 11 d, and ECM-coated dishes were prepared by alkali treatment in the usual way. BASM cells plated on each of these ECM-coated

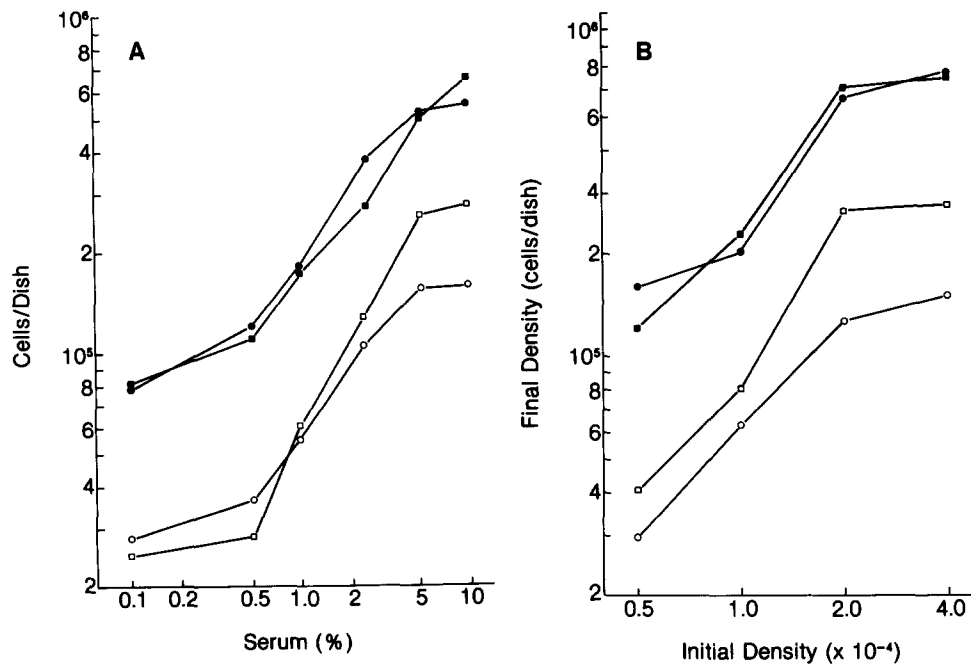


FIGURE 2 The effect of serum concentration and initial plating density on BASM cell growth. (A) Cells were plated at a density of 2.0×10^4 cells/35-mm well in tissue culture dishes which were either coated with ECM (solid symbols) or not (open symbols) in growth medium supplemented with 10% calf serum. 24 h later, the medium was changed to medium supplemented with variable concentrations of serum as shown and dexamethasone addition at a final concentration of 10^{-6} M (circles). Shown are the number of cells per well after 5-d growth. (B) BASM Cells were plated in 35-mm dishes at variable cell densities as shown. The figure shows the number of cells per dish 5 d after plating. The symbols are the same as in A

dishes were grown for 5 d and the number of cells per dish was counted. The growth of BASM cells on these DEX-treated BASM cell-derived ECM was identical to that on non-DEX treated matrix-coated dishes (data not shown). These results indicate that DEX has no effect on the deposition of those factors in the matrix which are required for its mitogenic activity.

Matrix and FGF Overcome the Glucocorticoid Regulation of Specific Protein Synthesis

The glucocorticoid regulation of a variety of cellular functions has been shown to be mediated by specific peptides whose rates of synthesis are either stimulated or repressed by the steroid. We have therefore examined this BASM clone to determine whether conditions that abolish the glucocorticoid inhibition of growth also abolish the regulation of specific peptides by glucocorticoids.

Shown in Fig. 3 are two-dimensional electrophoretograms of smooth muscle cells grown 48 h on either plastic or matrix in the presence and absence of DEX. For comparison, gels are also shown from an experiment where FGF was used instead of matrix. In both cases, cells were pulse-labeled 1 h with [³⁵S]methionine, and the nascent peptides were solubilized and then fractionated on two-dimensional gels. Of 10 peptides whose rate of synthesis was found to be regulated by glucocorticoids, a specific set of two peptides at pI 5.5, molecular weight 20,000 were consistently induced under conditions where glucocorticoid-induced growth inhibition occurred and were not induced by the steroid when the cells were grown on ECM-coated dishes or in the presence of FGF, conditions that abolish the glucocorticoid regulation of growth. Because this experiment was performed on cells in the early log-phase of growth, the glucocorticoid-regulated peptides shown in Fig. 3 do not reflect a change induced by the lower saturation density observed at later time points. They may, however, correlate with the slower growth-rate observed in glucocorticoid-treated BASM cells.

Growth of Primary Cultures of BASM Cells in Serum-free Media

Recent reports have shown that both bovine aortic endothelial and smooth muscle cells can be grown on ECM-coated dishes (derived from corneal endothelial cells) in the absence of serum if HDL is used to supplement the growth media (5, 15). To investigate the glucocorticoid response of primary BASM cells in serum free media, we grew cells on both ECM-coated and plastic tissue culture dishes under a number of growth conditions as shown in Fig. 4. Confirming the result shown in Table I, the primary BASM cells grew equally well on plastic and ECM in 10% serum, and the inhibition of cell growth by DEX occurred on both matrix and plastic, although it was more marked on plastic (68% inhibition) than on matrix (45% inhibition).

The addition of FGF (250 ng/ml) to primary BASM cells grown in 10% serum resulted in only slight growth stimulation on both ECM and plastic (1.2-fold). Although FGF was not potently mitogenic for this primary culture, it still abolished the inhibitory influence of DEX for cells grown on plastic, in agreement with our previously reported observation with cloned cells (7), and greatly reduced the DEX response for cells grown on ECM.

When primary BASM cells were plated on ECM-coated dishes in 10% serum for 24 h, followed by removal of this medium and replacement with DME containing human HDL (250 μ g/ml) and transferrin (50 μ g/ml), growth of the cells was ~40% that of cells grown in 10% serum. Cells which were plated directly in DME-HDL and transferrin grew to ~25% of the control value. Under both of these conditions, DEX inhibited growth by ~70% (Fig. 4A). The same conditions for cells plated on plastic (Fig. 4B) resulted in no significant growth, although the cells did attach to the plastic. Cells grown on ECM in heptane-extracted (cholesterol-low) HDL and transferrin grew as well as on nonheptane extracted HDL, implying that the growth-promoting role of HDL is not likely to be the provision of cholesterol to these cells. DEX addition

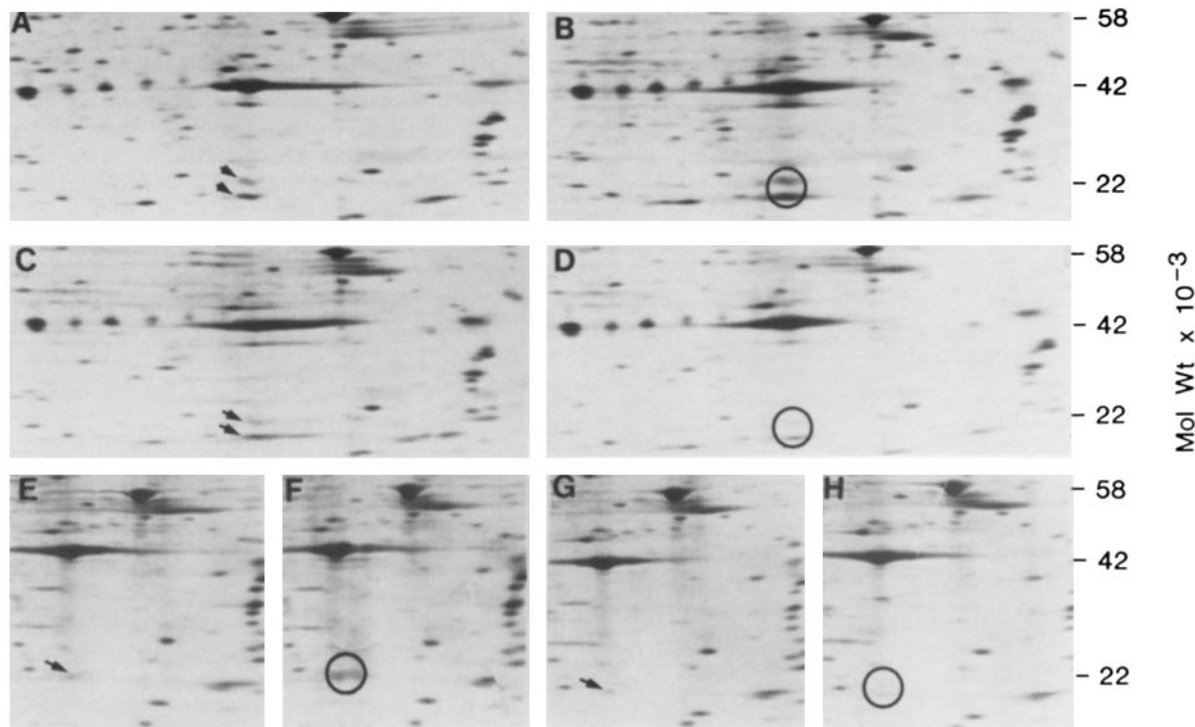


FIGURE 3 Two-dimensional gel electrophoresis of nascent smooth muscle cell peptides, following glucocorticoid treatment. Cells were plated and grown for 48 h in the absence (A, C, E, and G) or presence of 10^{-6} M DEX (B, D, F, and H). Cells were also plated on ECM-coated dishes (C and D) or grown in the presence of FGF (250 μ g/ml) (G and H). Cells were labeled for 1 h with [35 S]methionine, solubilized, and fractionated on two-dimensional gels as described in Materials and Methods. Circles indicate the glucocorticoid-induced proteins at pI 5.5, 20,000 mol wt.

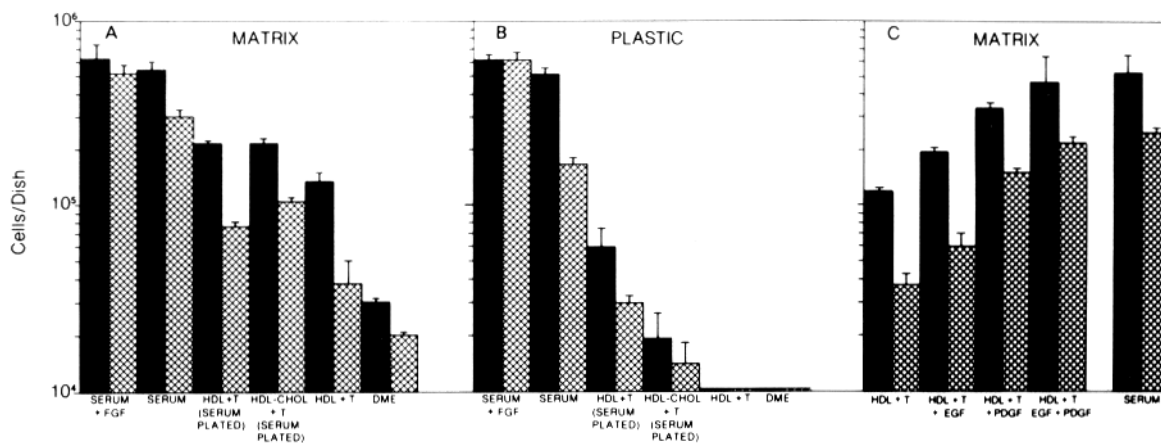


FIGURE 4 Growth of primary BASM cells in high density lipoprotein supplemented growth media vs. serum supplemented media in the presence and absence of dexamethasone. Primary cultures of BASM cells were grown using various supplements to DME-H16 on either matrix-coated (A) or plastic (B) culture dishes. Cells were plated at 2.0×10^4 cells/35-mm dish and grown for 5 d. Supplements to the DME were: 10% calf serum plus 250 ng/ml FGF; 10% calf serum; 250 μ g/ml HDL plus 50 μ g/ml transferrin; 250 μ g/ml of heptane-extracted HDL plus 50 μ g/ml transferrin; 250 μ g/ml HDL plus 50 μ g/ml transferrin (cells plated directly in this as opposed to plating in 10% serum for 18 h, then changing medium) and no addition to DME. In C, primary BASM cells were plated in 35-mm dishes coated with ECM in 10% serum. 24 h later, the medium was changed on those cells plated on matrix to 250 μ g/ml HDL and 50 μ g/ml transferrin with addition of EFG (50 ng/ml), PDFG (10 μ g/ml) or both, as shown. In all cases the cells were grown for 5 d. Hatched bars indicated the addition of 10^{-7} M dexamethasone. The average number of cells per dish \pm SD is shown for two determinations in triplicate.

to these cultures also inhibited growth by \sim 50%.

Influence of Exogenous Growth Factors on Serum-free BASM Cell Growth and the Glucocorticoid Response

It is clear from the data of Fig. 4 that factor(s) in HDL are

mitogenic for BASM cells, particularly when the cells are attached to an ECM. It is also clear that DEX is a potent inhibitor of HDL-stimulated BASM cell proliferation and that the growth promotion seen with only HDL and transferrin in the media is clearly not maximal, relative to that seen in the presence of serum-supplemented media. This suggests that other mitogens found in serum may also be required for

maximal growth rates and that their actions may or may not be influenced by glucocorticoids. We therefore tested two other circulating growth factors known to be mitogenic for smooth muscle cells, namely EGF and PDGF. As shown in Fig. 4C, smooth muscle cells on plastic in the presence of 10% serum were inhibited 62% by DEX. Growth in the presence of HDL and transferrin alone on ECM was only 30% of that seen with 10% serum, and the DEX-induced inhibition of HDL-stimulated growth was 73%.

The addition of EGF (50 ng/ml) to the HDL-transferrin supplemented medium stimulated growth by 1.6-fold relative to control and by 1.9-fold when DEX was present. The addition of PDGF (10 μ g/ml) resulted in a 2.7-fold growth stimulation in the absence of DEX, but a 5.4-fold stimulation in its presence. When both PDGF and EGF were added, the growth of BASM cells was comparable to that of 10% serum-supplemented media. Under these conditions, DEX produced a 45% inhibition of growth, similar to that seen for cells grown on ECM in 10% serum. The data show that DEX is still growth inhibitory in the presence of EGF and PDGF; however, the growth stimulatory effect of these mitogens, expressed as a fold increase in cell number, is retained or is greater than that seen with serum when DEX is present. Thus, the growth-inhibitory effect of DEX is not abolished by these mitogens under these growth conditions, as was shown to be the case for the noncirculating growth factor FGF (Fig. 4A and B).

DISCUSSION

The studies described above have examined the influence of the extracellular substrate on the glucocorticoid responsiveness of aortic smooth muscle cells. The use of both a cloned strain and primary mass culture shows that these steroids significantly decrease both the proliferative rate and saturation of density of BASM cells when examined on plastic tissue culture dishes. However, when ECM-coated dishes were used, the cloned strain was no longer sensitive to glucocorticoid treatment and the primary mass culture showed a significantly reduced responsiveness. Even so, the primary mass cultures when grown on ECM-coated dishes attained cell densities that were 40–50% less in the presence of DEX than in its absence. It is reasonable to consider the mass culture as a mixture of cloned strains, some of which are not responsive to glucocorticoids when plated on ECM-coated dishes and some of which remain responsive; the mass culture may therefore more closely reflect the growth responses displayed by these cells *in vivo*.

Studies with the cloned BASM cells showed that the homologous ECM increased the growth rate, especially at low cell densities, and allowed the cells to remain in logarithmic growth for longer periods, thus attaining higher saturation densities. The ECM also significantly increased the sensitivity of the cells to the mitogenic action of serum, such that cells grown in 2% serum attained cell densities which were as high as that of cells grown in 10% serum on plastic. Because the ECM was able to effectively overcome the glucocorticoid effect on saturation density, which showed a requirement for serum concentrations >1%, it may be argued that the glucocorticoid reduces the cellular sensitivity to some serum factor and that the ECM simply increases this sensitivity to an extent greater than the inhibition.

The cloned BASM strain also allowed an evaluation of the patterns of proteins regulated by glucocorticoids and the

possible correlation of this regulation with the growth responses. Two peptides in particular at pI about equal to 5.5, with molecular weights of 20,000, were strongly induced by DEX only under conditions that resulted in the inhibitory growth response. Cells grown on ECM or in the presence of FGF, both of which abolish the glucocorticoid growth response of this clone, did not show induction of these peptides by DEX although their constitutive level of expression was uninfluenced by the ECM or FGF. Although the identification, subcellular location, and role in BASM proliferation of these peptides remain to be determined, their pI and molecular weight as well as their migratory relationships to other peptides on this gel system are almost identical to those of a set of nuclear peptides observed in GH₃ pituitary tumor cells whose phosphorylation is regulated by glucocorticoids (16). It should be noted that not all glucocorticoid responses are abolished by maintenance of these cells on ECM. As described in the accompanying report (17), the glucocorticoid regulation of collagen and noncollagen production by this clone remains in the absence of a growth response to glucocorticoids. Thus, the ECM selectively regulates an array of steroid-induced responses.

The studies with primary mass cultures of BASM cells have allowed the investigation of serum mitogens that may be required for the glucocorticoid responsiveness of a mixed population of smooth muscle cells. The use of serum-free conditions and the selective addition of the serum mitogens, HDL, EGF, and PDGF, has shown that glucocorticoid treatment specifically inhibits the mitogenic action of HDL on smooth muscle cells. The mitogenic effect of both EGF and PDGF when added alone or in combination was as great or greater in the presence of DEX. Under these serum-free conditions, smooth muscle cell growth was comparable to that observed when the cells were grown on ECM in the presence of 10% serum, and the magnitude of the glucocorticoid inhibition was also identical.

At present, it is not known how vascular smooth muscle cell proliferation is regulated *in vivo*. However, these findings and those of our previous studies (7) suggest that endogenous circulating levels of glucocorticoids may significantly alter the responsiveness of vascular smooth cells to mitogenic stimulation by circulating growth factors. Indeed, myointimal proliferation in response to endothelial denudation in the rabbit has been shown to be significantly decreased by exogenous glucocorticoid treatment (8).

The mitogenic activity of HDL observed with smooth muscle cells in culture is also of great interest to the study of atherogenesis. HDL is known to be mitogenic for endothelial cells (19) and to protect against low density lipoprotein (LDL) cytotoxicity, and an HDL-LDL balance may be a critical factor in the susceptibility of individuals to vascular disease (20, 21). It is of interest that in the present studies heptane-extracted HDL was found to be equally mitogenic, indicating that provision of cholesterol is not the major role of HDL in promoting BASM cell proliferation.

Our initial motivation for initiating these studies was to investigate a direct glucocorticoid-vascular cell interaction that might explain the considerable clinical evidence that hyperglucocorticoidism constitutes a significant risk factor in the development of vascular disease (22–24). We reasoned that glucocorticoids could be considered pro-atherogenic if they inhibited vascular endothelial cell proliferation and/or stimulated smooth muscle cell proliferation. The results of

our previous studies have shown that, although various factors influence the proliferation of both cell types, glucocorticoids, per se, have no significant effect on vascular endothelial cell growth (7, 13) and can only be judged to be growth inhibitory with respect to smooth muscle cells (7). Although all of these studies have been carried out *in vitro*, the use of a variety of different tissue culture conditions, including the use of ECM, serum-free conditions, clonal, and primary mass cultured cells, have all confirmed a large (50–70%) inhibition of BASM cell proliferation mediated by a direct action of glucocorticoid hormones on these cells. On the basis of these observations we conclude that these steroids are not pro-atherogenic in terms of their ability to influence vascular wall cell proliferation; however, they may be important in negatively regulating the sensitivity of vascular smooth muscle cells to mitogenic stimulation by circulating growth factors.

The authors wish to acknowledge Dr. Denis Gospodarowicz for his generous gift of FGF and EGF, Dr. Fredric Kraemer for preparation of the HDL purified from blood donated by J. P. Longenecker and L. K. Johnson, and Mr. David Colbert, Ms. Diane Jarest and Ms. Eliza Pond for their assistance in preparation of the manuscript. J. P. Longenecker was supported by a fellowship from the National Heart, Lung and Blood Institute. HL06354-01. This work was supported by a grant from the Veterans Administration and by grant RR5353 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health (to L. K. Johnson).

Received for publication 13 December 1982, and in revised form 3 October 1983.

REFERENCES

- Ross, R., and J. A. Glonset. 1976. Pathogenesis of atherosclerosis. *N. Engl. J. Med.* 295:420–425.
- Ross, R., J. A. Glonset, B. Karinger, and L. Harker. 1974. A platelet dependent serum factor that stimulates the proliferation of arterial smooth muscle cells *in vitro*. *Proc. Natl. Acad. Sci. USA.* 71:1207–1210.
- Gospodarowicz, D., J. S. Moran, and D. L. Braun. 1977. Control of proliferation of bovine vascular endothelial cells. *J. Cell. Physiol.* 91:377–386.
- Gajdusek, C., R. DiCorleto, R. Ross, and S. M. Schwartz. 1980. An endothelial cell-derived growth factor. *J. Cell Biol.* 85:467–472.
- Gospodarowicz, D., K. Hirabayashi, L. Giguere, and J. P. Tauber. 1981. Factors controlling the proliferative rate, final cell density, and life span of bovine vascular smooth muscle cells in culture. *J. Cell Biol.* 89:568–578.
- Castello, J. J., M. L. Addonizio, R. Rosenberg, and M. J. Karnovsky. 1981. Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. *J. Cell Biol.* 90:373–379.
- Longenecker, J. P., L. A. Kilty, and L. K. Johnson. 1982. Glucocorticoid influence on vascular wall cell growth in culture. *J. Cell. Physiol.* 113:197–202.
- Gospodarowicz, D., H. Bialecki, and G. Greenburg. 1978. Purification of the fibroblast growth factor activity from bovine brain. *J. Biol. Chem.* 253:3736–3744.
- Savage, C. R., and S. Cohen. 1972. Epidermal growth factor and a new derivative: rapid isolation procedures and biological and chemical characterization. *J. Biol. Chem.* 247:7609–7611.
- Havell, R., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34:1345–1353.
- Camley-Campbell, J. H., G. R. Campbell, and R. Ross. 1981. Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *J. Cell Biol.* 89:379–383.
- Gospodarowicz, D., J. Moran, B. Braun, and C. Birdwell. 1976. Clonal growth of vascular endothelial cells: fibroblast growth factor as a survival agent. *Proc. Natl. Acad. Sci. USA.* 73:4120–4124.
- Longenecker, J. P., L. A. Kilty, J. A. Ridge, D. C. Miller, and L. K. Johnson. 1982. Proliferative variability of endothelial clones derived from adult bovine aorta. Influence of smooth muscle extracellular matrix and fibroblast growth factor. *J. Cell. Physiol.* 114:7–15(1983)
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
- Gospodarowicz, D., and J. P. Tauber. 1980. Growth factors and the extracellular matrix. *Endocrine Rev.* 1:201–227.
- Johnson, L. K., J. P. Longenecker, J. D. Baxter, M. F. Dallman, E. P. Widmaier, and N. L. Eberhardt. 1981. Glucocorticoid action: a mechanism-involving nuclear and non-nuclear pathways. *Br. J. Dermatol.* 107:6–23.
- Leitman, D. C., S. C. Benson, and L. K. Johnson. 1982. Stimulation of collagen and noncollagen protein synthesis by glucocorticoids in cultured vascular smooth muscle cells. *J. Cell Biol.* 98:541–549.
- Manthorpe, R., C. Garbarsch, and I. Lorensen. 1975. Glucocorticoid effect on repair processes in vascular connective tissue. *Acta Endocrinologica.* 80:380–397.
- Gospodarowicz, D., and C. R. III. 1980. The extracellular matrix and the control of proliferation of vascular endothelial cells. *J. Clin. Invest.* 65:1351–1364.
- Miller, G. J., and N. E. Miller. 1975. Plasma high density lipoprotein and development of ischaemic heart disease. *Lancet.* 1:16–19.
- Castelli, W. P., J. T. Doyle, T. Gordon, L. G. Hames, M. C. Hjortland, S. B. Hulley, A. Kagan, and W. J. Zubel. 1977. HDL, cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation.* 55:767–772.
- Kalback, K. 1972. Incidences of arteriosclerosis in patients with rheumatoid arthritis receiving long-term corticosteroid therapy. *J. Pediatrics.* 73:320–328.
- Bulkey, B., and W. Roberts. 1975. The heart in systemic lupus erythematosus and the changes induced in it by glucocorticoid therapy. *Ann. Rheum. Dis.* 31:196–200.
- Lieberman, E., E. Heuser, G. S. Gilchrist, G. N. Donnell, and B. Landing. 1968. Thrombosis, nephrosis and glucocorticoid therapy. *J. Pediatrics.* 73:320–328.