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Corticosteroid Effects and Senescence in Cultured Endothelium

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Corticosteroid Effects and Senescence in Cultured Endothelium

A dissertation submitted in partial fullfillment of the requirements for the degree Doctor of Philosophy at Virginia Commonwealth University.

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To my wife Sally and daughter Skylar

who have shown me the true meaning of life

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Table of Contents

List of Figures

List of Tables

Corticosteroid Effects and Senescence in Cultured Endothelium

ABSTRACT

A dissertation submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy at Virginia Commonwealth University

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Cultures of human umbilical vein endothelial cells were treated with heparin-corticosteroid combinations to determine effects on cellular growth. Standard proliferation assays, colony formation assays, and cytoflourometric analysis were the methods employed. Dexamethasone (DEX) and hydrocortisone (HC) were inhibitory to growth of HUVEC when EtOH was used as a solvent and fully supplemented medium (20% serum) was employed. When DMSO was the solvent, growth enhancement sometimes occurred when (DEX) was the test steroid; growth inhibition occurred with this steroid when a reduced (1%) serum component was used. Since significant inhibition of cell colony formation in response to DEX administration was observed, the results suggest that low density growth of HUVEC was inhibited by steroid treatment, while higher density growth was facilitated. Cytoflourometric analysis of HUVEC treated with DEX-heparin combinations indicated

that this treatment increased cellular size and possibly increased the number of endothelial cells in the S and/or G_2-M phases of the cell cycle in early passage HUVEC.

Senescent expression was studied in HUVEC using a cell counting method to determine the percentage of senescent cells in these cultures. The effects of culture gender and passage number on senescent expression were determined for various plating densities and passage split ratios. When primary cultures of HUVEC were passed at a 1:10 split ratio and subsequently passed at a 1:5 split ratio, male cultures expressed a greater degree of cellular senescence than to female cultures. When differences in confluent cell density between male and female primary cultures were corrected for by plating at standard density (1.25×10^5 cells/flask), there were no significant differences in total cells, total senescent cells, or percentages of senescent cells between male and female cultures, a phenomenon which was true in both five and seven day assessments. When cultures of both male and female HUVEC were passed at high density (1:4 split ratio), significantly greater senescent expression occurred in later passages vs. earlier passages, an effect not seen when the cultures were passed at a lower density (1:16 split ratio). There was a trend for both male and female cultures passed at a lower density to express less

senescence when contrasted with male and female cultures passed at a higher density. The results suggest that cellular density at passage may be more important than gender in the expression of senescence in HUVEC.

Part I

Effect of Corticosteroids on Cultured Human Umbilical Vein Endothelial Cells

Introduction

This study addresses the *in vitro* growth characteristics of human umbilical vein endothelial cells (HUVEC) subjected to treatment with corticosteroids and heparin. The rationale for the studies using steroids and heparin is based on *in vivo* studies; it has been demonstrated that tumor suppression by inhibition of tumor angiogenesis can be effected by combined treatment with heparin and selected corticosteroids (Folkman, 1985a). The mechanism by which angiogenesis is inhibited is unknown, although a recent report has suggested that the mechanism is related to heparin-corticosteroid induced dissolution of the vascular basal lamina (Ingber *et al.*, 1986). In reviewing the literature, however, it becomes apparent that the effects of steroids, or steroid-heparin combinations, on proliferation of endothelial cells (EC) are not completely documented. Since cellular proliferation, along with cellular migration, is a major cellular kinetic process necessary for the formation of blood vessels, studies of the effect of steroids and heparin on the proliferation of endothelial cells in culture were initiated. The rationale for the *in vitro* experiments is that these studies may help to define the mechanism of the anti-angiogenic effect seen *in vivo*.

Review of Literature

The following review is divided into four sections. The first section describes the results of *in vivo* studies which have examined the effect of corticosteroids and heparin upon angiogenesis. The second section summarizes findings regarding the effect of various corticosteroids upon cellular growth in general. The third section introduces the history and methodology of culturing HUVEC, the cells used in the present study. Lastly, the fourth section delineates the research addressing the *in vitro* effect of corticosteroids upon functional parameters of endothelial cells, with emphasis on HUVEC.

Corticosteroids and angiogenesis *in vivo*

Angiogenesis or neovascularization, the formation of new blood vessels from the established microvasculature, occurs in response to various pathologic or physiologic stimuli. Pathologic angiogenesis includes vessel growth occurring in response to inflammation, wound healing, tumors, and other disease processes such as psoriasis and scleroderma (Furcht, 1986). It is well recognized that neovascularization of tumors is necessary for their

continued growth. In fact, cell populations which acquire the ability to induce angiogenesis, but are normally devoid of this capacity, have an increased risk of neoplastic transformation (Zilch & Gullino, 1982).

The process of angiogenesis involves both proliferation and migration of EC lining the microvasculature (Sholley et al., 1984). Factors, then, which influence endothelial cellular proliferation and/or migration are of immediate interest in the study of new blood vessel formation. Intriguing recent reports have demonstrated that angiogenesis can be inhibited *in vivo* by selective treatment with heparin and various corticosteroids (Folkman, 1985a,b; Folkman et al., 1983; Crum et al., 1985). *In vitro* studies examining the positive or negative influence of various corticosteroids on the proliferation of EC would assist in understanding the cellular kinetics involved with the anti-angiogenic phenomenon. Any pharmacologic agents which might influence the proliferation and/or migration of EC could alter the growth of new blood vessels.

Proliferation of EC is an important component of many pathological reactions, such as inflammation, wound healing, and the formation of atherosclerotic lesions. Factors affecting the migration of vascular cells are also

central to both the repair of damage and pathological changes in the vascular structure (Gee & Minta, 1984). Due to the fact that endothelial damage and the extent of repair can affect the integrity of the existing endothelial barrier, determinants of endothelial cellular proliferation and migration are also integral aspects in the process of atherosclerosis, which may develop after injury to the endothelium (Schwartz et al., 1981). Factors which maintain viability of the vascular endothelium and protect it from injury may indirectly prevent or attennuate the atherosclerotic process (Folkman, 1984).

The identification of pharmacological agents which suppress proliferation of EC is of interest regarding possible therapeutic manipulation of diseases involving angiogenesis, such as growth of malignant tumors, diabetic retinopathy, neovascular glaucoma, corneal inflammation, and scleroderma. Corticosteroids are already used clinically to suppress ocular neovascularization, but their effectiveness is thought to be related largely to their anti-inflammatory properties. Whether these steroids directly affect proliferation of EC is uncertain, and information concerning their possible effects on the proliferation of EC would greatly facilitate understanding of the mechanism of corticosteroid suppression of neovascularization.

The concept of anti-angiogenesis as a means of controlling tumors by regulating their blood supply is not new. Recently Folkman (1985a) summarized studies in this area. Despite evidence that heparin alone can promote and facilitate angiogenesis, heparin in the presence of cortisone or hydrocortisone could inhibit angiogenesis, when used in the mouse skin or lung, in the rabbit cornea, or on the chick chorioallantoic membrane (CAM). The results of these studies utilizing in vivo models demonstrated that under certain conditions, heparin could become a negative regulator of angiogenesis. The possibility was also raised that the anti-angiogenic effect of hydrocortisone was independent of its glucocorticoid activity. Folkman (1985a) employed 11-alpha epicortisol, a stereoisomer of hydrocortisone having no glucocorticoid or mineralocorticoid activity, and found that anti-angiogenic activity was sustained.

Crum et al. (1985), in further studies, characterized seven corticosteroids with heparin dependent anti-angiogenic (angiostatic) activity but no glucocorticoid or mineralocorticoid activity, demonstrating the existence of a class of steroids for which inhibition of angiogenesis appears to be the principal function. They suggested that angiostatic

activity appears to be associated with certain alterations of the pregnane structure and is governed mainly by specific structural configurations on the D ring. The absence of either the 17-hydroxyl or both the 17-hydroxyl and carbons 20 and 21 on the D ring led to successive decreases in angiostatic activity. Also, Crum et al. (1985) found that dexamethasone, which originally exhibited no anti-angiogenic activity when used at concentrations maximally effective for hydrocortisone, displayed a potent angiostatic effect when given at a lower concentration. This finding stresses the importance of determining a broad and inclusive dose-response relationship in establishing optimally effective corticosteroid-heparin combinations in angiostatic activity.

Ingber et al. (1986) suggested that induction of capillary basement membrane dissolution may be involved in the mechanism of anti-angiogenesis observed in the heparin-corticosteroid treated chorioallantoic membrane (CAM). The CAM, when treated with combinations of angiostatic steroids and heparin, exhibited capillary basement membrane fragmentation and eventually complete loss of fibronectin and laminin from regions of capillary involution. The authors, however, admittedly pointed out that it could not be clearly determined whether the

basement membrane breakdown was a primary effect of corticosteroid treatment or secondary to the loss of endothelial cell viability. Thus, the necessity of ascertaining the effect of heparin-corticosteroids on proliferation of EC is further supported.

Corticosteroids and Cellular Growth in Vitro

There is a wide body of literature which explores the effect of corticosteroids, both natural and synthetic, upon the growth characteristics of various cultured cells. Many of the studies have been performed upon fibroblast cell lines in culture. The results of these *in vitro* studies have been equivocal. Some cell lines exhibit enhanced proliferation, DNA synthesis, and increased metabolism when treated with steroids; while others show an inhibition of cellular growth. It is thought that target cells may biotransform the molecular structure of corticosteroids in a specific unique way; a particular steroidal molecular structure is apparently essential for eliciting a particular biologic response (Berliner, 1965). Cells more resistant to cortisol effects, for example, will selectivity inactivate the hormone in greater quantities than will more sensitive cells.

Ponec *et al.* (1977) added various corticosteroids to logarithmically growing cultures of primary human skin

fibroblasts, and determined their effect on cellular growth using a cell counting assay. These steroids inhibited proliferation of the human fibroblasts at concentrations (10^{-5} M to 10^{-8} M) which fell in a range expected to occur during the topical treatment of skin disorders. Inhibition was independent of the source (baby foreskin or adult arm skin) and cell passage number of the fibroblasts. Building on this study, Ponec et al. (1979) found that when steroids were added at a later stage of cellular growth, when cell density was increased, fibroblasts showed a reduced sensitivity or even an insensitivity to growth inhibition. The inhibition in early growth stages was transient and most pronounced when the culture medium was not renewed. In contrast, Rosner & Cristafalo (1979), using the Coulter counter method to examine the effect of hydrocortisone on the proliferative activity of various vertebrate fibroblast-like cell lines, found that addition of hydrocortisone to human fetal foreskin and human fetal lung-derived cell lines (W-138) was growth enhancing. Serum was required in the medium for this enhancement to occur. However, they also found that steroids caused growth inhibition in a number of other vertebrate cell lines. Their results suggested that the proliferative response to hydrocortisone demonstrated a cell-type specificity, as well as steroid-molecular structure specificity, mediated by high affinity

glucocorticoid binding sites. That hydrocortisone acted to conserve the cycling state of human embryonic lung fibroblasts was suggested by Ban et al. (1980), who supported previous findings of Cristafalo (1972). They noted that hydrocortisone extended the population life span of these cells, without changing the maximum population doubling potential. It appeared that hydrocortisone delayed the progression of fibroblasts to a non-cycling state.

Baker et al. (1978) reported that dexamethasone, maximally effective at 0.25 uM, enhanced the mitogenic response of human diploid foreskin fibroblasts to epidermal growth factor (EGF). Binding studies with ^{125}I-labeled EGF suggested that dexamethasone caused this permissive effect by modulating cell surface receptors for EGF. Thus, glucocorticoids may modulate cell growth indirectly by altering cell responsiveness to growth factors or serum. Cristafalo (1979) delineated experiments looking at the specific binding of glucocorticoid hormones in human fibroblast WI-38 cells. They concluded that the stimulation of WI-38 cell proliferation by hydrocortisone involved specific glucocorticoid receptors whose concentration per cell is under cell cycle control. They also compared cells with a cumulative population doubling level (CPDL) of 30 with

cells with a CPDL of 57, and found that there was an age-associated decrease in specific glucocorticoid binding sites. The latter might explain a previously observed loss of responsiveness to hydrocortisone observed in aging cultures (Kalimi & Seifter, 1979; Rosner & Cristafalo, 1979). Also, significantly, hydrocortisone effected an increased life span for WI-38 cells, in terms of cumulative population doublings and chronological time. Because the effect of hydrocortisone was serum-dependent, it was suggested that the steroid was an amplifier of the serum stimulation of growth. It was later shown that the responsive period of WI38 cultures is limited to a brief period (up to 12 hours) following subcultivation, although cultures may remain responsive to glucocorticoid-conditioned medium throughout the growth cycle (Finlay et al., 1985).

Barrack & Hollenberg (1981) showed that the growth promoting action of serum in human skin fibroblast cultures can be attributed to the presence in the serum of a number of specific growth factors, and outlined their finding that glucocorticoids that had an 11 beta hydroxyl group (e.g., dexamethasone) enhanced several fold the mitogenic activity of synthetic polypeptides on fibroblasts. Supporting the findings of Rosner & Cristafalo (1979) above, Conover et al. (1983)

demonstrated that the increased [^{3}H]thymidine incorporation caused by insulin-like growth factor (somatomedin-C) in combination with human hypopituitary serum could be dramatically increased with further addition of dexamethasone or hydrocortisone. This synergistic effect was corroborated by cell replication studies. Wu et al. (1981) found that hydrocortisone enhanced cell growth in HeLa cells in serum free medium, yet inhibited cell growth in serum-supplemented cultures. Hydrocortisone increased twofold the ^{125}I-labeled EGF binding within 24 hours after the addition of pharmacological concentrations of this steroid. Since a higher concentration of steroid was needed to affect the binding capacity of labeled EGF than was required to elicit the growth response, the growth response appeared to be independent of EGF.

Further regarding glucocorticoid-serum-growth factor interactions, it is held that glucocorticoids can either sensitize or desensitize various cell types to the action of serum or growth factors added to cell cultures. Gospodarowicz and Moran (1975) found that at plateau values of fibroblast growth factor (FGF) (between 2.5 and 5 ng/ml), FGF, dexamethasone, and insulin added to cultures of BALB/c 3T3 fibroblasts were 82% as effective as saturating concentrations (10%) of serum in stimulating

DNA synthesis. Quinlan and Hochstadt (1977) extended these findings, demonstrating that FGF, dexamethasone, and insulin could induce mitogenesis and mitosis in quiescent 3T3 cells in 0.8% serum induced G_0-arrest. In contrast, Rubin (1977) found that hydrocortisone inhibited yet insulin stimulated the coordinate response of uridine uptake and thymidine incorporation into DNA in chick embryo fibroblasts.

Jarvelainen et al. (1982) determined the proliferative effects of hydrocortisone on cultured human aortic smooth muscle cells. At concentrations of 10 uM or greater, hydrocortisone retarded cell growth markedly, as judged by decreased incorporation of [^{3}H] thymidine by the cells, and decreased DNA content of the cultures. Longenecker, Kilty, & Johnson (1982) found that dexamethasone inhibited the growth of bovine smooth muscle cells (BSMC) in vitro when FGF was absent from the growth medium. Curiously, this inhibition was abolished when FGF was present. Thus, it again appears that glucocorticoids indirectly modulate cell growth in this cell type by changing the cellular responsiveness to various growth factors or serum. Dexamethasone inhibited maximal obtainable saturation densities in BSMC, but this effect could apparently be overcome by FGF.

Glucocorticoid induced inhibition of cell proliferation in murine growth cartilage *in vitro* was shown to be receptor-mediated (Silberman & Maor, 1985). Berliner *et al.*(1978) found that hydrocortisone could induce cell spreading in rat glial cells 24 hours after its addition to the culture medium; this spreading correlated with an increase in the fraction of the peripheral cytoplasm occupied by microfilaments.

As pointed out in this review, many authors have examined the effects of various steroids upon cellular growth in a variety of cells *in vitro*, although the majority of these studies have been performed upon fibroblasts. These studies have demonstrated that corticosteroids, both natural and synthetic, can either stimulate, inhibit, or have no effect on cellular growth in various non-endothelial human and animal cell lines (Finlay *et al.*, 1985). The presence of various concentrations of serum, and stage of cellular growth (i.e. passage number *in vitro*) are important variables which can alter the response of a particular cell line to corticosteroid influence, and they should be entertained in studies measuring the effect of corticosteroids upon cellular proliferation. The concentration of steroids, the time frame of steroid administration, and the presence of various additional growth factors all appear to be

modulating factors in the expression of proliferation effects upon cultured cells.

HUVEC in Culture

The human umbilical vein has been used as a source of endothelial cells for over two decades. Mayuma (1963) reported the use of this vessel as a source of endothelial cells in culture, and became the first individual to describe a pure culture of human endothelium. Using a trypsin incubation method for cell removal, he grew HUVEC in 20% umbilical cord serum or horse serum, 80% YLH medium, and 200 ug/ml streptomycin. Cells came to lie flat in 3-4 days, and spread out, forming an epithelial like sheet at 7-14 days. Unfortunately, Mayuma (1963) could not get the EC to replicate in vitro, and the cells began to degenerate in 14-21 days. Mitotic figures were rarely seen; there was some contamination with "fibroblast-like cells" (which, one can speculate, could have been smooth muscle cells or fibroblasts); and, curiously, some cells were binucleate or multinucleate. This pioneer investigation led to many subsequent researchers using the human umbilical vein as a source of uncontaminated and uniform cells for study. Mayuma (1963) described the beneficial reasons for using HUVEC as a source of EC for study: they are of fetal origin; they can be obtained fairly easily; the human umbilical vein has no

tributaries, making it easy to cannulate and handle; and the lumen of the vessel has an adequate volume for trypsin to dislodge the endothelia. Gimbrone (1976) summarized methods utilized in culturing endothelial cells, and agreed that the recovery of EC from the human umbilical vein was technically uncomplicated, and could provide a continuously available supply of normal human vascular tissue for experimental use.

Expanding on Mayuma's work, Fryer, Birnbaum, & Luttrell (1966) examined sections of the umbilical cord after trypsinization of the umbilical vein, and found that the endothelial cells detached readily, leaving the internal elastic lamina of the vein intact. The cells, grown on double cover slips and Rose chambers, were polygonal or slightly elongated in shape, and 40 to 50 um at their longest axis. These authors also did not observe replication or migration of the cells in culture. They also cultured the umbilical artery, outlining the difficulties in perfusion of this relatively smaller vessel. EC derived from the umbilical artery initially differed from those of the vein only by the presence of a small number of fibroblasts, which, in older cultures, overgrew the endothelial cells.

Jaffe et al. (1972) were the first authors to use collagenase rather than trypsin, and were able to obtain EC that grew to confluence in primary cultures. Saba, Zucker, & Mason (1973), following the method of Jaffe et al. (1972), also utilized collagenase to isolate EC from the human umbilical vein. The cells were exposed to platelets, neoplastic cells, or both to determine possible interactions. Although platelets did not adhere to EC, degenerating EC produced a slight aggregation of platelets. They concluded that, in response to certain stimuli, the EC release an activity or factor which inhibits platelet aggregation. But even more importantly, it was underscored that the availability of uniform EC grown in tissue culture would open numerous new avenues for investigation of the function and properties of these cells.

Gimbrone et al. (1974) found that control of digestion time and concentration of collagenase were critical for successful cell isolation and culture. Using standard cell proliferation studies utilizing [^{3}H] thymidine, they further characterized EC as behaving as a density dependent population with respect to DNA synthesis, and for this reason, stated that HUVEC could be used for studies examining the mechanism of endothelial cellular growth and regeneration. Using HUVEC with a

controlled scraping model to effect a wounded monolayer, Sholley et al. (1977) demonstrated that small defects in the endothelium could be repaired with migration alone, without proliferation of cells. Henrikson et al. (1975) found that the replicative ability of HUVEC was correlated with cell density at the time of seeding. Paradoxically, high cell seeding densities (greater than 1×10^4 cells/cm^2) inhibited cellular proliferation. They determined that the optimum cell seeding density for success in cellular proliferation was in the range of 500-700 cells/mm^2.

Wall et al. (1978), in order to more fully understand vascular repair mechanisms following endothelial injury, studied the proliferative response of HUVEC to cell free plasma derived serum, whole blood serum, fibroblast growth factor (FGF), and macrophage conditioned medium in vitro. They concluded that proliferation of EC in pre-confluent cultures was dependent on plasma factors, since all four parameters above enhanced EC proliferation. Interestingly, in HUVEC, proliferation, migration, and repopulation of irradiated and/or wounded cultures appeared to be independent of platelet factors. DeGroot et al. (1983) defined experimental conditions allowing HUVEC to grow in serum free medium. Although serum was needed for a short period

of time to allow attachment and spreading of EC after trypsinization, the cells could be grown in Medium RPMI-1640, supplemented with transferrin, insulin, and human serum albumin. Although ECGF enhanced growth, these authors concluded that it was not absolutely required for proliferation and subcultivation of HUVEC.

Cultures of HUVEC have the following characteristics: monolayer formation, production of Factor VIII antigen, production of prostacyclin, and the presence of Weibel-Palade (WP) bodies (Zetter, 1981). The ultrastructure of human umbilical vessel endothelium *in situ* was studied in detail by Parry & Abramovich (1972). There was a paucity of glycogen in the venous EC. Rough endoplasmic reticulum (RER) was more abundant than in the umbilical artery EC. The latter was particularly well developed in the 15 week fetus; almost all RER at that time was in the form of widely dilated channels and cisternae. Weibel Palade bodies, endothelial cell specific organelles (Weibel & Palade, 1964), were numerous throughout pregnancy in human umbilical vein EC, and the Golgi are well developed. These findings correlate well with studies of HUVEC ultrastructure *in vitro* (Haudenschild *et al.*, 1975). These authors described WP bodies in abundance, peripheral pinocytosis vesicles, and both thick (100 Å) and thin (60-70 Å) filaments in HUVEC

in culture. McDonald et al. (1973) also described primary and subcultured HUVEC as consisting of closely apposed large polygonal cells, 20-50 uM in diameter; under EM, they were phenotypically similar and revealed features consistent with EC in situ.

Cultured EC have provided a powerful approach to study the characteristics and function of the endothelium (de Groot et al., 1983). Mechanistic studies of the biology of HUVEC have been facilitated by the establishment of accepted in vitro cell culture conditions, the definition of endothelial cellular phenotypes, and the identification of polypeptide growth factors which promote the growth of EC. These advances in techniques have afforded methods which allow serial cultivation of HUVEC over many passages (Maciag et al., 1981; Thornton et al., 1983). The discovery of endothelial cell growth factor (ECGF) was a major breakthrough in culture methodology (Maciag et al., 1979). ECGF, originally a partially purified growth factor derived from bovine hypothalamus, is used by researchers to stimulate replication of EC (Maciag et al., 1979, 1981,1982; Gordon et al., 1983; Knaver & Cunningham, 1980). ECGF was found to contain a significant level of mitogenic activity when tested in a HUVEC growth assay (Maciag et al., 1979). Recently, the structure of ECGF

and the gene coding for ECGF have been described (Jaye et al., 1986). These authors showed that ECGF was an acidic form of fibroblast growth factor.

It was found that angiogenic basic FGF and FGF-like growth factors had a strong affinity for heparin (Shing et al., 1984). Apparently, these growth factors acted in a synergistic fashion with heparin (Thornton et al., 1983), and are protected and stabilized by heparin (Gospodarowicz and Cheng, 1986). It was suggested that heparin could be involved in the modulation of growth factor activity, thus indirectly participating in the control of cellular growth (Schreiber et al., 1985). These authors felt that ECGF induced a conformational change in the heparin molecule which increased or stabilized the biological activity of the growth factor mitogen. Exactly how heparin participates in the mitogenic events induced by ECGF is unknown. Since EC possess high affinity binding sites for ECGF, it was proposed that these same binding sites could be activated by an ECGF-heparin complex (Maciag et al., 1984). It is of interest that heparin binding angiogenic factors are found in both normal tissues and tumors (Shing et al., 1985). Folkman & Klagsburn (1987) have summarized the intense research productivity regarding angiogenic factors in the past few years, tracing the discovery of these

factors and describing their significance in understanding growth regulation of the vascular system *in vivo*.

Effect of Steroids upon EC *in vitro*

Maca *et al*. (1978a) studied the effects of various steroids, including dexamethasone, hydrocortisone, and prednisone on the morphology and growth characteristics of cultured HUVEC grown in serum-containing media, but in the absence of ECGF and heparin. They found that corticosteroids caused an increase in cell surface area and protein synthesis and content, without an increase in DNA synthesis or cellular replication. Dexamethasone was approximately 10 times more effective than hydrocortisone in causing these changes; prednisone was least effective. Other steroids that were tested, including aldosterone, testosterone, progesterone, estradiol, and estriol were ineffective in this manner. Maca *et al*. (1978b) also found that dexamethasone decreased adhesiveness of cultured leukemia and lymphoma cells to confluent cultured endothelium; hydrocortisone was also effective in this manner. The effects of corticosteroids on HUVEC have also been examined by Piovella *et al*. (1982), who found that dexamethasone-treated cells displayed larger cytoplasms and larger nuclei, the latter with more prominent

nucleoli. Preliminary studies in our laboratory using dexamethasone with HUVEC have corroborated these findings. Piovella et al. (1982) also observed that dexamethasone increased the amount of fibronectin matrix in cultured HUVEC, suggesting that this increased matrix was responsible for the more flattened aspects of cells and their better spreading on the culture dish.

Using the HUVEC culture system, Almasio et al. (1984) also found that dexamethasone induced production of a more cohesive and complete extracellular matrix, causing the EC to assume a more polygonal shape, with abundant cytoplasm. There was a greater degree of confluence and each cell covered a greater area. Earlier, Piovella et al. (1980) cultured HUVEC in the presence or absence of dexamethasone, and with daily counts reported a greater number of cells in the steroid-treated cultures in comparison to control cultures for the first five days. However, by day six both control and dexamethasone-treated cultures reached confluence without significant differences in cell counts. In comparison to the non-treated cultures, the confluent monolayer appeared tighter in the steroid treated cultures (Piovella et al., 1980). The preservation of adhesion in the dexamethasone treated cultures was possibly related to the increased fibronectin extracellular matrix observed, the latter

demonstrated by immunofluorescence. It is well known that the extracellular matrix plays a pivotal role in the modulation of EC behavior *in vivo* (Madri & Pratt, 1986). Within this conceptual framework, the same preservation of adhesion might have affected the cell counts in the experiment, with the relatively less adhesion in the non-steroid treated cultures reflected in a lower cell count, thus making the early increased cell number observed in the dexamethasone treated cultures more apparent than real. The availability of space into which cells can replicate is also an important factor, and crowded near-confluent EC cultures will necessarily have a lower growth rate if evaluated at that time (Goldsmith *et al.*, 1984). Although it appears that dexamethasone initially accelerated growth in cultured EC, this finding was neither repeated nor corroborated by others, nor were other corticosteroids tested.

Goldsmith *et al.* (1984) used cytofluorometric techniques and found that approximately 2-8% of EC in primary confluent HUVEC were in the proliferative phases (S, G_2 and M) of the cell cycle. Unfortunately, the proliferative characteristics of later passage HUVEC were not examined with cytofluorometry; no author has utilized this technique to assess the effects of corticosteroids on endothelial cellular kinetics.

Berliner (1981), repeating her earlier studies concerning the effect of hydrocortisone upon HUVEC, developed a defined medium which supported maximum levels of cell division. She found that both insulin and FGF increased the incorporation of ^{3}H thymidine in HUVEC when hydrocortisone was present- the steroid alone did not increase incorporation of the radionuclide. Hydrocortisone also increased cell spreading in serum containing medium. Although her defined medium did not contain serum, it was pointed out that plating and maintenance of cells in serum containing medium was necessary before transfer to defined medium, otherwise the cells would not divide. In this, she supported the findings of De Groot et al. (1983), who found serum containing medium necessary for initial plating before serum-free medium could be used to support growth of the cells.

Hoshi & McKeehan (1984) tested various hormones and growth factors, including steroids, on HUVEC proliferation. Although they inferred that steroids examined at concentrations reported to be active for other cells were not mitogenic for HUVEC, they did not indicate which steroids or concentrations were tested, nor were

quantitative data offered. Very little concerning steroid effects on HUVEC could be concluded from their report.

Longenecker, Kilty, & Johnson (1982) tested primary and cloned strains of bovine aortic endothelial cells (BAEC) for their response to glucocorticoids. The growth of EC was inhibited by dexamethasone in the presence of FGF. Since there was no inhibition of proliferation of EC in the absence of FGF, it is possible that glucocorticoids mildly inhibit the maximal response of EC to FGF. However, these influences were corticosteroid-specific and concentration dependent, but independent of serum concentration. Also, when depression of growth did occur, it was relatively mild (i.e., less than 20% of controls), and growth depression was inconsistent. Perhaps even more important, the authors pointed out that the steroid responses observed were due to relatively acute treatment periods (up to five or six days), and for that reason may potentially differ from responses that might occur to chronic exposure of EC to glucocorticoid excess throughout a culture life span, or from responses that might occur in the endothelium in vivo over the course of many months or years. Longenecker et al. (1983) later studied four clones of BAEC and found that the EC exhibited a rather striking clonal heterogeneity with regards to their sensitivity to glucocorticoid treatment.

Only one clone exhibited an FGF dependent dexamethasone induced inhibition of cellular growth, and that inhibition was abolished by the use of extracellular matrix (ECM) derived from bovine aortic smooth muscle cells (BASMC) as a growth substratum. The authors suggested that clonal variability, among other variables commonly encountered in the culture of endothelial cells, could be eliminated by the use of ECM coated culture dishes.

Harrison & McKee (1981) found that 17-beta estradiol caused an increased rate of replication in HUVEC, as measured by greater DNA content and [^{3}H] thymidine incorporation in the treated cultures. Corvazier et al. (1984), in contrast, found that 17-beta estradiol had no effect on endothelial cell proliferation, nor did progesterone. The latter authors did report that ethinyl-estradiol increased cellular proliferation when the plating density was 4 x 10^4 cells/flask or greater.

Using a ^{51}Cr release assay, Jarvelainen et al. (1985) tested hydrocortisone, dexamethasone, and prednisolone on HUVEC. All corticoids stabilized the EC monolayer as demonstrated by a decrease in the release of ^{51}CR. Paralleling other research studies employing dexamethasone, this particular steroid was maximally effective at a lower concentration than the other

steroids. They proposed that the beneficial effect of glucocorticoids observed empirically in the treatment of certain hemorrhagic disorders such as purpura might partially be explained by the strengthened endothelial integrity observed in their assay system.

A number of authors have studied the effect of corticosteroids upon prostaglandin release from EC. Rosenbaum et al. (1986), using cultured rabbit microvascular EC, found that dexamethasone effected a time and concentration dependent decrease in prostaglandin accumulation in the culture media, and a reduced basal and ionophore stimulated PGE_2 release. Hydrocortisone and corticosterone were less potent inhibitors of PGE_2 secretion, while aldosterone, progesterone, and dihydrotesterone did not inhibit PGE_2 release. Lewis et al. (1986) corroborated this finding in HUVEC, outlining the effects of dexamethasone and hydrocortisone on prostacyclin (PGI_2) and PGE_2 release. Dexamethasone reduced prostaglandin formation in cells stimulated by histamine, bradykinin, calcium ionophore, or mechanical agitation. Hydrocortisone had less potent, but similar effects. Here again, since neither testosterone nor progesterone affected prostaglandin production, the effect seemed to be glucocorticoid specific. Decaterina & Weksler (1986), also studying HUVEC, found that inhibition

of PGI_2 production in the presence of hydrocortisone averaged 15% less than the inhibition seen in the presence of dexamethasone. Corvazier et al. (1984) tested the natural sex hormone 17-beta estradiol and the synthetic hormone ethinyl-estradiol on prostacyclin release in HUVEC and found no effect with either steroid. Since vasodilatation precedes migration and proliferation of EC in angiogenesis in vivo (Folkman, 1984), and since prostacyclin (PGI_2) is a potent vasodilator as well as a platelet aggregation inhibitor, perhaps the inhibition of prostacyclin activity by corticosteroids may be partially responsible for the antiangiogenic effect of these corticosteroids observed in vivo. Seillan et al. (1983) stimulated secretion of prostaglandins from cultured female porcine EC by using the sex steroids 17 beta-estradiol and testosterone; male EC could not be stimulated in this manner. Since female, but not male, EC could convert testosterone to estradiol, the latter's induced stimulation of prostacyclin production may explain in part the beneficial role generally attributed to naturally occurring estrogens in cardiovascular diseases.

Mendsolsohn et al. (1982a, 1982b) studied the glucocorticoid induction of angiotensin converting enzyme (ACE) production in BAEC. Using confluent monolayers of BAEC, they found that dexamethasone increased ACE activity

six to seven fold over control cultures. Since ACE converts angiotensin I to angiotensin II, the latter a strong vasoconstrictor, the process might be facilatory to angiostasis (see above). Also, since ACE plays a role in the inactivation of bradykinin, a vasodilator, an increase in ACE activity would further contribute to the vasoconstrictive effect of corticosteroids, and favor the phenomenon of angiostasis. Lam et al. (1985) supported these findings, and reported that dexamethasone (1 uM) increased ACE activity in BAEC in a time-dependent fashion, and that this steroid and irradiation interacted to raise cellular ACE activity. However, dexamethasone and increased cellular ACE activity unfortunately did not appear to modify the endothelial cytotoxcity induced by irradiation.

In summary, due to the tremendous importance of the endothelium in inflammation and repair, tumor and other disease mediated angiogenesis, and permeability, a large body of literature has examined the effects of corticosteroids on endothelial cell function. Inhibition of prostacyclin release by EC and facilitation of ACE activity by corticosteroids, dexamethasone in particular, are processes whose single or combined effect would be to induce vasoconstriction in local vascular beds. Whether these mechanisms play a role in corticosteroid-heparin

induced angiostasis is not known. However, few published reports have explored the influence of corticosteroids upon endothelial cellular proliferation, a basic component of angiogenesis, and the results of these reports have been inconclusive. In addition, no authors have used cytofluorometry to address these effects upon HUVEC population kinetics. Experiments using cellular growth assays and cytofluorometry on heparin-corticosteroid treated EC will increase our understanding of the role of these substances in endothelial cell proliferation and hopefully provide a rationale for the clinical use of these pharmacological agents in pathological processes and disease syndromes in which endothelial cellular proliferation plays a significant role.

The primary aim of this research was to establish the effects of heparin and selected corticosteroids upon growth characteristics of HUVEC in culture. Monolayer cultures of HUVEC were used in these studies. Direct cellular proliferation studies, utilizing cell counting and analysis of population doubling, were performed in order to determine the effect of heparin and corticosteroids on the growth characteristics of EC. Specifically, corticosteroids known to be antiangiogenic *in vivo* were added to culture media at various times after culture plating to determine the effect on cellular

proliferation. A variable response by EC to corticosteroids was found, depending on a number of factors, including type of steroid solvent, time frame of steroidal administration, and length of time of corticosteroid exposure. Our results suggest that EC are influenced by many factors when responding to heparin-corticosteroid administration. The results do not lend unequivocal support to the hypothesis that heparin and corticosteroids induce angiostasis partly due to the suppression of endothelial cellular proliferation.

Materials and Methods

Experimental Methodology

Culture of HUVEC. Harvest of endothelial cells from umbilical cord veins was based on the method of Gimbrone et al. (1974). Term umbilical cords, detached from the placenta, were obtained from the Labor and Delivery Room of the Department of Obstetrics and Gynecology at the Medical College of Virginia. Using sterile technique, unclamped umbilical cord segments of at least 10 cm. in length were cannulated at both ends using one way stopcocks and blood was flushed by a perfusion of 200-400 ml. of phosphate buffered saline solution (PBS), pH 7.20. The lumen of the vein was filled with 0.1% collagenase (Clostridium histolyticum, type I, Sigma Chemical Company, St. Louis, MO) in PBS with calcium and magnesium [$CaCl_2$ (anhyd), 0.10 gm/L, and $MgCl_2 \cdot 6\ H_2O$, 0.10 gm/L], and the stopcocks closed. The cord segment was then incubated in a bath of PBS at 37 degrees centigrade for 15 minutes. The collagenase solution was collected in a 15 ml. conical centrifuge tube with an equal volume of Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. The EC

were then pelleted by centrifugation at 200 x g for five minutes.

Defined fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) was used in media preparation, as this serum is supplied with an analysis so that the types and amounts of endogenous steroids in the serum would be known. Other media and reagents were obtained from Gibco Laboratories (Grand Island, NY) or Meloy Laboratories (Springfield, VA). The pelleted EC were resuspended in complete Medium 199 (CM-199) containing the following ingredients: Medium 199 with Earle's Salts and L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml Fungizone, 20% FBS, 90 µg/ml heparin (sodium salt, grade 1, from porcine intestinal mucosa, Sigma Chemical Co., St. Louis, MO), and 20 ug/ml endothelial cell growth factor (ECGF, Tissue Culture Grade, Meloy Laboratories, Springfield, VA. ECGF was also a generous gift of Dr. Thomas Maciag of Jerome Holland Research Center of the American Red Cross, Rockville, MD). Thornton et al. (1983) described the use of low concentrations of ECGF along with heparin, since heparin potentiates the effects of ECGF. Cultures were grown in gelatin coated T-25 flasks (Corning). Gelatin (0.2% wt/vol) promotes the attachment and growth of HUVEC (Gordon et al., 1983; Folkman et al., 1979; Thornton et

al., 1983; Walker et al., 1984). Cultures were then continuously incubated at 37 degrees C in a humidified atmosphere of 95% air, 5% CO_2.

Identification of HUVEC. The endothelial nature of the cultured cells was determined by observing the typical "cobblestone" morphological appearance of the monolayer when viewed under phase contrast microscopy (Nikon Inverted Phase Contrast Microscope, Model M), and by immunofluorescent staining for the factor VIII antigen. Direct immunofluorescent staining for factor VIII antigen was performed on selected confluent cultures using fluorescein-conjugated rabbit anti-human factor VIII related antigen (Atlantic Antibodies, Scarborough, ME) according to the protocol of Maciag et al (1981).

Addition of Corticosteroids. Dexamethasone (DEX), dehydroepiandrosterone (DHEA), and hydrocortisone (HC) (Sigma Chemical Co., St. Louis, MO) were dissolved in either absolute ethyl alcohol (EtOH) or dimethyl sulfoxide (DMSO). The corticosteroid dilutions were adjusted so that the DMSO concentration in the media was standardized at 0.1%. When EtOH was used as a steroid solvent, the maximum concentration was 0.2% Control cultures were also run at these concentrations. For each steroid dosage,

triplicate flasks were evaluated; for control cultures, quadruplicate flasks were evaluated.

Trypsinization and Cell Counting. At selected times after subculturing, depending on the experimental paradigm being used, cultures were washed with HBSS without calcium or magnesium, rinsed briefly with 0.025% trypsin-0.01% EDTA, and incubated at 37 degrees C for six minutes. Either CM-199 or counting medium (Medium 199 plus 20% FBS, and antibiotic-antimycotic solution) was added and a single cell suspension was obtained by repeated aspiration. The cells were counted in quadruplicate using a hemacytometer. A trypan blue exclusion procedure (0.4% trypan blue mixed with the cell suspension at a 1:5 ratio) was used to determine the viability of harvested cells.

Percentage of Attachment and Population Doubling. For percentage of attachment (PA) determination, quadruplicate flasks for the selected experimental manipulation were seeded at established cell densities (1.25×10^5 cells/flask). After incubation at 37 degrees C and 5% CO_2 for 24 or 48 hours, several flasks were washed with appropriate media and the cells harvested and counted. The PA is the number of cells harvested divided by the number of cells seeded. This value determined the number

of cells present on the day steroids were added to the culture system, and was used in calculating growth rates and population doublings of HUVEC between either two and five or between two and seven days. The remaining flasks, after washing with media, were returned to the incubator until harvested according to the particular experimental protocol. The number of population doublings (PD) between seeding and ultimate harvesting was determined as follows:

$$PD = \frac{\log\left(\frac{\text{number of cells harvested}}{(\text{number of cells seeded})\ (PA)}\right)}{\log 2}$$

When corticosteroids were tested, the control flasks contained EC from the same subculture as the experimental flasks, and were subsequently handled in identical fashion, using medium including either EtOH or DMSO without corticosteroid. Control cultures without steroids or solvent were also studied. Population doublings were compared using ANOVA and a Duncan Multiple Range Test to test for significant differences.

Fixation of EC. Cultures were fixed for preservation of morphology according to the following procedure. After removing the medium from the flasks, each flask was washed

twice in fresh phosphate buffered solution with calcium and magnesium. Then, 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH = 7.3) was added to each flask, and the flask fixed at room temperature for one hour. After fixation, the flasks were rinsed once with 0.1 M sodium cacodylate buffer containing 7% sucrose. The cultures were then stored in the sucrose-cacodylate buffer at 4 degrees C until further evaluation was undertaken.

Absorbed Serum. Hyclone FBS was absorbed with a a charcoal-dextran mixture to remove endogenous steroids by the following procedure. The absorption material was prepared by mixing 3.75 gm. of activated charcoal with 0.375 gm. of dextran, and dissolving both in 100 ml. of deionized-distilled water. After thorough mixing, 10 ml. of the absorption preparation was removed and centrifuged at 2,000 g for five minutes. The pellet was recovered, and then added to 100 ml. of FBS. The serum-charcoal-dextran mixture was then refrigerated at 4 degrees C for 24 hours with constant, gentle stirring. At the end of this time period, the mixture was removed from refrigeration and centrifuged at 10,000 g for 20 minutes. The clear supernatant was collected and this absorbed serum immediately frozen at -16 degrees C until use in the appropriate experiment.

Colony Forming Assay. As an alternative method for assessing proliferation capacity of selected endothelial cell cultures, subcultured EC from a selected passage were harvested, counted, and seeded into T-25 flasks at clonal densities (1,000 cells/flask). After sufficient time to allow colony formation (eight to eleven days), the colony containing flasks were fixed as described above. Fixed cultures were rinsed twice in distilled water, and stained with 0.5% azure II and 0.5% methylene blue in 0.5% sodium borate. The number of colonies (at least 50 cells per colony) were counted for each flask, and experimental flasks and control flasks were compared using an ANOVA and a Duncan Multiple Range test to determine significant differences.

Cytofluorometry. The EC were prepared for cell cycle analysis according to the following procedure: The cells were trypsinized as described and suspended in 4 to 10 ml. of CM-199 in a gelatin-free plastic centrifuge tube, and counted. The cells were then centrifuged at 1000 x g for 5 minutes, and after removing the supernatant, the cells were vortexed and resuspended in 1.5 ml. of PBS without calcium or magnesium. While vortexing, 3 ml. of 95% ethanol was added dropwise. The cells were allowed to fix in this ethanol solution for one hour on ice. If desired, the cells could remain in this fixative up to one week

before additional processing. After centrifugation and supernatant removal, 10 to 20 ml. of staining solution (3.8×10^{-3} M sodium citrate, 2 mg/ml RNase, 0.05 mg/ml propidium iodide in distilled water) was added, and the cells stained on ice for three hours. The cells were then centrifuged, the supernatant removed, and the pellet resuspended in PBS at a concentration of 1×10^6 cells/ml. After filtering the cells through several sizes of nylon mesh (74 μm, 44 μm, 37 μm), the suspension underwent cell cycle analysis in a Coulter EPICS V Cell Sorter (Coulter Diagnostics, Hialeah, Fl.). DNA histograms were generated and the percentages of cells in the $G_0 + G_1$, S, and $G_2 + M$ phases of the cell cycle were determined using a computer algorithm designed for the EPICS V System (Parametric Analysis I, by C. Bruce Bagwell, Coulter Diagnostics, Hialeah, Fl.). Light scatter analysis using the cell sorter provided relative information on the average size of the cells in the cell population tested. The main purpose of using cytofluorometry was to determine the effect of corticosteroids on endothelial cellular size. The claim that corticosteroid administration increases the size of the EC (Maca et al., 1978) was addressed by obtaining cell size information from the cell sorter. Early experiments indicated that a dose related increase in EC size occurs after subculturing the cells in the presence of dexamethasone. Although limited flow

cytometric data regarding EC from the human umbilical vein were reported by Goldsmith et al. (1984), there are no reports in the literature describing the effects of corticosteroid treatment upon the cell cycle kinetics of HUVEC.

Experimental Designs

Effect of Corticosteroids Dissolved in EtOH. In order to determine the effects of corticosteroids, as well as the effects of ETOH as a solvent on the log phase growth of HUVEC, flasks were plated with first, second, or third passage HUVEC at standard density (1.25×10^5 cells / 25 cm^2 flask). CM-199 containing corticosteroids dissolved in EtOH at various doses {DEX (1 µM, 0.5 µM) or HC (1 µM)} were added to the culture flasks at two days after plating, just as the cells were about to enter the logarithmic phase of cellular growth. Three separate control groups were also examined: no EtOH, 0.2% EtOH, and 0.1 % EtOH. Cultures were refed with either control or steroid containing media on the fourth or fifth day after plating. The flasks were subsequently trypsinized and counted on the seventh day after plating. Cellular growth determinations between two and seven days after plating were made for the various control and corticosteroid

concentrations. The population doubling which occurred between two and seven days, which takes into account the number of cells present on the day of steroid administration, was calculated for each corticosteroid dosage.

Effect of Corticosteroids Dissolved in DMSO. In order to determine the effects of DMSO as a solvent, as well as a single steroid feeding on the second day on the log phase growth of HUVEC, flasks were plated with second or fourth passage HUVEC at standard density. DEX (5 µM, 1 µM, 0.5 µM, 0.1 µM); HC (20 µM, 10 µM, 5 µM, 1 µM); or DHEA (50 µM, 20 µM, 10 µM, 5 µM) dissolved in DMSO were added to the culture flasks at two days after plating. The DMSO concentration was adjusted to 0.1% in all steroid-containing media. HUVEC in CM-199 alone or CM-199 with 0.1% DMSO served as control groups. After this initial feeding with steroids or control media, the cultures were not fed for the remainder of the experiment. At seven days, the flasks were trypsinized, cells were counted, and cellular growth and population doublings between two and seven days were determined.

Effect of Charcoal Filtered FBS. In order to determine the effects of filtered FBS upon the growth of steroid treated HUVEC, CM-199 was prepared using Hyclone FBS which

was filtered twice through activated charcoal to remove endogenous steroids. All other media ingredients were identical to those used in preparing CM-199. Second passage HUVEC were plated at standard density, and DEX, HC, or DHEA dissolved in DMSO at the same doses as outlined immediately above were added to the culture flasks at two days after plating. Control flasks were plated with and without the DMSO solvent at the standard final concentration of 0.1%. The cultures were not refed during the experiment, and the flasks were trypsinized and counted on the seventh day after plating.

This experiment was repeated for DEX alone at the same dosages (5 µM, 1 µM, 0.5 µM, 0.1 µM) utilizing cells from the same cord but at a later passage (passage 4). In this experiment, both unabsorbed and charcoal filtration absorbed FBS containing media were employed and the results compared.

Comparison of Male and Female HUVEC. In order to compare the effects of corticosteroids on male and female HUVEC, a wider dosage range of DEX was used. Second passage cultures derived from paired male and female HUVEC harvested on the same day and passaged under identical conditions were used in these experiments. DEX (5 µM, 1 µM, 0,5 µM, 0.1 µM, 0.01 µM, or 0.002 µM) dissolved in

DMSO was added to either male or female cultures of HUVEC at two days after plating. There was no refeeding step involved. Since these cultures grew fairly rapidly, confluence was reached in many flasks by the sixth day. Due to this, the decision was made to evaluate the experiment on the sixth, rather than the seventh, day, lest cells be lost to the culture media.

Effect of Reduced (1%) FBS. DEX at the same doses used in the male-female comparison experiments was tested using media with a reduced serum component. One percent instead of the usual 20% FBS was used in preparing culture media for this experiment; all other media ingredients were identical to those used in CM 199. The six dosages of DEX, dissolved in DMSO, were added to second passage HUVEC at two days after plating. Again, the final DMSO concentration was 0.1%. The flasks were trypsinized and counted on the seventh day after plating with no refeeding step following the initial steroid administration. This experiment was repeated for 0.1 μM DEX and 0.001 μM DEX utilizing fourth passage cells derived from the same cord as cells used for the reduced serum experiments above. In this experiment, media containing either 1% FBS or 20% FBS was employed and the results compared. The experiment was again repeated for DEX at a slightly wider dose range (5

μM, 1 μM, 0.1 μM, or 0.01 μM), utilizing third passage cells obtained from a different cord.

Effect of Repeated Steroid Dosing. In all above experiments employing DMSO as a steroid solvent, cultures were fed only on the second day, with final count performed on the sixth or seventh day. In order to determine the effect of a second steroid feeding on the log phase of growth of HUVEC when treated with DMSO dissolved steroids , DEX (1.0 μM, 0.5 μM, 0.1 μM, or 0.01 μM) was dissolved in DMSO and added to second passage cultures of HUVEC on the second and fourth day after plating at standard density. The cultures were trypsinized and counted on the seventh day to ascertain the effect of a second steroid feeding on the log phase growth of HUVEC. This experiment was repeated for the same four DEX doses utilizing fourth passage endothelial cells derived from a different cord.

Effect of Steroids on Proliferation of HUVEC at Four Days. In order to determine if corticosteroid administration had an earlier effect on endothelial cellular proliferation than was seen at seven days, DEX at the same four doses used in the repeated steroid dosing experiments above was dissolved in DMSO and added to the culture flasks on the second day after plating. Without any further refeeding,

the flasks were trypsinized and counted on the fourth day, two days after steroid administration. The purpose of these interventions was to determine if DEX had any early effect on the proliferation of HUVEC that might be masked after growing for seven days as in preceding experiments. This four day cell counting experiment was repeated using the same four doses of DEX utilizing fourth passage endothelial cells derived from a different cord.

Dexamethasone and Colony Formation of HUVEC. Employing an alternate method of proliferation assessment, DEX (1 μM or 0.1 μM) was used in a colony forming assay to determine the effect of this steroid upon growth of HUVEC. Four variations were used in these experiments, in order to determine the influence of DEX on both plating and growth of HUVEC. Seventh passage HUVEC were used in all colony formation assay experiments. The first variation tested the effect of DEX on growth only as measured by colony formation. Triplicate flasks for the two DEX doses and for the control group were plated at colony formation density (1,000 cells/flask). At four hours after plating, the flasks were washed in plain medium 199, and immediately thereafter fed with CM-199 containing the desired DEX concentration or with CM 199 with 0.1% DMSO alone (control). The cultures were fed twice more at three day intervals with the appropriate steroid or DMSO

media, and fixed and evaluated on the ninth day after plating.

The second variation tested the effect of DEX on plating only. Triplicate flasks for 1 uM DEX or 0.1 uM DEX and control flasks were plated at colony formation density in CM 199 with the appropriate DEX dosage or CM 199 with 0.1% DMSO alone, respectively. At four hours after plating, the cultures were washed with plain medium 199, and immediately thereafter fed with CM-199. The cultures were fed twice more at three day intervals with CM-199, and fixed and evaluated on the ninth day after plating. The third variation tested the effects of dexamethasone on both plating and growth. In this experiment, triplicate flasks were plated at colony formation density in CM-199 with either 1 μM DEX or 0.1 μM DEX, or CM-199 with 0.1% DMSO alone. At four hours after plating, the cultures were washed with plain medium 199 and immediately thereafter fed with CM containing the desired DEX concentration or with CM-199 with 0.1% DMSO (control). The cultures were fed twice more at three day intervals with the appropriate steroid or DMSO medium, and fixed and evaluated on the ninth day after plating.

The fourth variation was designed to test the effect of the wash step four hours after plating. In this

experiment, the cultures were plated and fed in CM-199 exclusively. Two groups were evaluated, one with and one without the wash step with plain medium 199 at four hours after plating, prior to the first feeding with CM-199. Otherwise, the treatment of the groups was the same: the cultures were fed twice more with CM-199, and fixed and evaluated on the ninth day after plating.

Cytoflurometry Experiments. In order to determine the effect of corticosteroids on the percentage of cells in the various phases of the cell cycle and on cellular size in HUVEC, cultures were plated at standard density. DEX (1 μM, 0.5 μM) was dissolved in EtOH and added to the culture flasks on the second and fourth day after plating. The final concentration of EtOH in the steroid-containing media was 0.2% for 1 μM DEX and 0.1% for 0.5 μM DEX. Two control groups were also examined: no EtOH and 0.2% EtOH. Cells were harvested on the seventh day and prepared for flow cytometry as described. The percentages of cells in the $G_0 + G_1$, S, and $G_2 + M$ phases of the cell cycle were determined; light scatter analysis provided information concerning the relative size of the control and steroid treated cells.

Results

Effect of Glucocorticoids Dissolved in Alcohol

The purpose of our experiments was to determine if dexamethasone-heparin or hydrocortisone-heparin combinations affected the log phase of cellular growth in HUVEC. In early experiments in our laboratory, EtOH was used as a corticosteroid solvent, and its final concentration in the medium was 2.0%, 0.2% or 0.1%. When 2.0% EtOH was used as a solvent, it was toxic to the cells, and the concentration was lowered for subsequent experiments. For 1 μM DEX, the EtOH concentration was 0.2%; for 0.5 μM DEX, it was 0.1%. Controls were run at both concentrations, and the cultures were fed with corticosteroid-containing media on the second and fifth day after plating. The cell number harvested at seven days and the number of population doublings between two and seven days are shown in Figures 1a and 1b respectively. The graphs illustrate the pooled cell counts and population doublings from four experiments at the above steroid doses and control conditions. An ANOVA and Duncan's multiple range test were the test statistics used to examine the population doublings in this and all

other experiments reported here. Both steroid doses were associated with inhibition of cellular growth at seven days. Regarding population doublings, DEX at 1 μM caused a significant decrease in population growth compared to the control. DEX at 1 μM also effected a significant inhibition of population doubling when compared to either 0.1% or 0.2% EtOH solvents, which both caused slight, but not significant, inhibition of population doubling when compared to controls. Thus, alcohol was only slightly toxic to the cells at the concentrations employed, causing some minor growth suppression. The results indicate that 1 uM DEX demonstrated a greater inhibition of growth than 0.5 uM DEX, suggesting a dose response. However, only two doses were tested; a wider dose range was needed to substantiate a graded, dose-related inhibition of cellular and population growth.

Hydrocortisone (HC) was tested at 1 μM, in 0.2% EtOH. Controls were run at this alcohol concentration, and also without alcohol. Cultures were fed with steroid containing or control medium on the second and fifth day after cell plating, with cell harvest on the seventh day. The results from pooled cell counts and population doublings from three experiments conducted at 1 μM HC and control conditions are shown in Figures 2a and 2b, respectively. Cultures exposed to alcohol at 0.2%

displayed only slightly suppressed cellular growth at seven days. At the 1 μM concentration, HC caused growth inhibition, but this was not significant. Although 1 μM HC effected a slight decrease in the number of population doublings at seven days, it was not significant (Figure 2b). Since only one dose was tested, it cannot be ascertained whether there was a dose response. In a single experiment, 10 μM HC dissolved in a final media concentration of 0.1% EtOH was tested. Both 0.1% EtOH control and HC at 10 μM led to a significant inhibition of population growth, compared to the EtOH-free control group, so it could not be concluded that HC at this relatively high dose was growth inhibitory to HUVEC (data not shown).

A limited number of doses were used initially, especially for HC. It was clear from these findings that more extensive dose-response information was needed. In these initial experiments, the cultures were fed twice with steroid containing medium, and this factor may have potentiated the inhibitory effect of corticosteroids upon HUVEC. Also, the use of EtOH as a solvent was questioned. Although used by previous researchers (Almasio et al., 1984; Maca et al., 1978a; Piovella et al., 1980;), it was possible that EtOH as a solvent might affect the response of HUVEC to corticosteroid intervention.

Dimethylsulfoxide (DMSO) was proposed as a better solvent, due to its relative non-reactivity with steroids, and its lack of effect upon various cellular functions (M.Y. Kalimi, personal communication). All subsequent experiments reported in this paper were performed with a uniform DMSO concentration (0.1%) in the media used for the DMSO-control and all steroid doses. Each experiment also contained a control group not exposed to DMSO or steroids. In addition, all experiments were performed with heparin included in the media.

Effect of Corticosteroids Dissolved in DMSO

To determine the effect of DMSO as a solvent, of a single feeding with steroids on day two, and of a wider range of doses on the growth of HUVEC in the log phase, several experiments were carried out. In addition to DEX and HC at a wider dose range, another corticosteroid-dehydroepiandrosterone (DHEA) - was tested. Unlike DEX and HC, DHEA is not a glucocorticoid. DHEA is a quantitatively major steroid secreted by the adrenal cortex (15-30 mg/day in humans), and plays a role in adrenal androgen metabolism. DHEA is converted to androst-4-ene 3,17, dione, which in turn is converted to either testosterone or estrogen or is excreted as androsterone and etiocholanolone in the urine.

CM-199 containing DEX at four doses, containing DMSO (0.1%) without steroid, or containing no added steroid or solvent, was administered to HUVEC in culture on the second day after plating, with cellular harvest and counting performed on the seventh day. The additional steroid feeding used in the EtOH solvent experiments was omitted. The data represent the pooled results of two experiments conducted under identical parameters and conditions. For flasks receiving HC and DHEA, which were run simultaneously and are reported below, the basic experimental procedure regarding cell plating, steroid addition, and cell harvest was the same. There was little difference between control and 0.1% DMSO control cultures, suggesting that DMSO as a solvent had little effect on the growth of HUVEC. The control groups served as controls for all three steroids. The data illustrating the cell number for DEX at seven days are depicted in Figure 3a. All four doses of DEX (5 μM, 1 μM, 0.5 μM, & 0.1 μM) effected a paradoxical increase in cellular growth. The population doublings are shown in Figure 3b. Compared to DMSO control, all four DEX doses caused a significant increase in population growth at seven days, although there was not a dose response.

The four doses of HC employed in DMSO solvent experiments were relatively higher than those used for DEX. Since HC is a less potent glucocorticoid than DEX, slightly increased doses of the former were used. Also, since 1 μM HC in a previous experiment (Figure 2b) effected a decrease in growth, albeit non-significant, higher doses might have been expected to manifest a similar inhibitory effect. However, as was the case with DEX, all four HC doses demonstrated a paradoxical increase in cellular growth at seven days (Figure 4a). All four HC doses caused a significant increase in population doublings at seven days, compared to DMSO control (Figure 4b).

The significant increases in population doubling observed following both DEX and HC treatment led to the idea that the cells possibly overcome the initial inhibitory effects of the corticosteroid, and rebound strongly, therefore leading to a relative increase in growth at seven days. This conceptual question was addressed in later experiments, when the proliferation of steroid-treated HUVEC was measured at four days.

As with DEX and HC, DHEA at the doses tested caused an increase in cell number (Figure 5a). Statistical analysis of the population doublings (Figure 5b) indicated

that the PD achieved with DHEA 50 µM, 20 µM, and 5 µM were significantly higher than those attained by the DMSO control group. A dose response was not found. DHEA at 10 µM, although causing growth enhancement, was not significantly different from the DMSO control group.

In the first set of corticosteroid-heparin treatment experiments using DMSO as a solvent, all three steroids tested caused growth enhancement in HUVEC. Since the same two cell lines derived from the same two umbilical cords were used for all three steroids, it could be possible that these cell lines responded to corticosteroid treatment by growth facilitation. It cannot be ruled out, however, that an initial decrease in cellular growth may have occurred, and was overcome by a subsequent increase in cellular growth as the cells recovered from the pharmacological treatment by seven days. Note these findings are in contrast to those found by Piovella et al. (1980) and Maca et al. (1978a), who reported an early increase in cell numbers under the influence of DEX which stabilized by the sixth day after plating so that cell numbers in control and treatment flasks were comparable.

Effect of Absorbed Serum

The purpose of charcoal-dextran absorption of the Hyclone FBS used in these experiments was to remove endogenous steroids that might affect the interaction of the cells with the test steroid. Theoretically, freeing the media of endogenous steroids would free receptors on the endothelial cell, and make it more responsive to exogenously added steroids. Both absorbed and unabsorbed FBS were used in a final concentration of 20%. In the first of these experiments, DEX was tested in this absorbed medium and compared to DEX administered to HUVEC in CM-199 with unabsorbed serum. DEX at the same doses used in the initial DMSO solvent experiment was added to HUVEC growing for two days. As in previous experiments using the DMSO solvent system, only one steroid feeding was done. At seven days, the cell number and population doublings (Figures 6a and 6b respectively) were compared between the absorbed and the unabsorbed group.

In comparison to absorbed serum, obviously greater growth in the HUVEC occurred when the unabsorbed serum was used. This could suggest that endogenous steroids in the serum used in routine media preparation might be facilitative to cellular growth in HUVEC. Alternatively, other growth factors in serum might be altered by the

charcoal filtration process. Using absorbed serum, very little variation from the DMSO control was observed when DEX was added to the cultures. For the absorbed group, DEX at 5 μM and 1 μM was associated with a significant decrease in population doublings compared to controls, but not to DMSO controls. Therefore, it cannot be concluded that the relative decrease seen in the DEX treated flasks was due to the corticosteroid alone. The difference between control and DMSO control is greater for the absorbed group than for the unabsorbed group. Apparently the charcoal filtered serum, with its endogenous steroids removed, allowed DMSO to exert a more suppressive effect on cellular growth.

In the unabsorbed group, all four doses of DEX caused a significant decrease in population doublings at seven days (Figure 6b). This is an interesting finding, since DEX at the same doses was growth enhancing in previous experiment (Figures 3a and 3b). Growth inhibition occurred in some DEX doses in absorbed serum-containing media, although it was significant only in the media containing unabsorbed serum.

Since the use of absorbed serum suggested a possible trend toward inhibition of growth, it was decided to retest DEX, and, in addition, to examine the effects of

absorbed serum upon HC-heparin and DHEA-heparin administration. In followup experiments, DEX, HC, and DHEA were again tested using the dosages employed in the initial DMSO solvent experiments, but this time employing media containing absorbed serum. A simultaneous comparison with unabsorbed serum was not done in this experiment. The results using DEX are shown in Figures 7a and 7b, which display the cell number and population doublings at seven days, respectively. In this experiment, DEX at 5 μM and at 1 μM caused growth inhibition and population growth inhibition while DEX at 0.5 μM and at 0.1 μM caused growth facilitation. These differences are not significant. DEX at 5 μM and 1 μM doses caused population doublings to be significantly lower than controls, but not lower than DMSO controls (Figure 7b). As in Figure 6b, then, the decrease in population doublings observed was not due to corticosteroid intervention alone. As they did in the preceding absorbed FBS experiment, the two highest doses of DEX again caused growth inhibition. It is interesting that in both experiments, the higher DEX doses (5 μM, 1 μM) caused growth levels that were significantly lower than controls, but not DMSO controls. Again, the results may well reflect a greater suppression of cell and population growth by DMSO in the absorbed serum.

When HC was used (20 μM, 10 μM, 5 μM, 1 μM) with absorbed serum, all four doses effected an increase in cellular and population growth at seven days (Figures 8a and 8b). A dose response was not observed. The population doublings for all HC doses except 10 μM were significantly greater than the DMSO control group. However, the 10 μM dose of HC still caused an increase in population growth. Apparently, the facilatory effect of HC upon cellular growth of HUVEC, demonstrated for unabsorbed serum (Figure 4b), does not appear to be influenced by using absorbed serum.

When DHEA (50 μM, 20 μM, 10 μM, and 5 μM) was used with absorbed serum, a variable response was found. Figures 9a and 9b display the cellular and population growth respectively at seven days for the various doses of DHEA. In absorbed serum, 10 μM DHEA inhibited population growth which was significantly less than control, but not DMSO control groups; therefore this inhibition was not due solely to corticosteroid intervention. Addition of DHEA (20 μM) caused a non significant increase in cellular and population growth at seven days, compared to DMSO control, but the population growth observed at this dose was still less than that for the control group. For two doses of DHEA, 50 μM and 5 μM, neither growth inhibition nor facilitation occurred. This is in contrast to the

facilitory effect of DHEA when unabsorbed serum was used (Figures 5a and 5b). Since the experiments with absorbed serum were run simultaneously for DEX, HC, and DHEA, comparison for each steroid was to the same control and DMSO control treatment groups.

In summary, when DEX, HC, and DHEA were tested in media containing absorbed serum, the inhibitory effect of DEX, at least at higher doses, appeared to be somewhat sustained, while the facilitory effect of HC seen in DMSO solvent experiments (albeit with unabsorbed medium) was unaffected by the absorbed serum. DHEA at the doses used resulted in a variable response. In this second group of experiments employing medium containing absorbed FBS, a non significant difference between control and DMSO control groups was again observed (Figure 6b). This difference was relatively greater than that observed when unabsorbed serum was used.

Comparison of Male and Female HUVEC

For some time in our laboratory, it had been observed that female and male HUVEC appeared to grow quite differently in culture. It was noticed that female HUVEC tended to grow more rapidly yet enter the senescent phase of a culture's life span later, and overall senesce at a

slower rate when compared to male HUVEC. Experiments to assess this observation quantitatively are in progress. It was decided to compare the cellular and population growth response of DEX-heparin treated female and male HUVEC. Also, two additional doses of DEX (0.01 μM and 0.002 μM) were examined for their effects in both the female and male cultures. Steroids were dissolved in DMSO and added to the cultures on the second day after plating. For both the female and male treatment and control cultures, cell harvest was on the sixth, instead of the customary seventh, day. The cellular and population growth was thus assessed slightly earlier than previous assays. However, the cultures were confluent at the time of trypsinization and further delay would have resulted in lost cells. Cell lines for the female and male HUVEC used in this experiment were derived from cords started on the same day, and passed identically. The male and female cells utilized were of the same age and passage number.

For the female HUVEC (Figures 10a, 10b), there was no dose-response effect when six doses of DEX were used. However, the trend was again noticed that the higher doses of DEX (5 μM, 1 μM, and 0.5 μM) caused a slight decrease in population growth (Figure 10b), while the lower doses of DEX (0.1 μM, 0.01 μM, and 0.002 μM) caused a slight increase in population growth. These differences,

however, were not significant. Again, it was also observed that when using unabsorbed serum there was very little difference between control and DMSO control flasks regarding cellular or population growth assays.

It can be pointed out that the cell number and population doublings in the male treatment and control groups (Figures 11a, 11b) were somewhat lower than the corresponding values in the female treatment and control groups (Figures 10a, 10b). This observation lends support to the suggestion that female and male HUVEC grow at different rates. There was little difference between the control and DMSO control groups when male cells were utilized. Apparently, the slight inhibition of cellular and population growth that occurs in 0.1% DMSO alone in the medium, when compared to DMSO-free control groups, is not sex-specific and is a property shared by cells derived from either gender umbilical cord. A variable response was found among the male HUVEC treated with the six DEX doses. DEX at doses of 0.5 μM and 0.002 μM caused a slight (n.s.) inhibition in cellular growth at six days. The remaining DEX doses (5 μM, 1 μM, 0.1 μM and 0.01 μM) caused a facilitation in cellular growth at six days. DEX at 0.01 μM caused a significantly greater increase in population doubling when compared to DMSO control. In addition, the increase in population growth effected by

DEX at 0.01 μM was significantly different from control, 0.5 μM, and 0.002 μM DEX. Here, compared to female HUVEC, the higher doses of DEX (5 μM, 1 μM) caused an increase, rather than a decrease in cellular growth. However, a low dose of DEX (0.01 μM) caused a significant increase in cellular and population growth, which corroborates previous findings with lower doses of DEX (Figures 6b, 7b).

To summarize, in female HUVEC, the differences between treatment and DMSO control groups were not significant, but it was observed that the higher doses of DEX were manifested in a slight decrease in population growth, while the lower doses of DEX in a slight increase. The results for male HUVEC were less clear, but nonetheless DEX at 0.01 μM caused a significant increase in population growth, which supports a trend for lower doses of DEX enhancing growth, a finding suggested by previous experiments.

Effect of Dexamethasone in Reduced (1%) Serum

Growth of HUVEC in culture does not occur when serum is omitted from the media, unless serum substitutes are used. While optimal growth of these rather fastidious cells occurs with heparin, ECGF, and 20% FBS, reduction of

the serum component in the medium to a concentration as low as 1% can support low level growth of the cells. In order to determine whether DEX could affect cells under suboptimal growth conditions, six doses of DEX were used in combination with 1% FBS. As in all previous experiments using DMSO as a solvent, the cultures were fed with experimental media on the second day. The cell numbers (Figure 12a) demonstrated that all six doses of DEX caused a decrease in cell number at seven days, when compared to the DMSO control group. All doses of DEX except 0.5 μM caused a population growth that was significantly lower than DMSO control and control. The results are similar to our earlier experiments with DEX when ETOH was the steroid solvent. Apparently, using medium with a reduced serum component (1%) allows the inhibitory effect of DEX treatment to be more fully expressed.

Next, two doses of DEX (0.1 μM and 0.01 μM) were employed in a comparison experiment using fully supplemented media (20% FBS) or reduced serum medium (1% FBS). The same cell line as used in the 1% FBS experiment immediately preceding (Figures 12a, 12b) was used in the comparison experiment, except that the cells were at a later passage. A regular control group was not run, and all statistical comparisons were made with the DMSO

control group. Figures 13a and 13b depict the effect of DEX in media containing 20% and 1% FBS upon cellular and population growth, respectively. There was much greater growth in 20% FBS as expected. With 1% FBS in the culture medium, a non-significant decrease in cellular and population growth was observed for both DEX doses. With 20% FBS, growth enhancement at seven days was observed. Both the 0.1 μM and the 0.01 μM doses of DEX caused a significant increase in the number of population doublings, when compared to DMSO controls. A dose response over this range was not observed. Again, the reduced serum (1%) appeared to unmask an inhibitory effect of DEX or to prevent the stimulation observed with 20% FBS. The growth enhancement seen with 20% FBS was consistent with that found earlier when DMSO was used as a solvent (Figures 3b, 11b).

The 1% vs. 20% FBS comparison experiment was repeated using a slightly wider dose range of DEX (5 μM, 1 μM, 0.5 μM, 0.01 μM) using third passage cells from a different cord. There was little difference in cellular or population growth between 0.1% DMSO controls and controls when either the 1% or the 20% FBS was used (Figure 14a, 14b). Using 1% serum, growth was slightly, but not significantly, facilitated at seven days at the lower doses of DEX (0.1 μM, 0.01 μM); however, DEX at

doses of 5 μM and 1 μM had no effect. When 20% FBS was used, a relatively high dose of DEX (5 μM) caused a significant increase in population growth compared to DMSO controls (Figure 14b). Actually, all doses of DEX when used with 20% FBS enhanced population growth. This was consistent with earlier DEX experiments when 20% serum containing medium was used (Figures 3b,11b,13b).

In summary, a dose response was not observed in the above three experiments employing a reduced serum component in the culture medium. Using 1% serum allowed DEX to exert an inhibitory effect on the growth of HUVEC in two of three experiments, although this was not significant in one of them. When 20% FBS was used in comparison experiments, cellular growth and population growth were enhanced, much as it was in the initial DMSO solvent experiments. Even when using higher doses of DEX (5 μM, 1 μM) that had proven inhibitory previously (Figures 3b,7b,9b), growth enhancement occurred with 20% FBS containing medium. Since DEX in medium containing 20% FBS either facilitated or inhibited growth, depending on the cell line used, these findings can be related to the clonal specificity theory of Longenecker et al. (1982), who reported that EC exhibited a clonal heterogeneity regarding their response (inhibitory or facilitory) to glucocorticoid treatment.

Effect of Repeated Steroid Dosing

When EtOH was used as a solvent in our early experiments, the cultures were fed twice, at two and five days, with corticosteroids. In all previous experiments employing corticosteroid-heparin combinations and DMSO as a solvent, the cells were fed with steroid containing medium only once on the second day after plating. Cells were counted at seven days. The purpose of the next experiments was to determine the effect of a second steroid feeding on the log phase growth of HUVEC when DMSO was used as a solvent. In these experiments, cultures were fed on the second and fourth day after plating, and counted on the seventh day. DEX at doses of 1.0 μM, 0.5 μM, 0.1 μM, 0.01 μM was used. Table 1 shows that at seven days, all four doses of DEX caused a decreased cell count. Apparently the second steroid feeding at four days after plating sustained the inhibitory effect of DEX seen in some previous experiments. However, while all four doses of DEX caused a decreased population growth at seven days, none were significantly different from DMSO controls.

When the experiment was repeated using later passage cells from a different cord, growth enhancement occurred (Table 2) despite the second steroid feeding. Three of the four doses of DEX caused a significantly greater

population growth (Table 2) when compared with DMSO control. Thus, it was observed that when cultures of HUVEC were fed twice with steroid medium, a non-significant inhibition occurred in one experiment, while a significant enhancement of cellular and population growth occurred in a second experiment. Since these experiments were conducted under identical conditions (except for the passage number), the clonal specificity response again appears to be supported (Longenecker et al., 1982).

Effect of Steroids on Proliferation of HUVEC at Four Days

Following the observations of Piovella et al. (1980), who noticed an enhancement of growth for the first five days of steroid treatment of HUVEC but level growth at seven days, and our own conflicting findings using seven day assays, it was decided to determine if steroids had an earlier effect on cellular proliferation and population growth than could be seen at seven days. These experiments were run simultaneously with those examining the effects of a second steroid feeding, using the same cultures at the same passage numbers. The purpose of these lines of inquiry was to determine if DEX had any early effect on the growth of HUVEC that might be masked after the cultures were allowed to grow for several additional days. DEX at doses used for the second steroid

feeding experiment just described were employed; cells were steroid-fed at two days and harvested at four days. All four doses caused inhibition of cellular growth at four days (Table 3); of these doses, 1 μM and 0.1 μM were significantly different from DMSO controls. The relatively less inhibition caused by the lower steroid dose (0.01 μM) was noted. It was possible, then, that DEX caused an early inhibition of cellular growth in HUVEC that might well have been overcome and not be evidenced at a later time, say seven days.

The four day cellular and population growth assays were repeated using the same steroid doses, but with later passage cells from a different cord. Early growth inhibition was again observed (Table 4), as all doses of DEX effected a decrease, albeit non-significant, in cell number and consequently in population doublings at four days. Again there appeared to be less inhibition of cellular and population growth at the lower DEX dose (0.01 uM). The conclusions from this second experiment were similar to those made above: DEX at the doses tested causes an early inhibition of cellular growth in HUVEC that might well be overcome, and not be evident, if the cultures are harvested at a later time (seven days).

Colony Forming Assay

A colony forming assay, an alternate method of cellular proliferation assessment, was used to test DEX at doses of 1 μM and 0.1 μM HUVEC. A colony forming assay may be a more sensitive growth assay, since it measures the ability of single cells to grow and form a colony of cells following plating at low density. In the first colony formation variation, the effect of DEX upon growth, and not upon attachment, was examined. The cells were plated at colony formation density (1,000 cells/25 cm^2) in CM- 199, and after four hours were washed and then fed with the appropriate steroid containing medium. The cultures were fed a total of three times with either steroid or DMSO control medium. Both DEX doses caused effective and significant inhibition of colony formation, i.e. there were significantly fewer colonies in the DEX treated flasks after nine days of growth (Figure 15).

In the second colony formation variation, the effect of DEX on attachment only was examined. In this experiment, the cultures were plated at colony formation density for four hours in CM-199 with the appropriate DEX dosage (1 μM or 0.1 μM) or CM-199 with 0.1% DMSO alone. After the four hour incubation with steroid, the cultures were washed and then fed with CM-199 without steroids or

DMSO. After a total of three such feedings, the cultures were fixed and evaluated at nine days. No difference in the number of colonies of HUVEC was observed at nine days, suggesting that a short attachment period in steroid medium would not affect subsequent colony growth. If possibly there was a slowing of the growth of HUVEC in this initial four hour period, it did not effect the number of viable colonies observed at nine days.

In the third variation, the effect of DEX on both attachment and growth was determined. In this experiment, cells were plated in steroid or DMSO containing medium for four hours, washed, and subsequently fed three more times with the steroid or DMSO control medium. The steroid treatment caused significantly fewer colonies to form when compared to the DMSO control group (Figure 15). There appeared to be a dose-response, but this assertation is made cautiously. Note the lower number of colonies in the steroid treated flasks both plated in and subsequently fed with steroid containing media (Variation 3), when compared to steroid treated flasks in the first experiment (Variation 1), when the effect of DEX was assessed for growth alone. These data suggest that plating with steroid media even for the relatively short period of four hours further inhibits colony formation in HUVEC, when steroid treatment is then continued.

Cytofluorometry

Phase contrast microscopy revealed that the dexamethasone-treated cultures contained EC with larger cytoplasmic and nuclear areas and more prominent nucleoli, supporting the observation of Piovella et al. (1982). The increase in cellular size was confirmed by light scatter analysis using the cytofluorometer. Figures 16, 17, 18, and 19 show the effect of dexamethasone on relative endothelial cellular size for control, control plus 0.2% ETOH, 0.5 μM dexamethasone, and 1 μM dexamethasone, respectively. From these data, it can be seen that an increase in concentration of dexamethasone paralleled an increase in cellular size. This effect may be dose related (Figures 18, 19).

Goldsmith et al. (1984) used flow cytometric analysis of both bovine aortic endothelial cells (BAEC) and HUVEC to examine the proliferative characteristics of repeatedly subcultured endothelial cells. They found that confluent primary HUVEC had approximately 2-8% of the cells in the proliferative phases (S,G_2, and M) of the cell cycle. Although the authors evaluated the effect of cell passage upon cell cycle profiles for BAEC, only primary HUVEC were studied. Preliminary work in our laboratory, utilizing cytofluorometric analysis of EC

cultures treated with dexamethasone in the presence of heparin, demonstrated that this treatment increased the number of EC in the S and/or G2-M phases of the cell cycle in early passage HUVEC (Table 5). The decreased population doubling observed when certain cultures were treated with DEX-heparin combinations was paradoxical, then, since greater population growth would have been anticipated with more cells in the proliferative cell cycle phases.

Discussion

Since angiogenesis can be inhibited *in vivo* by treatment with heparin and various corticosteroids (Folkman, 1985a,b; Folkman *et al.*, 1983: Crum *et al.*, 1985), *in vitro* experiments examining the effect of corticosteroids on cultured HUVEC were undertaken to determine if the mechanism of anti-angiogenesis involves suppression of cellular proliferation. Several papers (Maca *et al.*, 1978; Piovella *et al.*, 1980, 1982; Longenecker *et al.*, 1983) have dealt with steroid effects on cultured EC. In order to elaborate further the effect of these steroids upon growth characteristics of HUVEC, the current study has extended previous research by testing the glucocorticoids DEX and HC in the presence of heparin and ECGF under a variety of conditions. This study has also examined the effects of DHEA, a corticosteroid not used by previous researchers, upon cellular proliferation and population growth in HUVEC. In our study, a variable response of HUVEC to corticosteroids was found, depending on a number of factors, including the time frame of steroidal administration, the type of steroid solvent, and the length of exposure to corticosteroids. These equivocal findings involving HUVEC

were similar to those of Longenecker et al. (1983), who suggested that bovine aortic endothelial cells (BAEC) exhibit a rather striking clonal heterogeneity regarding their sensitivity to glucocorticoid treatment.

Using HUVEC grown under optimal conditions with FBS, ECGF, and heparin (Thornton et al., 1983), our results have corroborated some aspects of previous inquiries involving steroid effects; in other areas the findings are in contrast to earlier reports. The increased growth observed in HUVEC in many of our experiments (when steroids were dissolved in DMSO) was also reported by Piovella et al. (1980, 1982), who found that DEX caused an initial increase in cell number, but that the confluent density of HUVEC matched controls at six days. However, the increases we observed were sustained at confluence (seven days). Growth inhibition, as observed in some of our experiments, was also noted by Longenecker et al. (1983) in DEX-treated BAEC. However, they found that DEX inhibited cellular growth in only one of four clones that they studied. Growth inhibition of HUVEC treated with DEX and HC, which occurred consistently when EtOH was used as a steroid solvent in this study, stands in contrast to the findings of Piovella et al. (1980) as well as to our findings when DMSO was the solvent. The present study also has corroborated and, by employing cytofluorometric

light scatter analysis, added a quantitative dimension to, earlier reports (Maca et al., 1978; Piovella et al., 1980, 1982) that dexamethasone increases cellular size in HUVEC.

It was clear that when EtOH was used as a solvent for corticosteroids in the HUVEC culture system employed here, growth inhibition occurred that was reproducible in several experiments terminated at seven days. Growth inhibition significantly greater than the slight amount caused by the EtOH solvent was evident using two doses of DEX, (1 μM, 0.5 μM), and was consistently, although not significantly, inhibited when HC at 1 μM was used. The fact that the cultures were fed twice with steroid containing media in contrast to a single feeding when DMSO was the solvent may have sustained early growth inhibition, but we cannot conclude this unequivocally, since an earlier (four day) growth assessment was not done in experiments utilizing EtOH as a solvent. Also, since single feeding treatment regimens were not employed in the EtOH solvent experiments, we cannot ascertain whether recovery from early growth inhibition or even subsequent enhancement might have occurred if the cultures were fed once and harvested at seven days, as in most of the DMSO solvent experiments.

When DMSO was used as a solvent for DEX, HC, and DHEA, cellular and population growth enhancement was frequently observed. In these experiments, there was a slight and insignificant depression of growth caused by the DMSO solvent itself, suggesting that this solvent did not stimulate growth of HUVEC. In contrast to the growth inhibition seen when DEX was used with EtOH as a solvent, when four doses of DEX dissolved in DMSO were used, significant growth enhancement was initially seen at seven days. Subsequently, when unabsorbed and absorbed serum were compared regarding responses to DEX treatment, the unabsorbed group, which was comparable to the first DMSO solvent experiment except that a different culture was used, exhibited growth inhibition. And in a later experiment comparing 20% and 1% FBS containing media, growth enhancement occurred when the 20% FBS containing media was used. Thus, it seems clear that DEX is not consistent in its effect on cellular and population growth at confluence (seven days) in HUVEC when fully supplemented media, a DMSO solvent, and a single steroid feeding are employed.

Since 1 μM HC (Figure 2b) effected a non-significant decrease in growth when ETOH was the solvent, higher doses might have been expected to manifest a similar inhibitory effect. However, when DMSO was the solvent, all four HC

doses demonstrated a paradoxical increase in cellular growth at seven days. This enhancement with HC was significant, and was sustained when absorbed serum was used in the media. Thus, the growth facilitory effects of HC upon HUVEC when DMSO was used as a solvent did not appear to be affected by using absorbed serum. This finding suggests that hydrocortisone, a natural glucocorticoid, might be more consistent than dexamethasone, a synthetic glucocorticoid, under different experimental conditions in enhancing growth in HUVEC.

When DHEA was used with DMSO as a solvent, significant growth enhancement also occurred. In contrast, when DHEA was tested using absorbed serum in the media, a variable response was found. Since neither growth facilitation or inhibition occurred when two doses of DHEA were used, it appeared that DHEA had little definitive effect on the growth characteristics of HUVEC when absorbed serum was used.

To summarize, in contrast to the experiments employing EtOH as the solvent, when DMSO was used as the solvent, there was a tendency for growth enhancement to occur, but this was not observed in all experiments. In earlier research (Piovella et al., 1980), an early increase in cell number was seen but this enhancement was

not sustained at seven days. In the present study, this enhancement was sustained at least for five days after steroid administration on the second day of culture. However, Piovella et al. (1980) administered steroids at the time of cell plating; that is, two days earlier than in the present experiments, so the steroid-treated cells may have reached confluence earlier, allowing the control cultures to catch up by the end of the six day assessment period, therefore causing the cell numbers in control and steroid treated cultures to be comparable. In comparison, in our culture system, the cells were exposed to steroids for shorter periods of time, allowing early growth stimulation to still be operational at the end of the seven day assessment; thus growth enhancement was observed. However, since only a seven day assessment was carried out in these experiments, the possibility of an early growth inhibition occurring during the first few days following steroid treatment followed by a later growth enhancement manifesting at seven days cannot be completely ruled out. The results of our four day assessment experiments, which showed steroid-induced cellular growth inhibition two days after steroid treatment, support this contention.

It should be noted that in the first experiments in which DMSO was used as a solvent, cells derived from the

same umbilical cord were used for all three steroids tested. It may be that these cells respond to corticosteroid treatment by facilitation of growth, and that this response might be culture specific, much like the clonal variability responses seen in BAEC by Longenecker et al. (1983). One might speculate that the majority of cells from this cord would respond to steroid treatment with growth facilitation, and that a minority of cells would respond with inhibition, yielding an overall increase in culture growth following treatment with DEX, HC, or DHEA.

There was a consistent lack of a dose response in most of the experiments reported in this study. This was particularly true for experiments which employed media containing a full serum supplement (20% FBS) and steroids dissolved in DMSO. The full serum supplement in the media possibly may serve to down regulate steroid receptors on the endothelial cellular surface; the receptors may then not be in a propitious state to respond to exogenously administered steroids. Possibly for this reason, at the steroid doses that we employed, a dose response regarding proliferative effects was not observed. In addition, cells more resistant to steroid effects might selectively inactivate these steroids in greater quantities than more sensitive cells (Berliner, 1965). Since the human

umbilical vein cell populations used in these studies were uncloned, it is likely that our cultures contained groups of cells with varying sensitivity to steroid effects. This may partially explain the lack of a dose response, as well as the variable and equivocal nature of our findings, concerning the effects of steroids on endothelial cellular proliferation in vitro.

When the effects of corticosteroids in medium containing absorbed serum were compared to those in medium containing unabsorbed serum, the slight inhibition caused by the DMSO solvent was somewhat greater for the former than for the latter group. Apparently, the use of absorbed serum in the growth media allows the DMSO solvent to exhibit a greater degree of inhibition upon cellular and population growth.

Although our data suggest that endogenous steroids in the serum used in routine media preparation might be facilitative to cellular growth of HUVEC, the use of media containing absorbed serum appears to modify somewhat the response of HUVEC to corticosteroid intervention. The mechanism underlying this modification might concern the regulation of steroid receptors on the endothelial cellular surface, alluded to above in partially explaining

the lack of a dose response regarding proliferation in steroid-treated EC.

In using media containing unabsorbed serum, the already high steroid (ligand) concentration present in the serum might serve to down regulate steroid receptors on the endothelial cellular surface. Therefore, exogenously added steroids may further down regulate the steroid receptors. On the other hand, a lower steroid (ligand) concentration, as would be the condition when media containing absorbed serum was used, would serve to up regulate the steroid receptors on the cell surface. Thus, added steroids would have an effect on cellular proliferation, as the up regulated steroid receptors would then be more apt to bind the hormone, internalize it, and effect a growth response. According to Evans (1988), in steroid-cellular interactions, transcriptional regulation of cellular DNA derives from the binding of the hormone-receptor complexes to hormone response element sites on the DNA. Apparently the hormone binding region normally prevents the domain for DNA binding and transcriptional activation from functioning. The addition of steroid apparently relieves this inhibition (Evans, 1988). One can speculate that the use of media containing absorbed serum would increase the likelihood of this disinhibition occurring.

In the experiments using absorbed serum, there was a trend for higher doses of DEX (> 0.5 μM) to inhibit growth, and lower doses of DEX (<.5 μM) to facilitate growth. It is possible that the higher doses of DEX, added to cells whose steroid receptors were up regulated due to the presence of media containing absorbed serum, were more effective than the lower doses in engaging a greater number of steroid receptor sites, thus resulting in net inhibition of growth. In comparison, the lower DEX doses might not have been as effective in engaging as many steroid receptor sites on the endothelial cellular surface, thus even though the steroid receptors were in an up regulated state due to the presence of media containing absorbed serum, the binding of added steroids to cellular receptors was insufficient to effect a growth inhibition response; neutral growth or even slight growth enhancement occurred.

Alternately, the use of absorbed serum might modulate the number or character of ECGF and other growth factor binding sites on EC, and thus it would be the altered response by EC to growth factors per se, and not to corticosteroids, that would ultimately effect the level of cellular or population growth that was observed.

In the male vs. female experiments, the cell harvest was on the sixth day post plating, slightly earlier than in other experiments in this study. Although dexamethasone had one day less to exert any effect, this probably was not a significant factor in the responses observed, as the cells were tightly confluent when they were trypsinized and counted. When female and male HUVEC were compared regarding the effect of DEX on cellular and population growth, no consistent inhibition or stimulation of growth was observed in either group. It was not possible to definitively conclude from these experiments that gender plays a crucial role in the response of HUVEC to exogenous steroid administration.

In comparing the responses of female and male cells (Figures 10a and 11a), it was noted that there was a trend for cellular and population growth of control cultures for female cells to be greater than that for male cells. This observation lends support to the suggestion that female and male HUVEC grow at different rates. Further evidence for this phenomenon can be found in the four day growth experiments. When the four day assessments were carried out, two experiments were performed. Although these experiments were not designed to test cellular gender as a factor in steroid response, it is informative to examine the results for control cultures here also. The results

in Table 3 are those for male cells, and in Table 4, for female cells. The greater population growth of HUVEC under control cultures for female cells compared to male cells is noted. Also, from these tables, it can be observed that for each steroid dosage, the cell number for female cells was approximately twice as great as that for male cells, with both types of cells responding to steroid treatment with growth inhibition. In these experiments, all culture flasks were plated at standard density two days prior to steroid administration. The results seemed to indicate a trend for female HUVEC to exhibit a greater growth ability than male cells. However, more culture pairs will have to be tested specifically for the gender effects upon cellular growth to substantiate this trend.

DEX appears to be inhibitory when greatly reduced FBS (1% vs. the usual 20%) is employed in the media used to feed the cultures. When 1% FBS was used in the media, DEX consistently, but not always significantly, inhibited population growth. The use of medium containing 1% serum might lead to up regulation of the steroid receptors on the endothelial cellular surface in the same manner as when media containing absorbed serum was employed. The reduced serum component in the medium would effectively lower the concentration of steroid (ligand) available to the steroid receptors; added steroid would then be more

likely to engage the available receptors, thereby possibly exerting an effect on cellular proliferation. In this way, a reduced serum component in the media might make the HUVEC more susceptible to the possible inhibitory effects of heparin-corticosteroid combinations.

The response characterized by higher doses of DEX leading to growth inhibition and lower doses of DEX leading to growth enhancement, observed in two experiments when the absorbed serum paradigm was employed, was not seen with media containing reduced FBS except in one experiment. But when culture medium containing a full serum supplement (20% FBS) was used, either growth facilitation or inhibition could occur depending on the cell line used. These results as displayed in the comparison experiments could be related to the clonal specificity theory of Longenecker et al. (1982), who reported that EC exhibited a clonal heterogeneity regarding glucocorticoid sensitivity. Further testing employing medium with a full serum supplement, using cultures derived from cloned cells might confirm this or perhaps demonstrate a more definitive relationship of either growth inhibition or enhancement in cloned HUVEC treated with corticosteroids. Also, other steroids, in addition to DEX, should be studied using both a full and a reduced serum supplement in the media to determine if a

trend toward cellular and population growth inhibition observed with DEX would be expressed.

In the study of Longenecker et al. (1983), who emphasized clonal variability of BAEC, it was interesting that inhibition of proliferation of EC occurred only in the presence of FGF. It is possible also in our study that the response to glucocorticoids was also growth factor dependent. It might be that steroids mildly inhibit the maximal response of EC to growth factor, in our case, ECGF. Since ECGF is believed to be the acidic form of FGF (Jaye et al., 1986), it would be interesting to determine if FGF would cause the same effects, i.e. inhibition of the endothelial cellular proliferation response to the growth factor in HUVEC as well as BAEC. Alternately, different cultures or clones of HUVEC may display different responses to steroid-heparin combinations. Moreover, it cannot be ruled out that a given cell clone of HUVEC can simply respond to corticosteroid administration by either growth enhancement or inhibition.

Based on the results from two separate experiments where cultures of HUVEC were fed twice with medium containing DEX dissolved in DMSO, a non-significant inhibition in one experiment and a significant enhancement

of cellular and population growth in a second experiment, occurred. Since these experiments were conducted under identical conditions, the clonal specificity response again appears to be supported (Longenecker et al., 1983). However, in early experiments using EtOH as a solvent, a second steroid feeding was routinely employed, and cellular and population growth inhibition was rather constant. Perhaps then, the solvent is as important a factor as the steroid effects themselves.

It is conceivable that certain cultures of HUVEC could overcome the growth inhibition that was seen at four days of culture, that is, two days after steroid treatment. At this point, after cessation of inhibition, the inhibited cultures (at a lower density) might proceed to grow more rapidly during the final days of culture than the more dense controls, with ultimate confluent density consisting of similar numbers of cells in treated and control flasks. In experiments that employed a seven day growth assay, a cellular and population growth enhancement was a frequent finding, suggesting that if an initial steroid induced growth inhibition did occur, it could be overcome. Further support for this contention is supplied by the observations in the four day growth experiments and repeated steroid dosing experiments (DMSO solvent). Table Two and Table Four illustrate findings

that were observed using the same cell line, at the same passage number, and run simultaneously. The cultures exhibited cellular and population growth inhibition at four days, but enhancement at seven days. Even with a second feeding (Table Four), significant cellular and population growth enhancement was seen at seven days; replicate cultures had displayed inhibition of growth just three days previously.

The findings employing a second steroid feeding support the suggestion made above that a given culture of EC can respond to steroid treatment with either growth facilitation or growth inhibition. This is further evidence in support of the clonal specificity theory regarding endothelial cellular response to steroid treatment (Longenecker et al., 1983). However, even in a controlled in vitro experiment there are a number of factors that can determine the type of response by the EC. It is also possible that, depending on other factors, such as cellular density and medium composition, HUVEC may reach a point in their growth cycle when they are less responsive to any steroid induced inhibition of their growth characteristics. Alternately, exogenously introduced steroids may continue to bind to the surface of the EC, and when this is reinforced and further maintained by a second steroid feeding, the response of the EC may be

one of increased growth. This might explain the growth enhancement observed when the cultures were trypsinized and counted on the seventh day after plating.

Henriksen et al. (1975) found that the replicative ability of HUVEC was correlated with cell density at the time of cell seeding. Colony formation is an alternate method of growth assessment in culture systems and is a way of examining clonal growth of cells plated at very low density. Colony formation was employed to determine if corticosteroids administered to sparsely plated HUVEC had a possibly different effect than when administered to a denser, yet still subconfluent and growing culture of HUVEC. In the first colony formation variation, the effect of DEX upon growth only was assessed; a significantly lower number of colonies was observed in DEX-treated cultures compared to controls. In the second variation, HUVEC were plated in steroid containing media for four fours only, then subsequently fed with CM-199 without steroids; there was no difference in the number of colonies between DEX-treated and control cultures. In the third variation, the effect of DEX on both plating and growth was determined. Compared to the control, there were significantly fewer colonies in the steroid treated flasks when the cells are both plated in and fed with steroid containing media. It is noted, in comparing the

DEX-treated cultures in the first and third variations, that there were fewer colonies in the DEX-treated cultures in the latter. The data suggest that plating with steroid media even for a relatively short period of time can potentiate the inhibition of colony formation in HUVEC seen if the cells continue to be grown in the steroid containing media.

Since steroid-induced inhibition of growth was prominent in the colony formation experiments, where plating density is only 1,000 cells/flask (vs. 1.25×10^5 cells/flask in the usual growth assay experiments), low density growth of HUVEC may be inhibited by heparin and DEX, whereas high density growth is unaffected or slightly increased. A low number of cells, given a uniform steroid dosage, may not be able to metabolize it as rapidly; therefore, growth inhibition may ensue when cell density is low, the relatively higher steroid concentration acting on a smaller number of cells. This might be a possible mechanism for the inhibition seen with the four day growth assessments and the colony formation experiments. Now, at a later time, say seven days after plating, when the culture may be approaching confluence, there are more cells to metabolize the steroid and the concentration of the steroid has been reduced, so that an effect on growth does not occur. This could possibly explain the lack of a

consistent response to a second steroid feeding when DMSO was used as a solvent. Any inhibitory effects that might have resulted if the cellular density were lower might be minimized if the steroids were added at five days, as during the second steroid feeding experiments.

High density cultures of HUVEC seem to respond differently to steroid intervention. It should be noted that in several experiments the cultures were crowded and postconfluent at the termination of the experiment at seven days. It was for this reason that the experiments comparing the response of male and female HUVEC to corticosteroid administration were terminated at six, instead of seven, days. A crowded, densely populated culture of HUVEC may exhibit a deceleration of the cellular growth rates in the later period just prior to cell harvest; growth during this period may not be responsive to exogenous steroid administration. In our experimental paradigms involving administration of fresh steroids during later growth periods (i.e., experiments employing a second steroid feeding) there might have been little effect on growth rates during this crowded period. It appears then that the density of a culture during the later stages of steroid treatment may have a considerable effect on cell number assessed at a postconfluent time. Since the availability of physical space in the monolayer

into which the EC can replicate is an important factor in the growth of EC in vitro, crowded near confluent cultures will have a lower growth rate than less dense, younger cultures exhibiting the characteristic numerous mitotic figures indicative of rapid growth.

Another possible mechanism for the inhibition at low density might be related to the proportion of the endothelial cellular surface mechanically available to exogenously administered steroids. It is possible that crowded, more confluent cultures may be less responsive to inhibitory effects of steroid treatment because only the receptors on the apical surface of the cell would be exposed directly to exogenous steroids. Therefore only a limited number of receptors on the endothelial cellular surface would be likely to respond to steroid treatment. In the intact endothelium in vivo, this may be a factor where perhaps only the luminal surface of the EC would be exposed to circulating steroids. The clonal specificty hypothesis (Longenecker et al., 1983) could also be related to the inhibitory effects of DEX on colony formation. In colony formation, clones that are inhibited and do not grow are therefore not counted; in a mixed culture, loss of the cells contributed from a clone that is totally inhibited could be compensated for by the

greater growth potential of a neighboring clone allowed to grow to confluence.

On the other hand, steroids administered to low density, rapidly growing EC under *in vitro* experimental conditions might prove to be too artificial a situation to compare the responses to those which might possibly occur during *in vivo* conditions. However, when corticosteroid preparations are administered pharmacologically to treat various diseases where angiogenesis is an ongoing process, the effect on open, rapidly growing microvessels where migrating and dispersed EC are present might consequently differ from that seen in an intact endothelium where angiogenesis is not occurring.

Cell cycle analysis by cytoflourometry revealed that 1 uM DEX (EtOH solvent) almost doubled the percentage of HUVEC in the G_2-M phase of the cell cycle (Table 5). Since the number of population doublings was significantly suppressed by 1 uM DEX in these early growth experiments, it is possible that the mechanism of suppression involved arrest in the G2 phase of the cell cycle and therefore delay of division (Goldsmith *et al.*, 1984). Running additional cytofluorometric analysis simultaneously with growth studies could enable further correlation of the level of growth enhancement or suppression with the

relative distribution of cells in the various phases of the cell cycle. This may provide more information to answer questions concerning the cell cycle dependency of the growth response of HUVEC to corticosteroid-heparin treatment. Light scatter analysis of DEX treated cells revealed that this steroid caused an increase in cellular size. It is known that glucocorticoid treatment increases protein content of cultures of HUVEC (Maca et al., 1978); therefore, increased structural protein synthesis could be responsible for this increase in cellular size. Alternatively, although the exact mechanism for this phenomenon is unclear, it may be related to an increase in microfilament formation in association with the cell membrane, as reported for steroid treated C6 glial cells by Berliner et al (1978). Quantitative studies employing electron microscopic procedures might reveal whether a relative increase in microfilament formation occurs in steroid treated EC.

Rosner and Cristafalo (1979) studied a number of non-endothelial cell lines and observed both growth enhancement and growth inhibition when hydrocortisone was used in the medium. Perhaps in EC also, in this case HUVEC, the proliferative response of the cells to corticosteroids demonstrates a cell-line specificity, as well as a steroid-molecular structure specificity. In

this light, it cannot be concluded that a given culture or clone of HUVEC will respond with either growth facilitation or inhibition to all steroids.

Steroid-endothelial cell reactions are conceivably mediated by high affinity glucocorticoid binding sites on the endothelial cellular surface (Berliner, 1981). If the binding sites are altered in some way, the endothelial cellular response to corticosteroids will also be altered. Perhaps the study of factors which can compete, either positively or negatively, with the steroid binding sites would yield more fruitful information concerning cellular growth responses to corticosteroids than study of the corticosteroids per se. It is conceivable that various growth factors and media components can affect the function or regulation of these corticosteroid binding sites on the endothelial cellular surface. Berliner (1981) found that although HC did not appear to have a direct effect on thymidine incorporation in EC, the presence of the steroid was permissive for the effects of insulin and FGF. Although the mechanism of action of the steroid induced enhancement of the growth factor and insulin interaction is not completely understood, Baker et al. (1978) felt that it might be related to the increase in insulin binding observed when EC were treated with HC. It is entirely possible, according to the work of Baker et

al. (1978), that corticosteroids, dexamethasone in this case, cause a permissive effect on growth by modulating cell surface receptors for the growth factor. To this effect, DEX may enhance the mitogenic response of HUVEC to growth factors, and this may be the mechanism by which growth enhancement occurs. Therefore, the modulation of growth by steroids may be indirect. One might speculate that the level of corticosteroids in the blood exerts some control over the growth or quiescent state of the endothelium in vivo. Such an effect may be mediated by alteration of the character or structure of binding sites for heparin and growth factors on the surface of the endothelial cell.

Glucocorticoids can either sensitize or desensitize various cell types to the action of serum or growth factors added to the cell cultures, and the role of these growth factors needs to be further understood. Perhaps a time factor is relevant, with growth factors being more inhibitory in some phases of the cell cycle than others. However, the possible interaction of added steroids and growth factors is even more intriguing. Longenecker et al. (1982) reported that DEX inhibited the growth of bovine smooth muscle cells (BSMC) but only when FGF was absent from the medium. This suggests that the inhibition of cellular growth following exogenous steroid

administration may be dependent on the absence of FGF. However, smooth muscle cells were tested above; when steroids were administered to BAEC (Longenecker et al., 1983), growth inhibition was dependent on the presence of FGF. It is conceivable, then, that the steroids and growth factors may be competing with the same or interdependent binding sites on EC, sites which are responsible for the modulation of cellular growth. Whether steroids compete with growth factors for binding sites or render the latter inactive by binding to separate but physiologically related sites is unknown. Alterations of the location, size and/or character of these binding sites would also be important factors in determining endothelial cellular responses to steroid treatment.

Since heparin was utilized in all experiments in our cultures system, its role in steroid induced proliferative effects in EC needs further delineation. It is known that heparin interacts structurally with ECGF and potentiates the mitogenic activity in EC by altering immunological epitopes within the structure of ECGF, stabilizing its polypeptide structure, and increasing the affinity of ECGF for its cell surface receptors (Schreiber et al., 1985). The interaction and possible interdependence of these three factors (ECGF, heparin, steroids) in modulating proliferation of EC are topics for future study.

Note that Longenecker et al. (1983) also found that the steroid induced inhibition seen in one clone of BAEC was FGF dependent. In our culture system, we did not test whether the inhibition seen in some experimental conditions was ECGF dependent, as this growth factor was a component in all experiments conducted. It may be that the responses of HUVEC to steroid intervention would differ if ECGF were omitted from the culture media. However, without ECGF, cultures of HUVEC grow slowly and sporadically, and impaired growth could affect the response to steroid treatment. Further, regarding culture conditions, an alternative to completely eliminating the growth factor would be to modify the amount of heparin in the media. However, for optimal growth of HUVEC, this would necessitate increasing the amount of ECGF (Thornton et al., 1983). If it is indeed true that the mechanism behind steroid-induced inhibition or enhancement of growth lies in the interaction of the steroid with the growth factor, then increased concentrations of ECGF could play a role in any growth response to exogenously administered steroids.

Indirect support for steroid induced enhancement of proliferation comes from the work of Jarvelainen et al. (1985), who found that DEX and HC stabilized the monolayer

of EC, strengthening the adhesion of cells to the extracellular matrix, reducing their susceptibility to separation by enzymatic agents. Stabilization of the structural integrity of the endothelial cellular monolayer might render the cells less susceptible to factors that would inhibit growth via several mechanisms. Pursuant to this concept, since an increase of migratory activity precedes proliferation of cells in stimulated cultures of EC (Sholley et al., 1977), then the steroid induced stabilization of the monolayer observed with steroid treatment could play a role in the inhibition of proliferation by preventing the antecedent migration or chemokinesis.

Since angiotensin converting enzyme (ACE) activity is increased in steroid-treated cultures of EC (Mendelsohn et al., 1982a, 1982b; Lam et al., 1985), an *in vivo* role may exist for this phenomenon in steroid induced inhibition of cellular proliferation. Since the ACE converts angiotensin I to angiotensin II, the latter a potent vasoconstrictor, an *in vivo* correlation can be theorized. ACE induced vasoconstriction might constrict vessels *in vivo*, altering the cellular surface area, and consequently modifying the character of steroid or growth factor binding sites, making the cells less sensitive to

positive growth factors. Thus vasoconstriction would support the phenomenon of angiostasis.

The role of extracellular matrix (ECM) is an additional variable that should be explored in examining factors which may be operative in effecting *in vivo* anti-angiogenesis. Concerning this, three studies are relevant. Piovella *et al.* (1982) found that DEX increased the amount of fibronectin matrix in cultured HUVEC, and suggested that the phenomenon was responsible for better spreading of the cells on the culture surface. Whether this increased spreading affects cellular proliferation is not known. Longenecker *et al.* (1983) reported that the FGF dependent DEX induced inhibition of cellular growth in BAEC could be abolished by employing ECM derived from bovine aortic smooth muscle cells. Whether the gelatin matrix used in our culture system or the ECM laid down by HUVEC affects the response of the cells to steroid treatment is unclear, but certainly is a factor that deserves further consideration. Lastly, Ingbar *et al.* (1986) stated that basement membrane dissolution played a role in anti-angiogenesis mediated by angiostatic steroids *in vivo*. However, exactly when this degradation of basement membrane occurred in the sequence of events in

steroid induced angiostasis was not pinpointed in their study.

It must be remembered that the steroid responses seen in the present *in vitro* studies were observed during relatively acute treatment periods, and, as pointed out by Longenecker *et al.* (1983), might differ significantly from responses which might ensue due to more chronic exposure of EC to excess glucocorticoids, such as would occur in cells treated throughout a culture lifespan. They speculated that responses of the endothelium *in vivo*, exposed to exogenous glucocorticoids for months or years, would also be potentially different from those seen in cultures treated with steroids in more acute time periods. Perhaps also, in the *in vivo* situation, a homeostatic environment prevails in the endothelium, that is, there is a balance between growth facilitation and growth inhibition, and the response to physiological doses of glucocorticoids might be such so as to provide conditions favorable to the maintenance of endothelial integrity.

The following major findings in the present study regarding the effect of heparin-corticosteroids on HUVEC can be summarized as follows:

1. Both DEX and HC were inhibitory to growth of HUVEC when EtOH was used as a solvent and fully supplemented media was employed. The fact that these cultures were fed twice with steroids prior to harvest might have potentiated the effects of DEX and HC upon the growth of HUVEC.

2. When a full supplement of CM-199 was used as the media and DMSO was used as a solvent, there was a tendency toward enhancement of cellular growth when DEX was used.

3. The cellular and population growth enhancement seen when HC was used (DMSO solvent) was significant and was sustained when absorbed serum was used in the media.

4. Employing absorbed serum in the media, treatment with DEX and DHEA, but not HC, led to inhibition of cellular and population growth. The inhibition seen when absorbed serum is used may not be due solely to corticosteroid intervention.

5. Although gender does not appear to be a factor in the proliferative response of HUVEC to corticosteroid and heparin treatment, there was a trend for female EC to display better growth rates than their male counterparts.

6. Using a reduced serum component (1% FBS), inhibition of cellular and population growth was observed following treatment with DEX, but this was not always significant.

7. There was a trend for higher doses of DEX to effect cellular and population growth inhibition, while lower doses effected cellular and population growth enhancement. This was true for HUVEC grown in absorbed media and for female HUVEC.

8. There was a trend for DEX to effect an early inhibition of cellular and population growth in treated HUVEC, an inhibition that may well be compensated for by a subsequent cellular and population growth recovery during the later stages of culture.

9. Significant inhibition of cell colony formation in HUVEC in response to DEX administration was observed. Since cell colony experiments are conducted with a very low density of cells (1,000 cells/T-25 flask), it may well

be that low density growth of HUVEC is inhibited by steroid treatment, while higher density growth is facilitated. Alternatively, clonal assay may be a more sensitive method than mass culture for detecting suppressive effects on sensitive cells.

10. Cytoflurometric analysis of HUVEC treated with DEX in the presence of heparin indicated that this treatment increased cellular size and increased the number of EC in the S and/or G2-M phases of the cell cycle in early passage HUVEC.

Our experiments utilizing corticosteroids and heparin in cultures of EC demonstrated that treatment did not always decrease proliferation as postulated on the basis of in vivo findings (Folkman et al., 1983; Crum et al., 1985); by contrast DEX and HC sometimes caused an increase in proliferation. It might well be that the mechanism for inhibition of angiogenesis by angiostatic steroids observed in vivo does not involve inhibition of endothelial cell proliferation, but rather entails some alternative process, such as the induction of capillary basement membrane dissolution, as suggested by Ingber et al. (1986). But even in their work, it was not possible to conclude definitively that basement membrane alterations were a primary, rather than secondary effect;

basement membrane degradation might be a primary effect of corticosteroid treatment, or perhaps secondary to the loss of endothelial cell viability, the latter effected by steroid-induced growth inhibition. Studies such as the present one, designed to elucidate the effect of heparin-corticosteroid administration on the proliferation of HUVEC, hopefully may have assisted in clarifying their point to a somewhat greater degree.

It is unknown whether cellular migration in HUVEC is affected by corticosteroid treatment. Since endothelial cellular migration precedes proliferation both *in vitro* (Sholley *et al.*, 1977) and *in vivo* (Sholley *et al.*, 1984), factors which affect endothelial cellular migration may indeed by important in determining the response of HUVEC to heparin-corticosteroid treatment. Although both heparin and ECGF have been found to be chemotactic for HUVEC (Maciag *et al.*, 1984), the effect of corticosteroids upon directed migration of EC has not been studied. Migration assays utilizing various corticosteroids as possible chemotactic stimuli would provide information regarding any steroid mediated mechanisms involving endothelial cellular movement, which could greatly influence cellular proliferation *in vitro*.

Also, in this vein, it is known that vasodilation precedes both migration and proliferation of EC during *in vivo* angiogenesis (Folkman, 1984). Perhaps the inhibition of prostacyclin (a potent vasodilator) activity in cultured EC by corticosteroids (Lewis *et al.*, 1986; Deuterina & Walker, 1986) may be partially responsible for the anti-angiogenic effect of these steroids observed *in vivo*.

Additional studies, designed to elucidate further the role of corticosteroids upon either endothelial cellular growth or the endothelial basement membrane integrity, could provide answers to these remaining questions concerning corticosteroid mediated modifications in the vascular system, and offer more rationale for the use of corticosteroids in diseases where angiogenesis is a prominent feature.

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Appendix

Figure 1a. Effect of dexamethasone (DEX) on the growth of HUVEC (alcohol solvent). T-25 flasks were plated at standard density (1.25 x 10^5 cells/flask). Cells in the first through third passage were used. The cultures were fed with medium containing DEX dissolved in EtOH on the second and fourth or fifth day after plating. The final concentration of EtOH in the steroid containing media was 0.2% for 1 μM DEX and 0.1% for 0.5 μM DEX. For each experiment, cells were harvested and counted in quadruplicate in a hemocytometer on the seventh day after plating. The results shown are the combined pool of four separate experiments; each bar represents the cell counts from 12 flasks, with the standard error of the mean (SEM) drawn above the bar. Dexamethasone treatment has resulted in an inhibition of cellular growth.

Figure 1a. Effect of Dexamethasone on Growth of HUVEC (Alcohol Solvent)

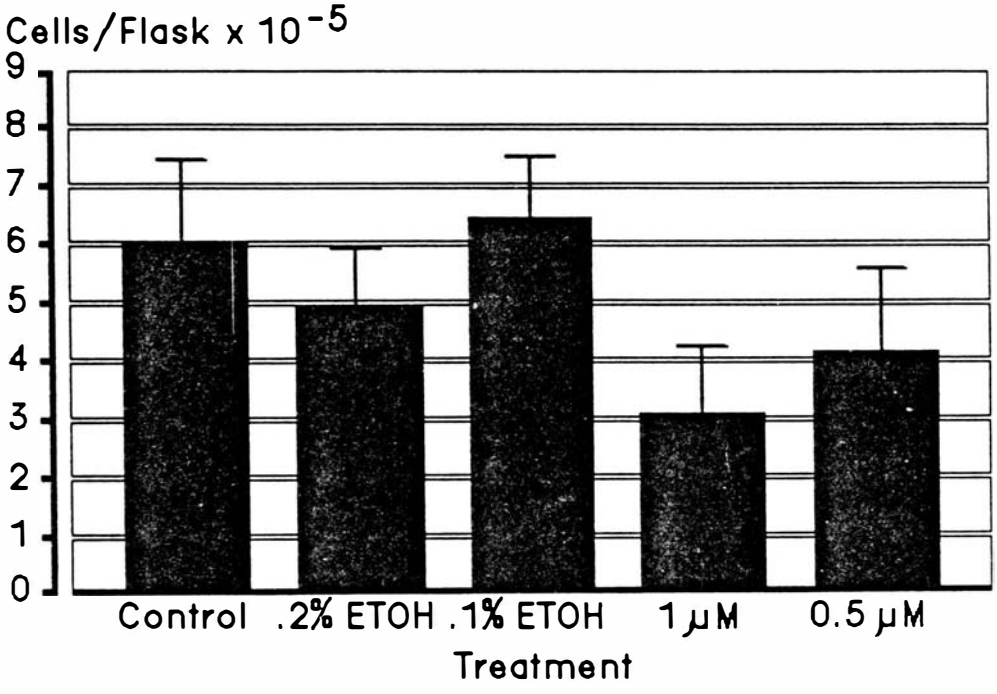


Figure 1b. Effect of dexamethasone on population growth (alcohol solvent). These bar graphs demonstrate the population doubling characteristics of HUVEC for the corresponding steroid doses and cell number data found in Figure 1a. Each bar represents the mean population doubling level from 12 flasks from four separate experiments, with the SEM above the bar.
* Significantly different from control, 0.2% ETOH, and 0.1% ETOH, ($p < 0.05$).

Figure 1b. Effect of Dexamethasone on Population Growth (Alcohol Solvent)

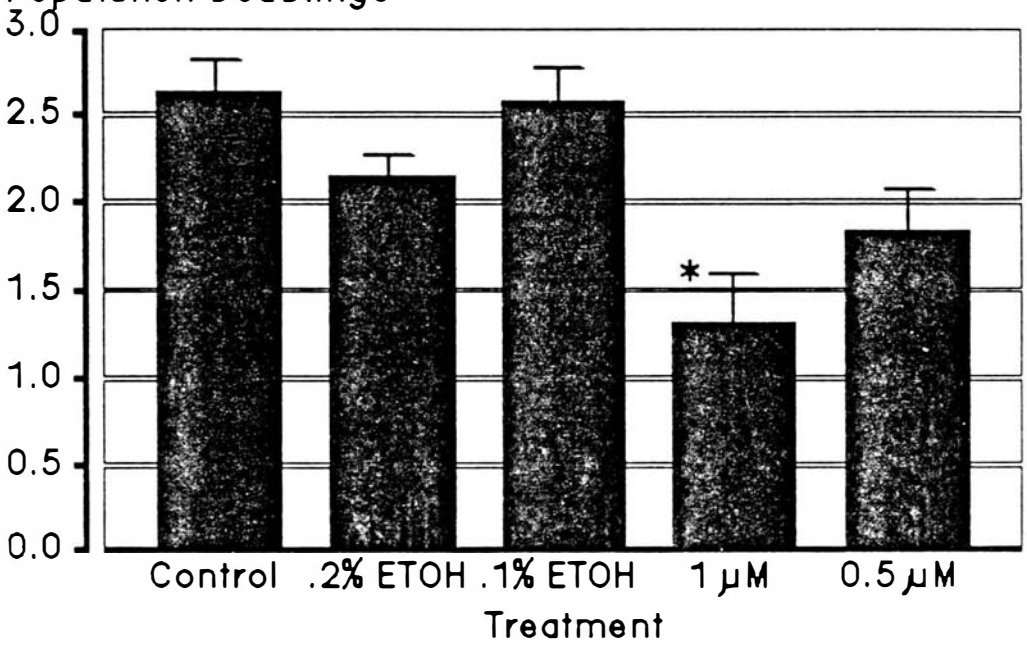


Figure 2a. Effect of hydrocortisone (HC) on growth of HUVEC (alcohol solvent). T-25 flasks were plated at standard density (1.25 x 10^5 cells/flask). Cells in the first through third passages were used. The cultures were fed with media containing HC dissolved in EtOH on the second and fourth day after plating. The final concentration of EtOH in the steroid containing media was 0.2%. For each experiment, cells were harvested and counted in quadruplicate with a hemocytometer on the seventh day after plating. The results shown are the combined pool of three separate experiments. Each bar represents the mean cell counts from nine flask determinations, with the SEM drawn above the bar.

Figure 2a. Effect of Hydrocortisone on Growth of HUVEC (Alcohol Solvent)

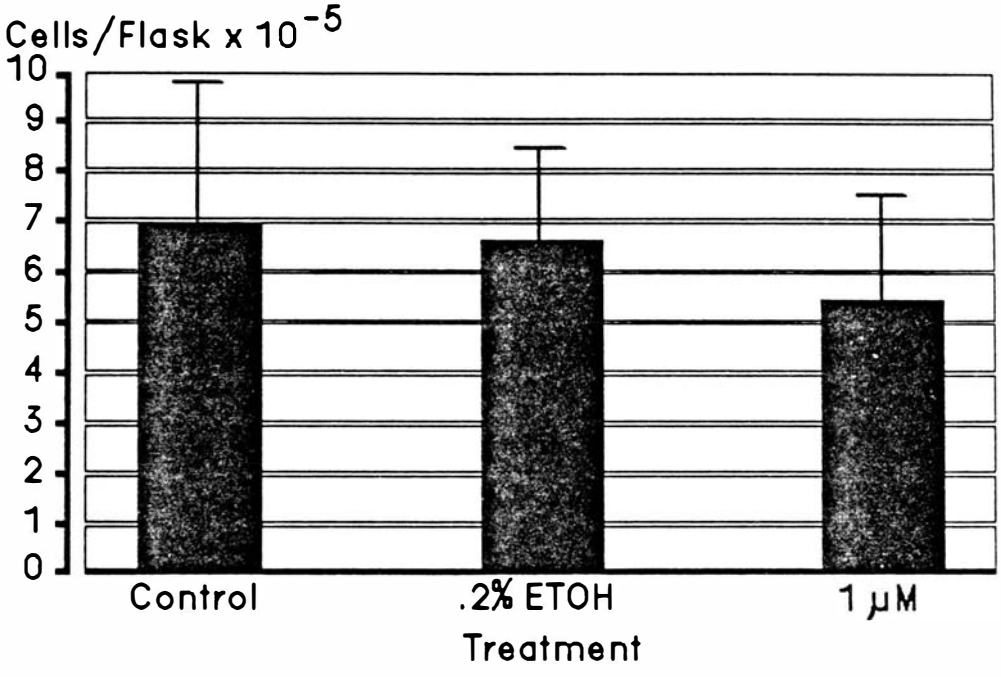


Figure 2b. Effect of hydrocortisone (HC) on population growth (alcohol solvent). The bars indicate the growth characteristics of HUVEC for the corresponding steroid doses and cell number data given in Figure 2a. Each bar represents the mean population doubling value from nine separate determinations, with the SEM drawn above the bar. The population growth inhibition effected by HC was not significant.

Figure 2b. Effect of Hydrocortisone on Population Growth (Alcohol Solvent)

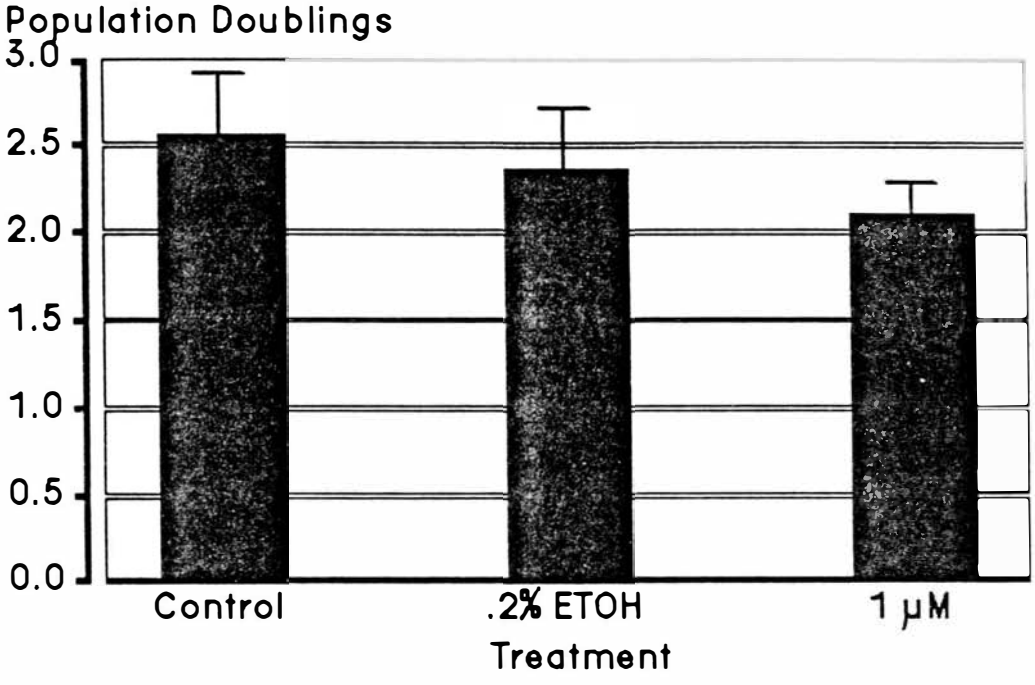


Figure 3a. Effect of dexamethasone (DEX) on growth of HUVEC (DMSO solvent). T-25 flasks were plated at standard density with second or fourth passage HUVEC. On the second day after plating, the culture media was removed and replaced with fully supplemented media (CM 199) containing DEX dissolved in dimethylsulfoxide (DMSO) at the dosages indicated. The final media concentration of DMSO for DMSO control and all steroid treated cultures was 0.1%. Cells were harvested and counted at seven days. The results shown are the combined pool of two experiments; each bar represents the mean cell counts from six separate determinations, with the SEM above the bar. Growth enhancement is evident.

Figure 3a. Effect of Dexamethasone on Growth of HUVEC (DMSO Solvent)

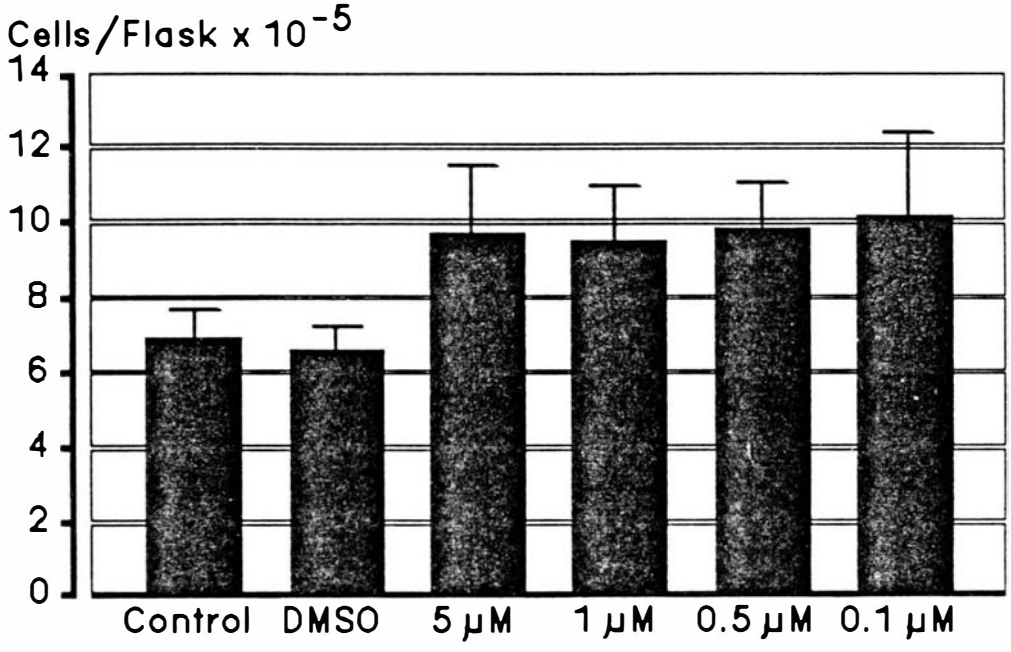


Figure 3b. Effect of dexamethasone on population growth (DMSO solvent). This graph depicts the growth characteristics of HUVEC for the corresponding steroid doses and cell number data given in Figure 3a. Each bar represents the mean population doubling value calculated from six separate determinations, with the SEM above the bar. A non-dose response increase in population growth was effected by all doses of DEX employed. * Significantly different from DMSO control, $p < 0.05$.

Figure 3b. Effect of Dexamethasone on Population Growth (DMSO Solvent)

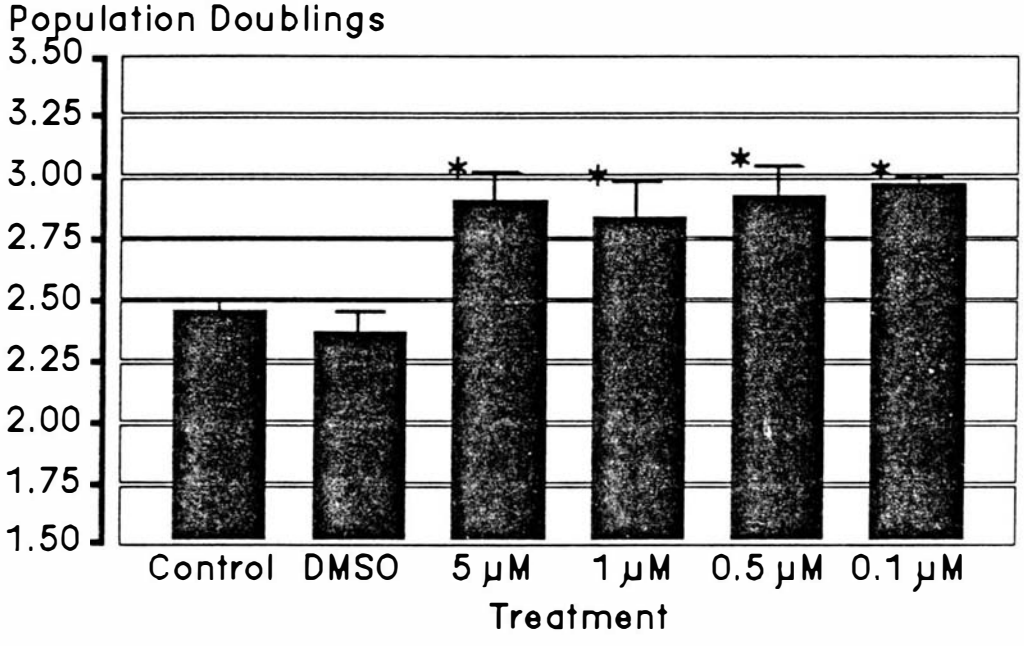


Figure 4a. Effect of hydrocortisone (HC) on growth of HUVEC. (DMSO solvent). T-25 flasks were plated at standard density with second or fourth passage HUVEC. On the second day after plating, the culture media was removed and replaced with fully supplemented media (CM 199) containing HC dissolved in DMSO at the dosages indicated. The final media concentration of DMSO for DMSO control and all steroid-treated cultures was 0.1%. Cells were harvested and counted at seven days. The results shown are the combined pool of two experiments; each bar represents the mean cell counts from six separate determinations, with the SEM above the bar. An increase in cellular growth occurred with all HC doses employed.

Figure 4a. Effect of Hydrocortisone on Growth of HUVEC (DMSO Solvent)

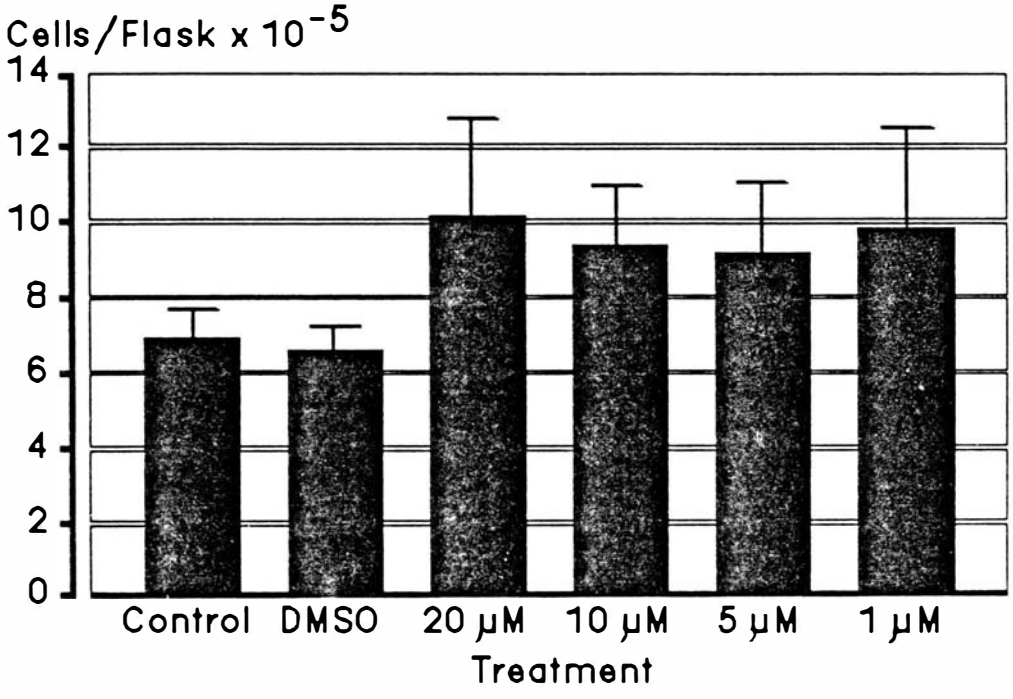


Figure 4b. Effect of hydrocortisone (HC) on population growth (DMSO solvent). This graph depicts the growth characteristics of HUVEC for the corresponding steroid doses and cell number data displayed in Figure 4a. Each bar represents the mean population doubling level calculated from the six separate determinations, with the SEM above the bar. A non-dose response enhancement of cellular growth was detected by all HC doses employed.
* Significantly different from DMSO control, $p < 0.05$.

Figure 4b. Effect of Hydrocortisone on Population Growth (DMSO Solvent)

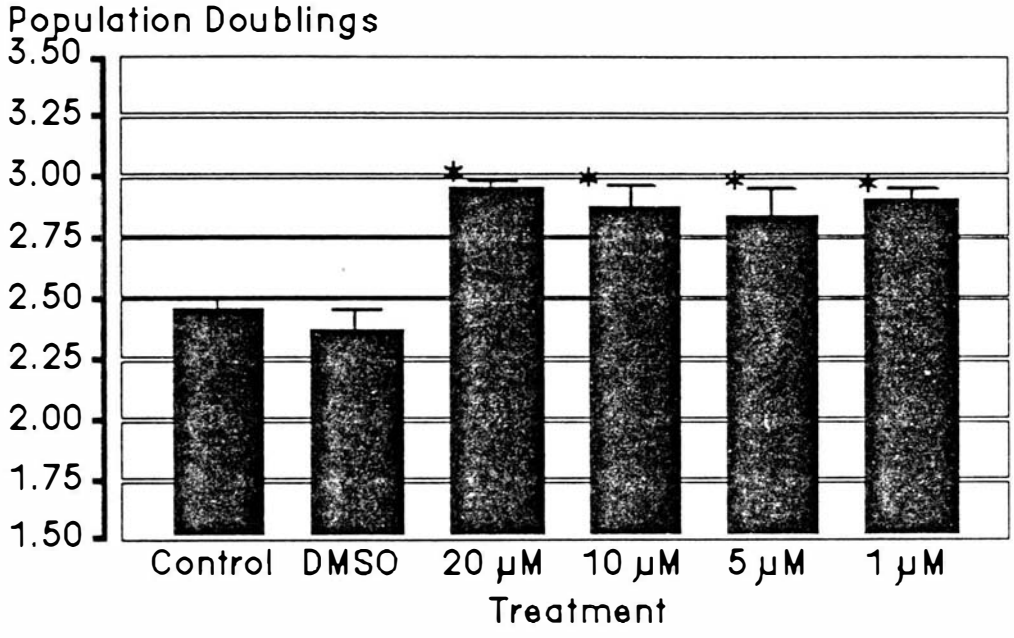


Figure 5a. Effect of DHEA on growth of HUVEC (DMSO solvent). T-25 tissue culture flasks were plated at standard density using second or fourth passage HUVEC. On he second day after plating, the culture media was removed and replaced with fully supplemented media (CM 199) containing DHEA dissolved in DMSO at the dosages indicated. The final concentration of DMSO for DMSO control and all steroid treated cultures was 0.1%. Cells were harvested and counted at seven days. The results shown are the combined pool of two such separate experiments; each bar represents the mean cell counts from six separate determinations, with the SEM above the bar. Again steroid-induced enhancement of cellular growth is seen.

Figure 5a. Effect of DHEA on Growth of HUVEC (DMSO Solvent)

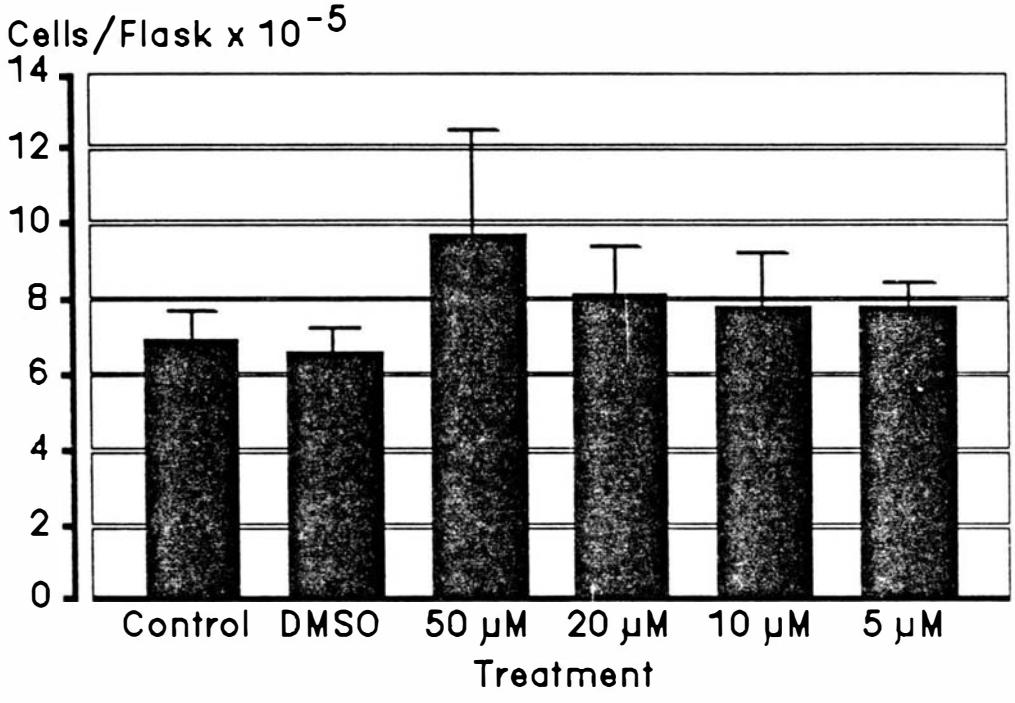


Figure 5b. Effect of DHEA on population growth (DMSO solvent). This graph depicts the growth characteristics of HUVEC for the corresponding steroid doses and cell number data given in Figure 5a. Each bar represents the mean population doubling value calculated from six separate determinations, with the SEM above the bar. The increase in population growth was significant in three of the doses employed. * Significantly different from DMSO control, $p < 0.05$.

Figure 5b. Effect of DHEA on Population Growth (DMSO Solvent)

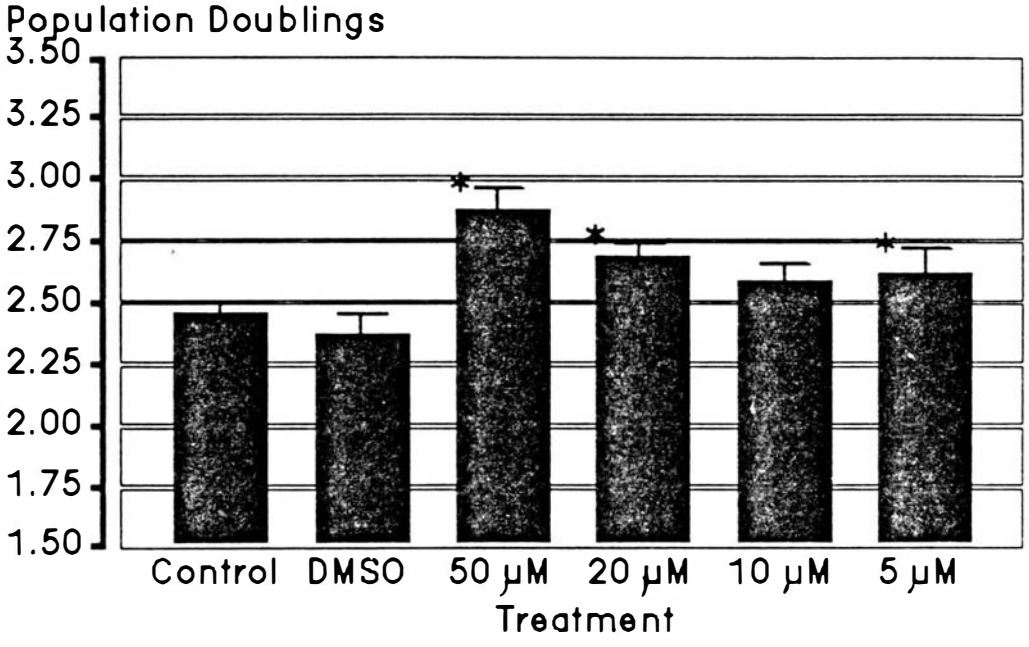


Figure 6a. Effect of dexamethasone (DEX) on growth of HUVEC (unabsorbed vs. absorbed FBS). The graphs compare the effects of media containing charcoal filtered (absorbed) FBS with those of media containing unfiltered (unabsorbed) FBS. T-25 culture flasks were plated at standard density with fourth passage HUVEC. On the second day after plating, CM-199 was replaced with either CM-199 made from absorbed FBS or CM-199 made from unabsorbed FBS, plus the appropriate DEX dosage. For both absorbed and unabsorbed media, the final concentration of DMSO in control or steroid groups was 0.1%. Cells were harvested and counted on the seventh day. The bars represent the mean cell counts of triplicate flasks, given along with the SEM. The increased growth with unabsorbed FBS-containing media is obvious. Also observed is a decreased growth in steroid treated cultures when absorbed FBS was used.

Figure 6a. Effect of Dexamethasone on Growth of HUVEC (Absorbed vs. Unabsorbed FBS)

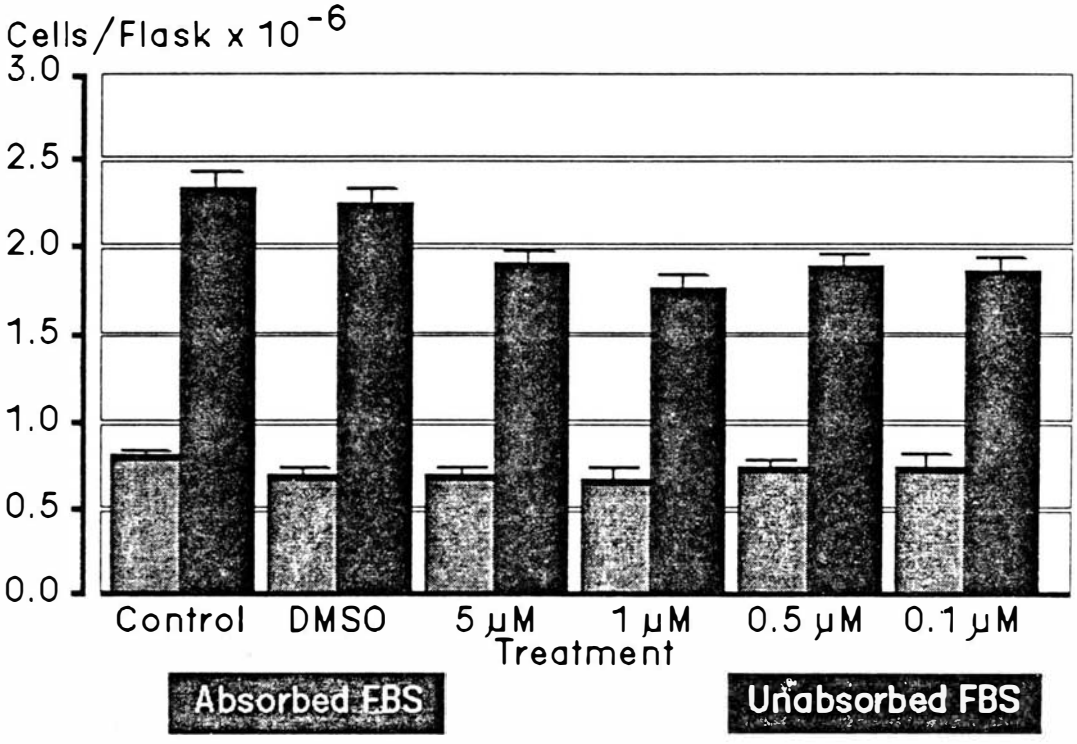


Figure 6b. Effect of dexamethasone on population growth (unabsorbed versus absorbed FBS). The graphs compare the effects of unabsorbed and absorbed FBS containing media on the population doubling characteristics of HUVEC for the corresponding to the data given in Figure 6a. Each bar represents the population doubling determined from triplicate flasks at the indicated FBS parameter, with the SEM above the bar. Note the greater difference between the control and DMSO control group when absorbed FBS was used. Also, fewer population doublings were achieved with the absorbed FBS in comparison to the unabsorbed FBS.
* Significantly different from unabsorbed DMSO control, $p < 0.05$.

Figure 6b. Effect of Dexamethasone on Population Growth (Absorbed vs. Unabsorbed FBS)

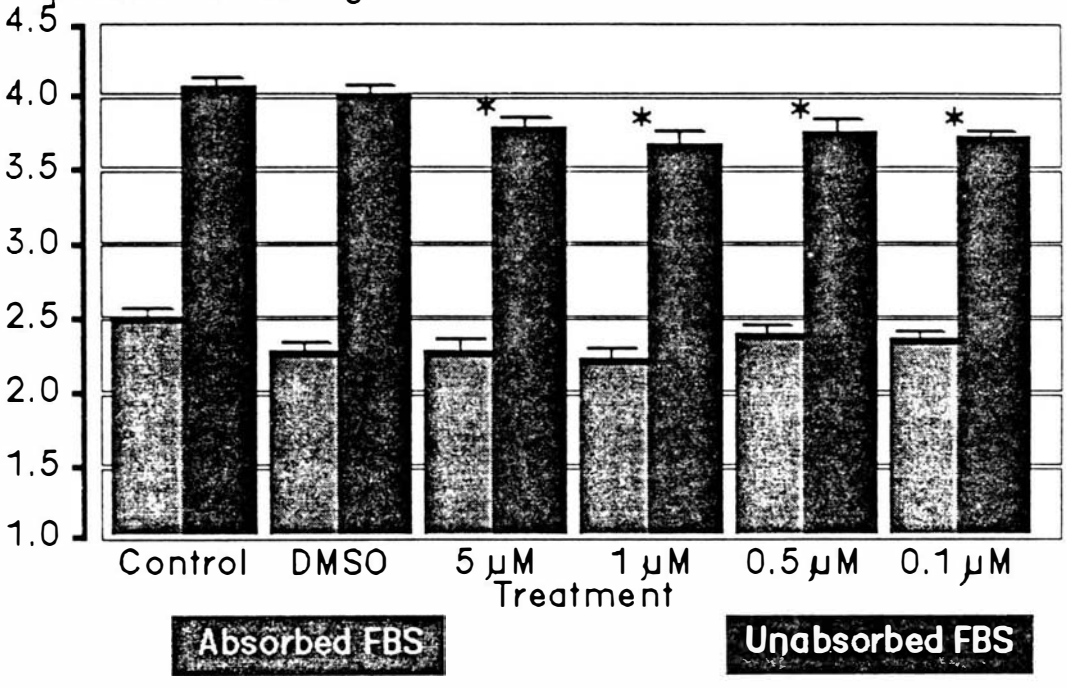


Figure 7a. Effect of dexamethasone (DEX) on growth of HUVEC (absorbed FBS). The cell counting experiments graphed in Figure 6a were repeated with DEX in a non-comparison experimental paradigm; only absorbed FBS was used. Second passage HUVEC were plated in T-25 flasks at standard density using regular CM-199. On the second day after plating, cultures were fed with culture media made from FBS from which endogenous steroids had been removed by double charcoal filtration, plus DEX dissolved in DMSO at the dosages indicated. The final concentration of DMSO in the DMSO control and all steroid flasks was 0.1%. The cells were harvested and counted on the seventh day. The bars represent the mean cell counts of triplicate determinations, with the SEM above the bar.

Figure 7a. Effect of Dexamethasone on Growth of HUVEC (Absorbed FBS)

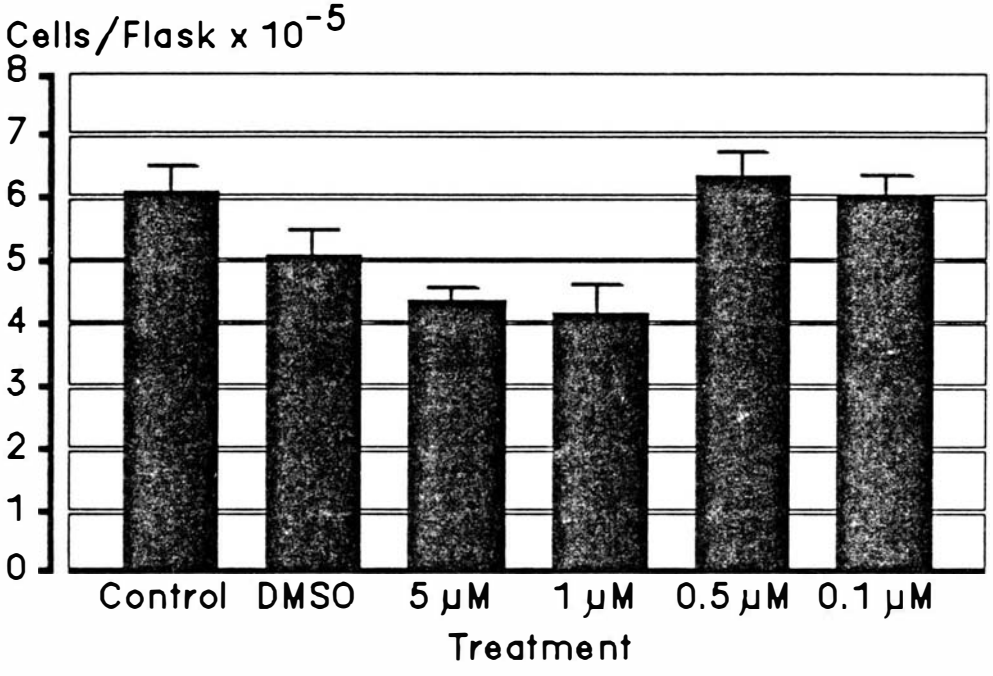


Figure 7b. Effects of dexamethasone (DEX) on population growth (absorbed FBS). The graph indicates the growth characteristics of HUVEC grown in absorbed FBS-containing media for the corresponding steroid doses and cell number data given in Figure 7a. Each bar represents the mean population doubling determined from triplicate flasks, with the SEM above the bar. Neither the population doubling inhibition effected by the higher DEX doses (5 μM, 1 μM) nor the population growth enhancement effected by the lower DEX doses (0.5 μM, 0.1 μM) was significant. The relatively greater difference between the control and DMSO control group when absorbed serum was used is again noted.

Figure 7b. Effect of Dexamethasone on Population Growth (Absorbed FBS)

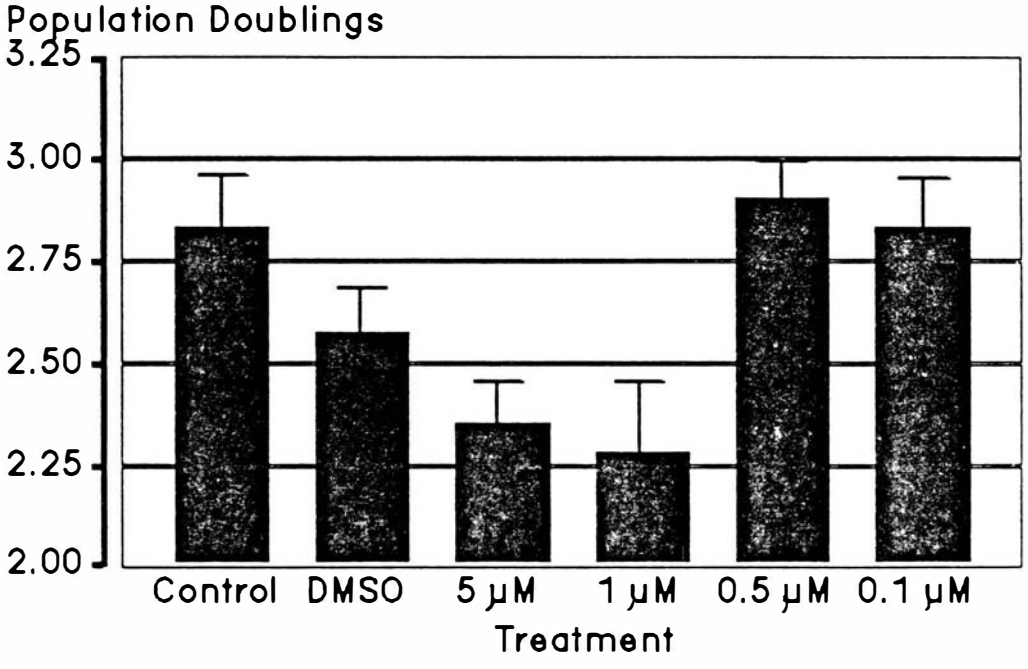


Figure 8a. Effect of hydrocortisone (HC) on growth of HUVEC (absorbed FBS). The cell counting experiments using absorbed serum graphed in Figure 6a were repeated with HC in a non-comparison paradigm; only absorbed FBS was used. Second passage HUVEC were plated in T-25 flasks at standard density. On the second day after plating, cultures were fed with culture media made from FBS from which endogenous steroids had been removed by charcoal filtration, plus DEX dissolved in DMSO at the dosages indicated. The final concentration of DMSO in the DMSO control and all steroid flasks was 0.1%. Cells were harvested and counted on the seventh day. The bars represent the mean cell counts of triplicate determinations, with the SEM above the bar. All doses led to growth facilitation.

Figure 8a. Effect of Hydrocortisone on Growth of HUVEC (Absorbed FBS)

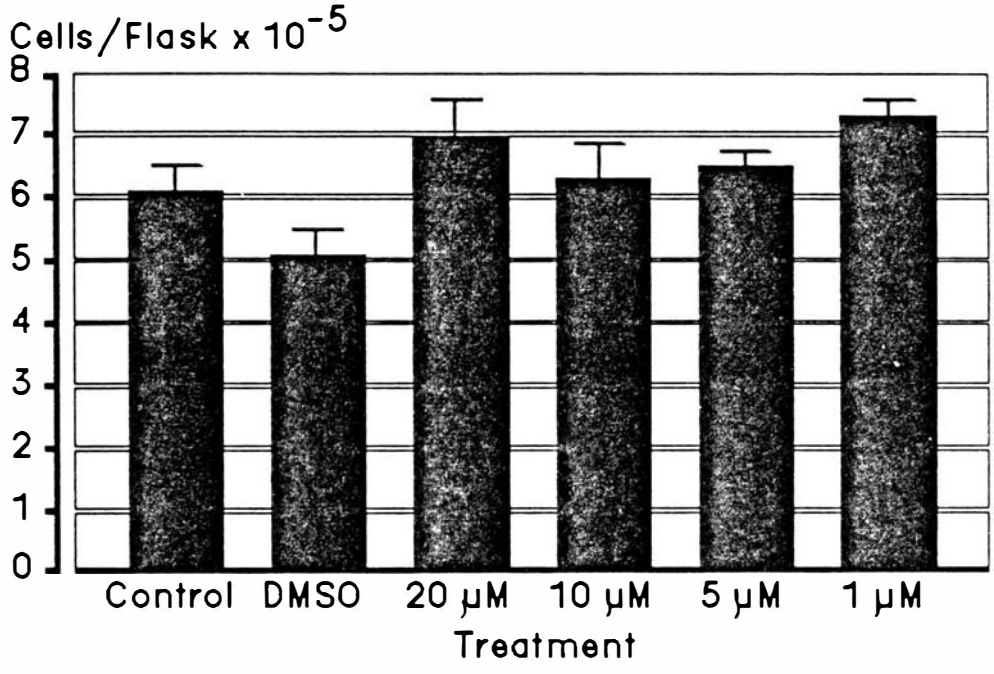


Figure 8b. Effect of hydrocortisone (HC) on population growth (absorbed FBS). The graph depicts the growth characteristics of HUVEC grown in media with absorbed FBS corresponding to the given in Figure 8a. Each bar represents the population doubling determined from triplicate flasks, with the SEM above the bar. In contrast to DEX, all four doses of HC employed effected growth enhancement. A relatively greater difference (n.s.) between the control and DMSO control group is again recognized. * Significantly different from DMSO control, $p < 0.05$.

Figure 8b. Effect of Hydrocortisone on Population Growth (Absorbed FBS)

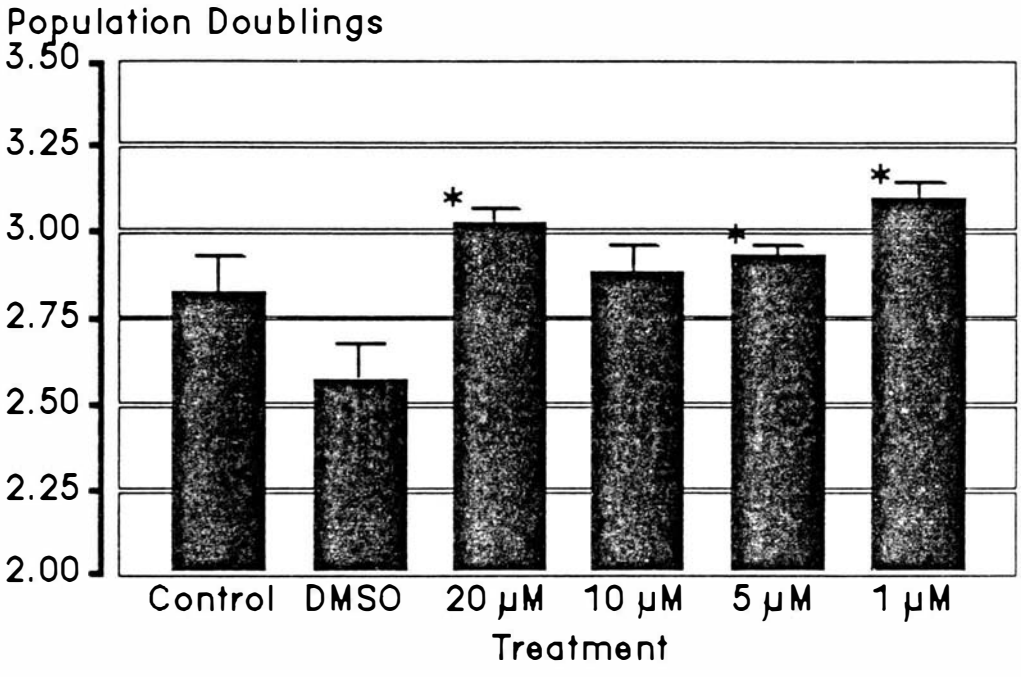


Figure 9a. Effect of DHEA on growth of HUVEC (absorbed serum). The cell counting experiments using absorbed serum graphed in Figure 6a were repeated once again with DHEA in a non-comparison experimental paradigm; only absorbed serum was used. Second passage HUVEC were plated in T-25 flasks at standard density. On the second day after plating, cultures were fed with culture media made from FBS from which endogenous steroids had been removed by charcoal filtration, plus DHEA dissolved in DMSO at the dosages indicated. The final concentration of DMSO in the DMSO control and all steroid flasks was 0.1%. Cells were harvested and counted on the seventh day. The bars represent the mean cell counts of triplicate determinations, with the SEM above the bar. The effect of DHEA on cellular growth when absorbed serum was used is negligible.

Figure 9a. Effect of DHEA on Growth of HUVEC (Absorbed FBS)

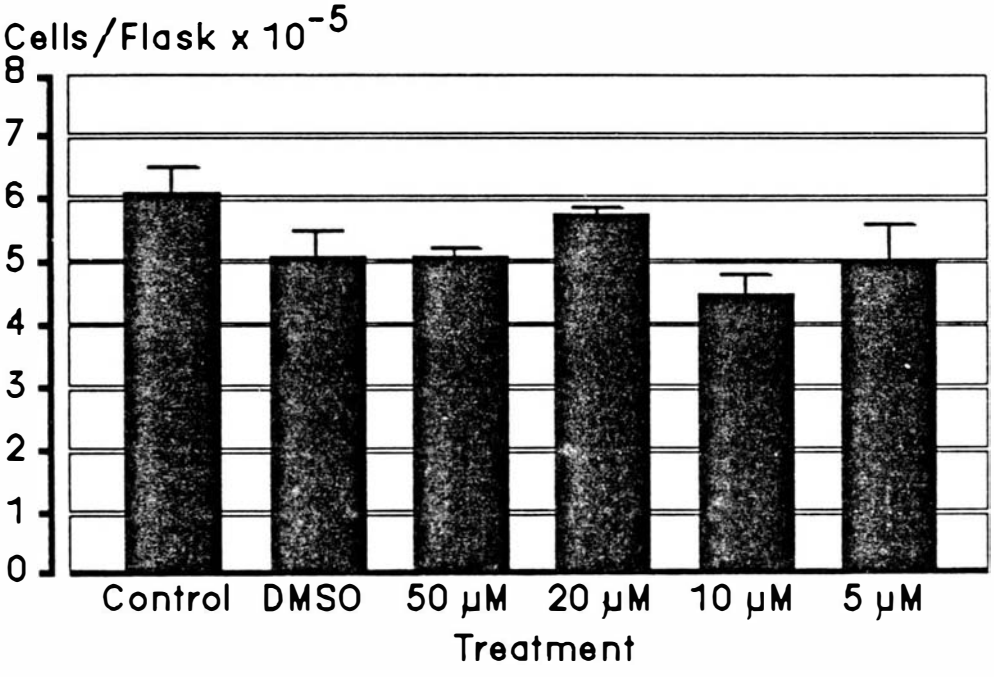


Figure 9b. Effect of DHEA on population growth (absorbed FBS). The graph depicts the growth characteristics of HUVEC grown in media containing absorbed FBS corresponding to the data given in figure 9a. Each bar represents the PD determined from triplicate flasks, with the SEM above the bar. As with DEX and HC above, the relatively greater difference between control and DMSO control groups when absorbed serum is used is noted. The effect of DHEA on population growth in HUVEC when absorbed serum was used is not significant.

Figure 9b. Effect of DHEA on Population Growth
(Absorbed FBS)

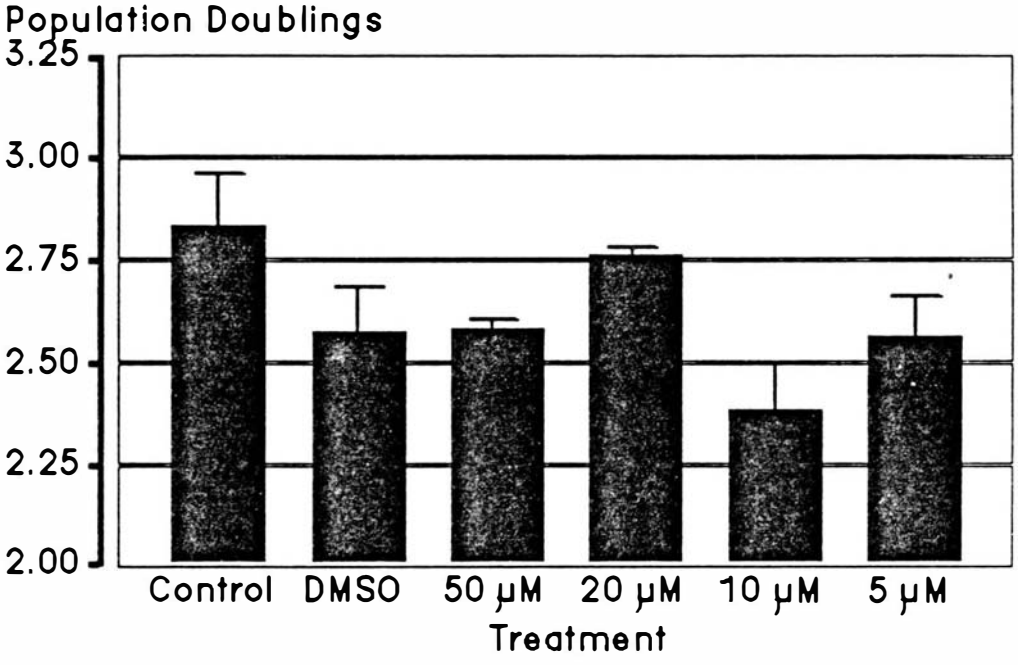


Figure 10a. Effect of dexamethasone (DEX) on growth of HUVEC (female cells). Second passage female HUVEC were plated in T-25 flasks at standard density. On the second day after plating, cultures were fed at a wider range of DEX doses as indicated. The final concentration of the DMSO solvent in the DMSO control and all treatment groups was 0.1%. Cells were harvested and counted on the sixth day. The bars represent the mean cell counts of triplicate flask determinations, with the SEM above each bar. Although not significant, the trend in female HUVEC was for higher doses of DEX to effect slight growth inhibition, while lower doses appeared to cause slight growth enhancement, compared to the DMSO control group.

Figure 10a. Effect of Dexamethasone on Growth of HUVEC (Female Cells)

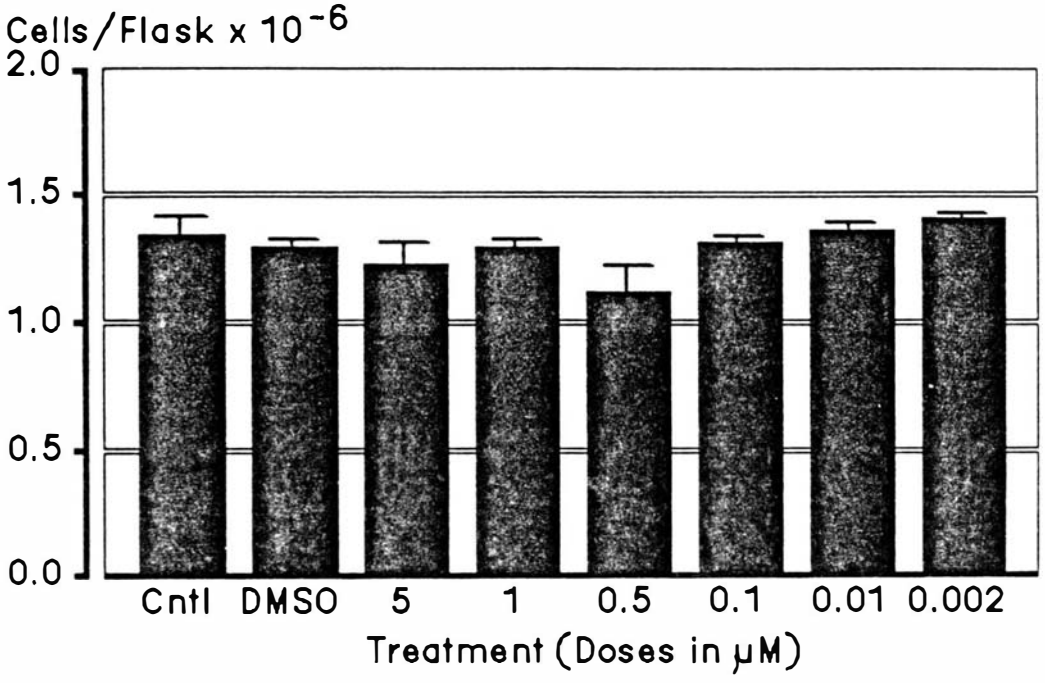


Figure 10b. Effect of dexamethasone (DEX) on population growth (female cells). These bars illustrate the population doubling characteristics of steroid-treated female HUVEC corresponding to the data given in Figure 10a. Each bar represents the mean population doubling from triplicate flask determinations, with the SEM above the bar. The population growth response was variable, but there was a trend for higher doses of DEX to cause slight growth inhibition, and the lower doses, enhancement in female cultures of HUVEC.

Figure 10b. Effect of Dexamethasone on Population Growth (Female Cells)

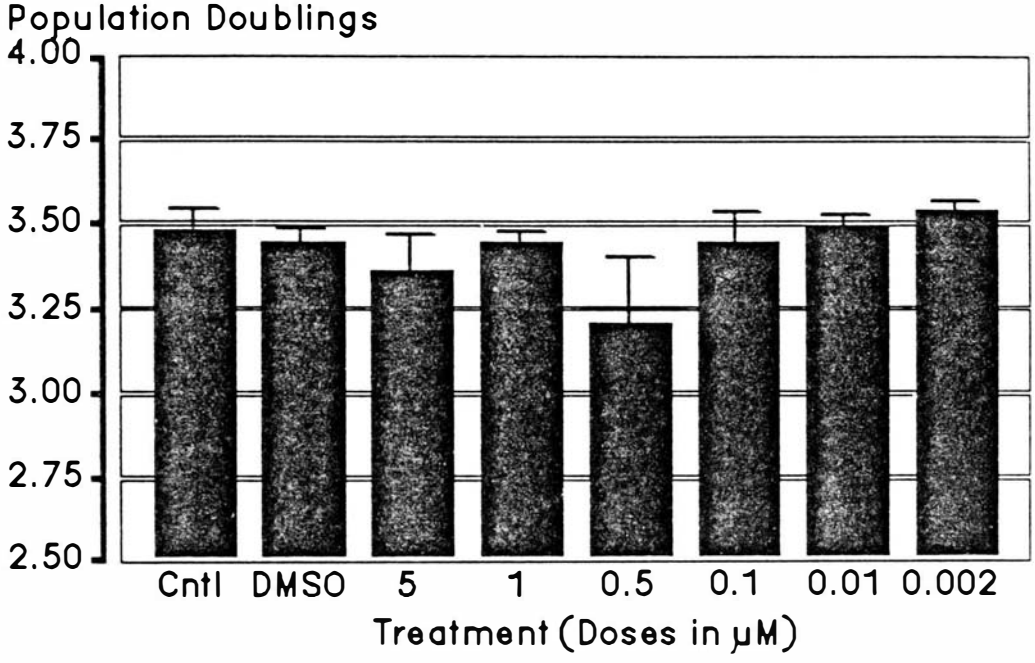


Figure 11a. Effect of dexamethasone (DEX) on growth of HUVEC (male cells). Second passage male HUVEC were plated at standard density. On the second day after plating, cultures were fed with DEX at the six dosages indicated. The final concentration of the DMSO solvent in the DMSO control and all treatment groups was 0.1%. Cells were harvested and counted on the sixth day. The bars represent the mean cell count of triplicate flask determinations, with the SEM above the bar. Lower doses of DEX caused slight growth enhancement, much the same as for female cells (Figure 10a), except for the slight inhibition at 0.002 µM DEX. Slight growth enhancement, rather than inhibition, occurred with higher steroid doses.

Figure 11a. Effect of Dexamethasone on Growth of HUVEC (Male Cells)

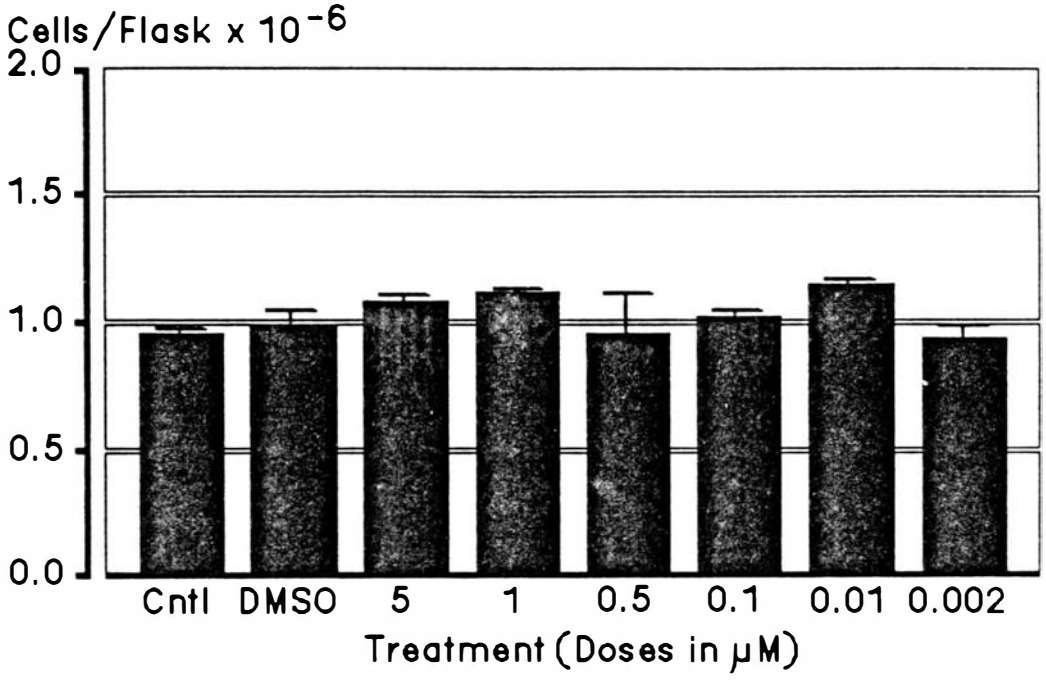


Figure 11b. Effect of dexamethasone (DEX) on population doublings (male cells). These bars illustrate the population doubling characteristics of male HUVEC, corresponding to the data given in Figure 11a. Each bar represents the population doubling from triplicate flask determinations, with the SEM above the bars. Although one dose of DEX (0.01 μM) effected a significant population growth enhancement, a lower dose (0.002 μM) was inhibitory. Also, high doses of DEX caused an enhancement of cellular growth. * Significantly different from DMSO control, control, and DEX at doses of 0.5 μM and .002 μM, $p < 0.05$.

Figure 11b. Effect of Dexamethasone on Population Growth (Male Cells)

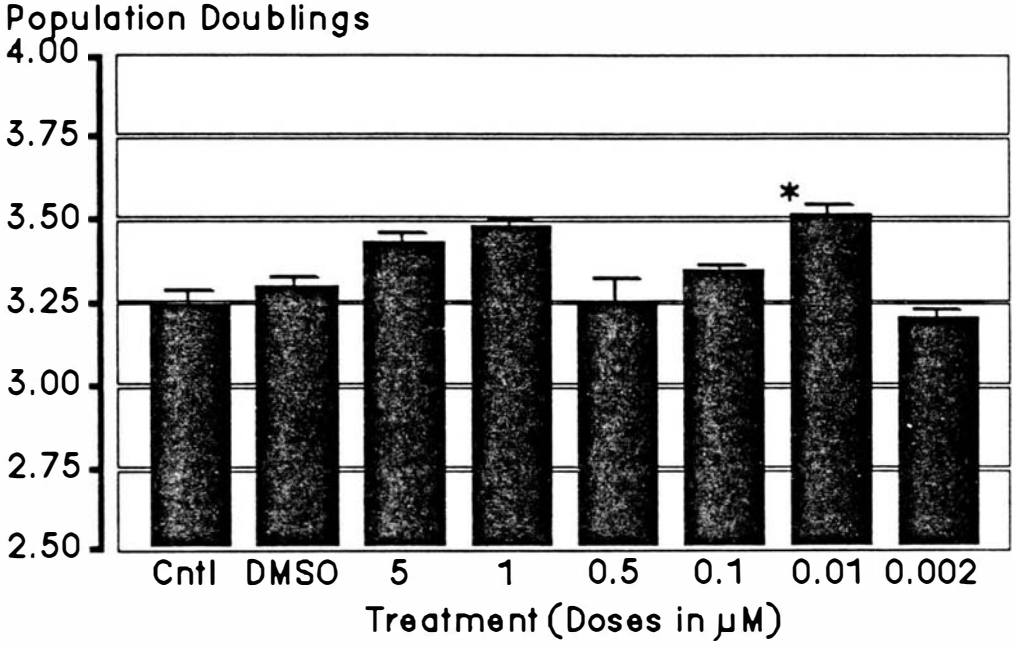


Figure 12a. Effect of dexamethasone (DEX) on growth of HUVEC (1% FBS). The bars depict the effect of six doses of DEX upon the growth of HUVEC when a reduced serum component in the culture medium was employed. Second passage HUVEC were plated in T-25 flasks at standard density using media made with 1%, instead of 20% FBS. All other media components were identical to CM-199. On the second day after plating, the cultures were fed with the appropriate DEX doses. The final concentration of DMSO solvent was 0.1% for DMSO control and all steroid flasks. Cells were harvested and counted on the seventh day. The bars represent the mean cell counts of triplicate flask determinations, with the SEM above the bar. Cellular growth inhibition occurred with all doses employed when 1% FBS was used in the culture media.

Figure 12a. Effect of Dexamethasone on Growth of HUVEC (1% FBS)

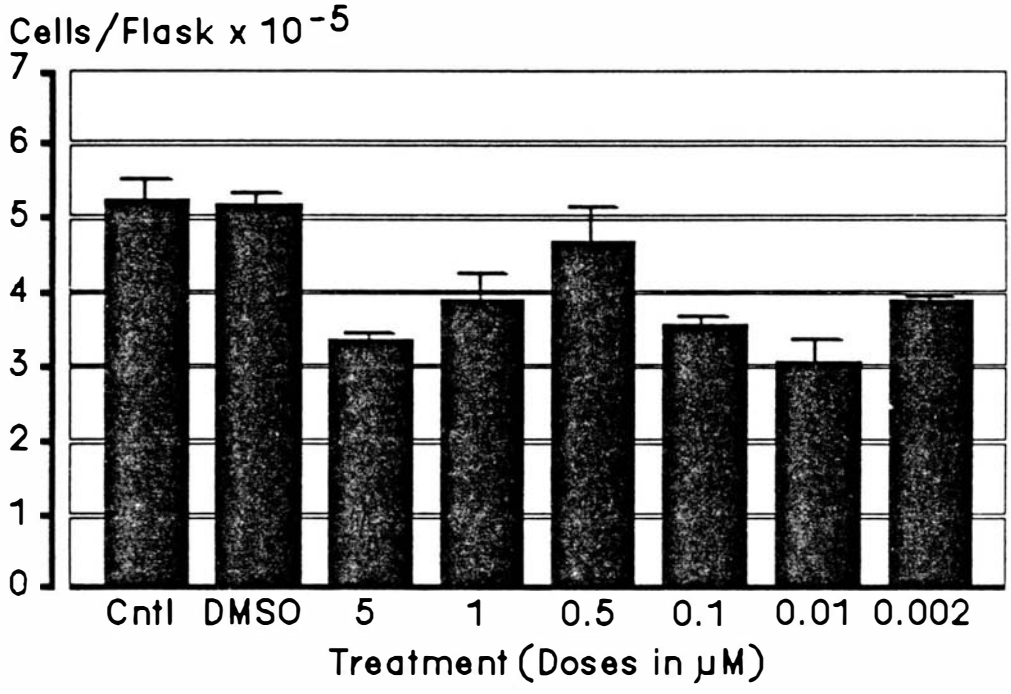


Figure 12b. Effect of dexamethasone (DEX) on population growth (1% FBS). The bars illustrate the growth characteristics of HUVEC corresponding to the data given in Figure 12a. Each bar represents the population doublings (PD) from triplicate flask determinations, with the SEM above the bar. There was a significant inhibition of population growth with all but one DEX dose (0.5 μM) when reduced serum was employed in the culture media.
* Significantly different from DMSO control, $p < 0.05$.

Figure 12b. Effect of Dexamethasone on Population Growth (1% FBS)

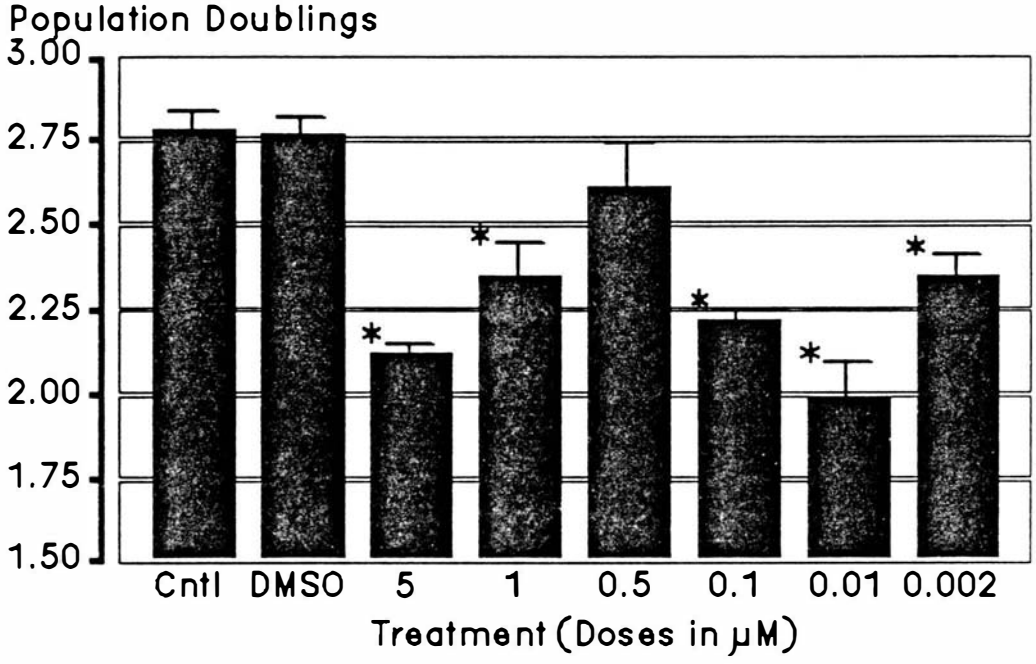


Figure 13a. Effect of dexamethasone (DEX) on the growth of HUVEC (1% vs. 20% FBS). These bars compare the effects of DEX in media containing reduced serum (1%) or a full serum component (20%). Fourth passage HUVEC were plated in T-25 flasks at standard density using media made with the designated serum concentrations. On the second day after plating, the cultures were fed with either 0.1 μM DEX or 0.01 μM DEX in the appropriate media. In either medium, the final concentration of DMSO in the DMSO control and DEX treated flasks was 0.1%. Cells were harvested and counted on the seventh day. The bars represent the mean cell counts of triplicate flask determinations, with the SEM above the bar. The decreased growth that occurred with the reduced serum component in the culture media is noted. Slight inhibition of cellular growth occurred in media with 1% FBS,, while an enhancement of cellular growth occurred in media with 20% FBS.

Figure 13a. Effect of Dexamethasone on Growth of HUVEC (1% Versus 20% FBS)

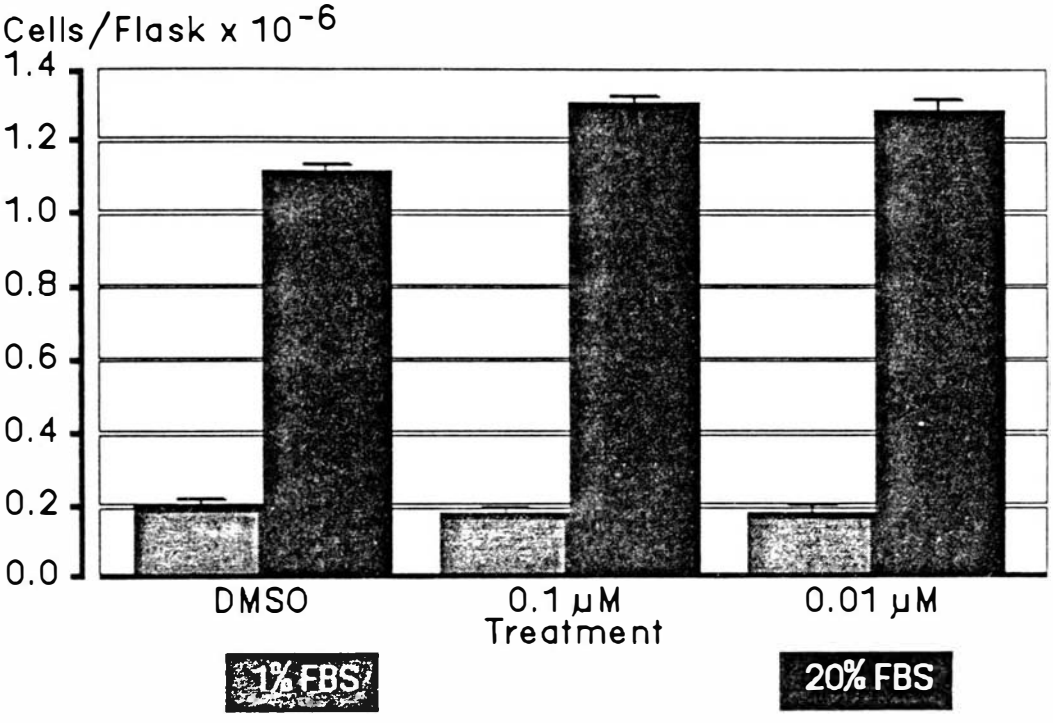


Figure 13b. Effect of dexamethasone (DEX) on population growth (1% vs. 20% FBS). The bars compare the effects of 1% and 20% FBS-containing media on the growth characteristics of steroid-treated HUVEC for the steroid doses and cell number data given in Figure 13a. Each bar represents the population doubling determined from triplicate flasks. In the reduced serum medium, DEX caused slight population growth inhibition, while the full serum supplement media caused a significant population growth enhancement. This finding was consistent for many experiments when full CM 199 was employed as the culture medium. * Significant from DMSO control, 20% FBS. ($p < 0.05$).

Figure 13b. Effect of Dexamethasone on Population Growth (1% Versus 20% FBS)

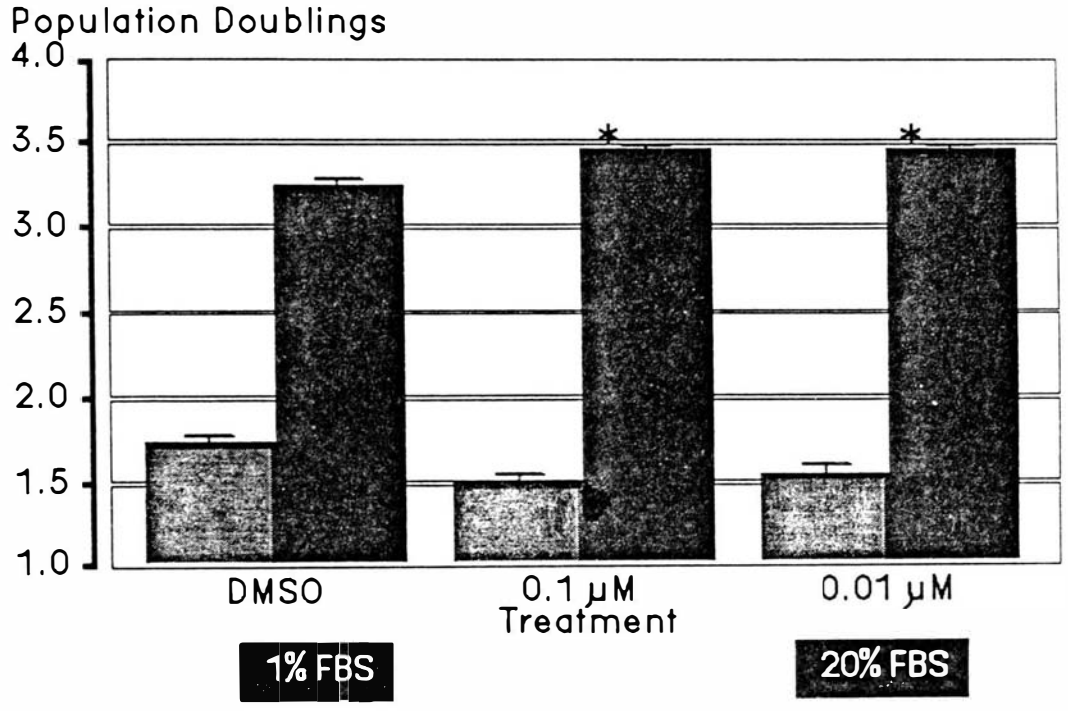


Figure 14a. Effect of dexamethasone (DEX) on growth of HUVEC (1% vs. 20% FBS). A comparison of the effects of media containing reduced serum with fully supplemented media was again performed with a wider DEX dose range. Third passage HUVEC were plated in T-25 flasks at standard density, using media with either 1% FBS or 20% FBS. On the second day after plating, the cultures were fed with the appropriate media containing DEX at the doses indicated. The final concentration of DMSO solvent was 0.1% in the DMSO control and all experimental flasks. Cells were harvested and counted on the seventh day. The bars represent the mean cell counts of triplicate flask determinations, with the SEM above the bars. The decreased cellular growth that occurred with the reduced serum component is again noted. The effect of DEX in the reduced media is inconstant; cellular growth enhancement occurred when 20% FBS containing media was used.

Figure 14a. Effect of Dexamethasone on Growth of HUVEC (1% Versus 20% FBS)

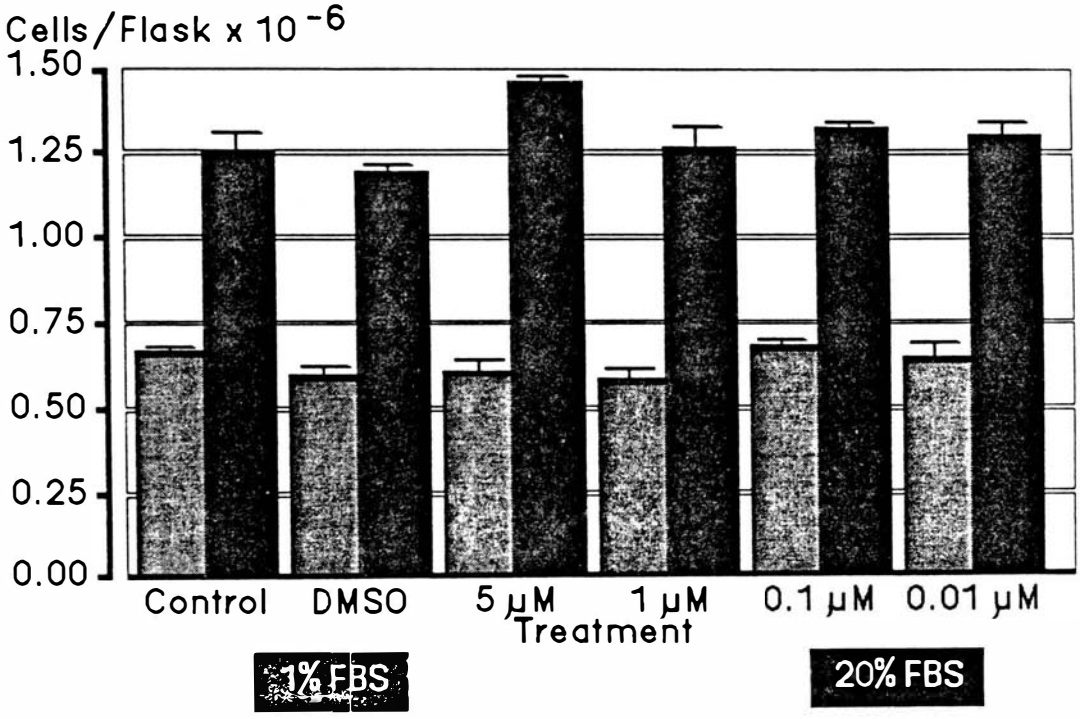


Figure 14b. Effect of dexamethasone (DEX) on population growth (1% vs. 20% FBS). The bars compare the growth in 1% FBS and 20% FBS-containing media for the corresponding steroid doses and cell number data given in Figure 14a. Each bar represents the population doubling value determined from triplicate flasks. As found previously (Figure 13b), population growth enhancement occurred when medium with a full (20%) serum supplement was used. The previously found growth inhibition with 1% FBS media is not demonstrated here. * Significantly different from DMSO control, $p < 0.05$.

Figure 14b. Effect of Dexamethasone on Population Growth (1% Versus 20% FBS)

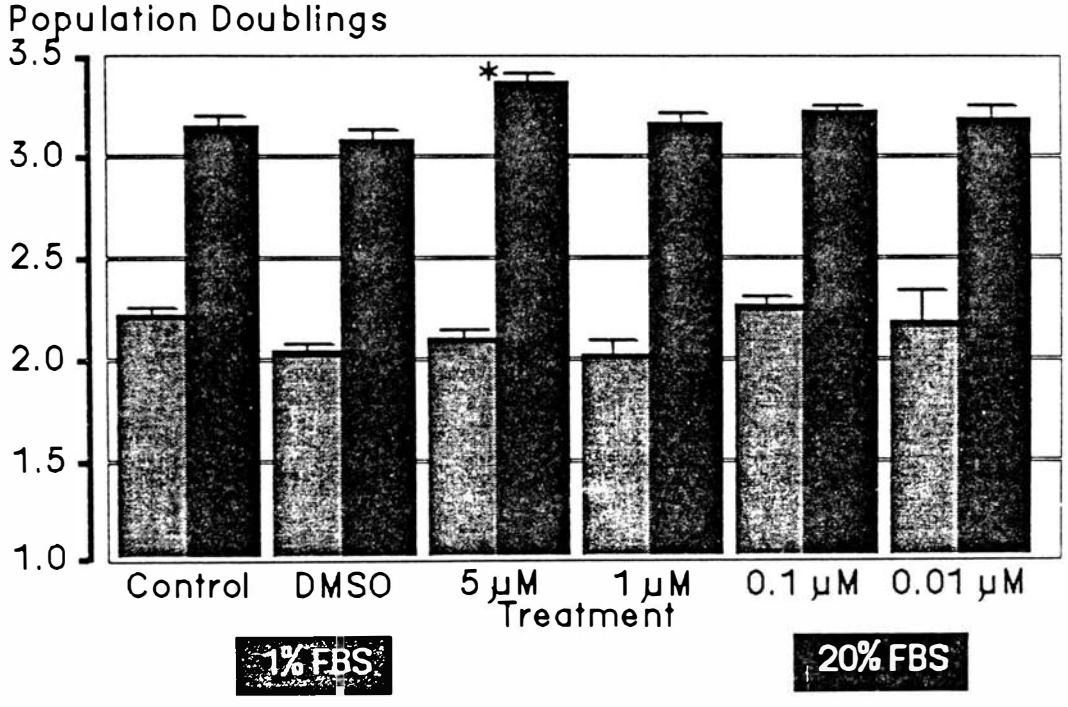


Figure 15. Effect of dexamethasone (DEX) on colony formation by HUVEC. These bar graphs illustrated the number of colonies of HUVEC achieved with two different doses of DEX using three different variations on attachment and growth. For all variations, seventh passage HUVEC were plated in T-25 flasks at colony formation density (1,000 cells/flask). After four hours, cultures were washed and then fed with steroid or control media according to the variation employed. Cultures were fed twice more at three day intervals with the appropriate steroid, DMSO control, or control media, and fixed and evaluated on the ninth day. The final concentration of DMSO solvent for DMSO control and all experimental flasks was 0.1%. Variation 1: To test the effect of DEX on growth only, cells were plated in CM-199 without steroids, and refed three times as described above with the appropriate DEX containing medium or DMSO control medium. Variation 2: To test the effect of DEX on attachment alone, HUVEC were plated with steroid containing or DMSO control medium, and then refed three times with CM-199 without steroids or DMSO. Variation 3: To test the effect of DEX on both attachment and growth. HUVEC were both plated and subsequently refed three times with the appropriate steroid containing or DMSO control medium. Bars represent in the mean colony counts from triplicate flask determinations, with the SEM above the bar. Colony formation is significantly inhibited by growth in DEX; plating in steroid media does not lead to colony formation inhibition unless subsequent feedings are with steroid containing media as well. * Significantly different from Variation 1 DMSO control group, $p < .05$. ** Significantly different from Variation 3 DMSO control group, $p < .05$.

Figure 15. Effect of Dexamethasone on Colony Formation by HUVEC

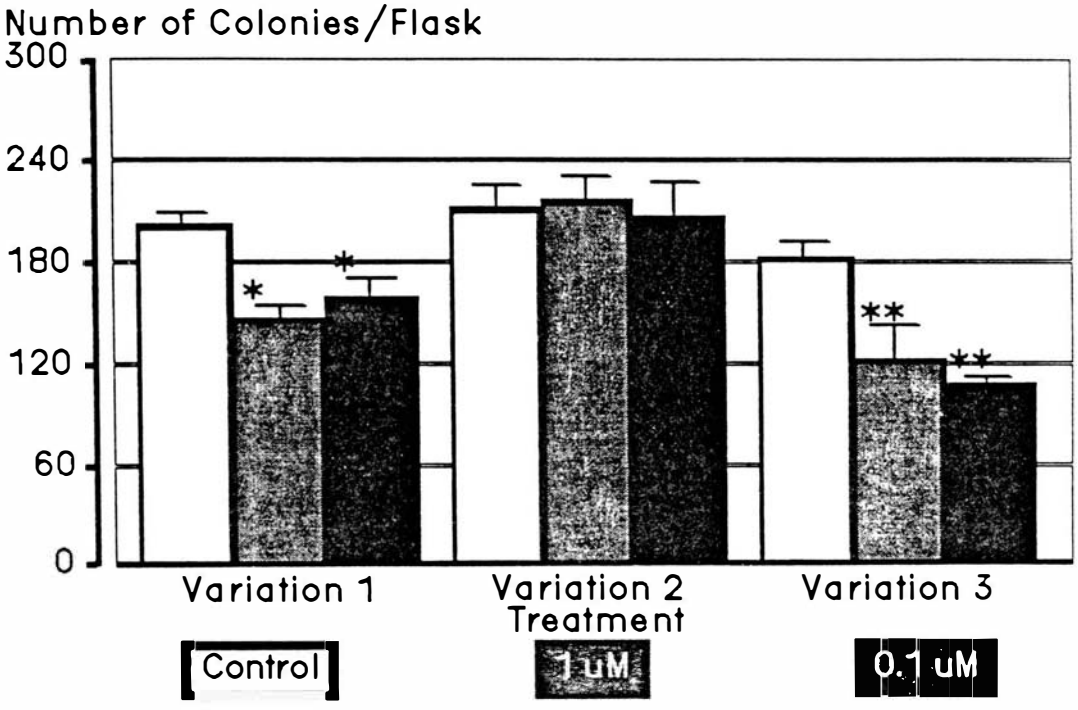


Figure 16. This plot shows the light scatter analysis of control HUVEC. HUVEC were plated in T-25 tissue culture flasks at standard density (1.25 x 10^5 cells/flask). They were fed with fully supplemented media on the second and fourth day after plating. Cell harvest was on the seventh day.

Figure 16

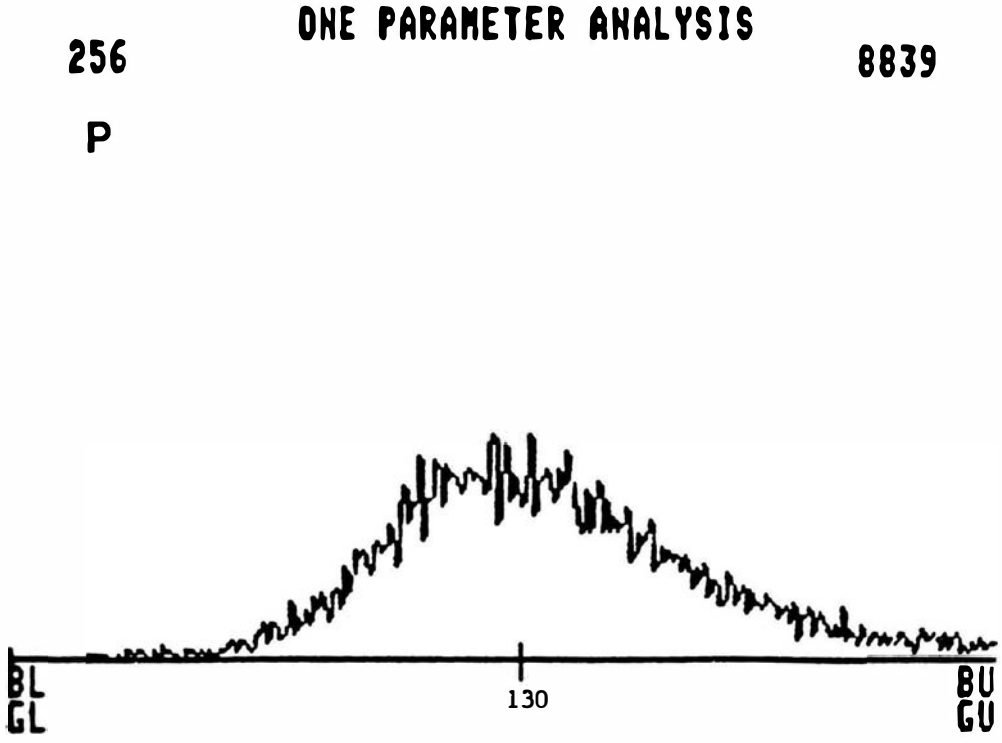
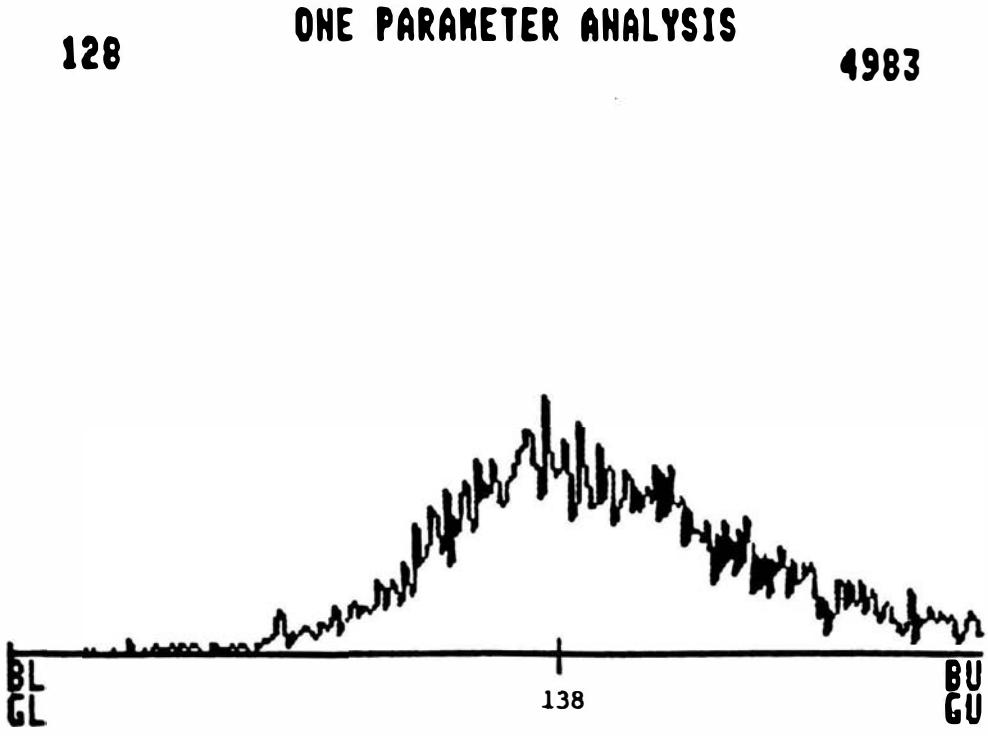

ONE PARAMETER ANALYSIS
256
8839
P
BL
GL
130
BU
GU
C18 P3-3-33 22/ 8/85 10:43
1P-256 LS

Figure 17. This plot demonstrates the light scatter analysis of HUVEC with 0.2% EtOH added to the culture media. HUVEC were plated in T-25 tissue culture flasks at standard density. The cultures were fed with media containing 0.2% EtOH on the second and fourth day, with cell harvest on the seventh day.

Figure 17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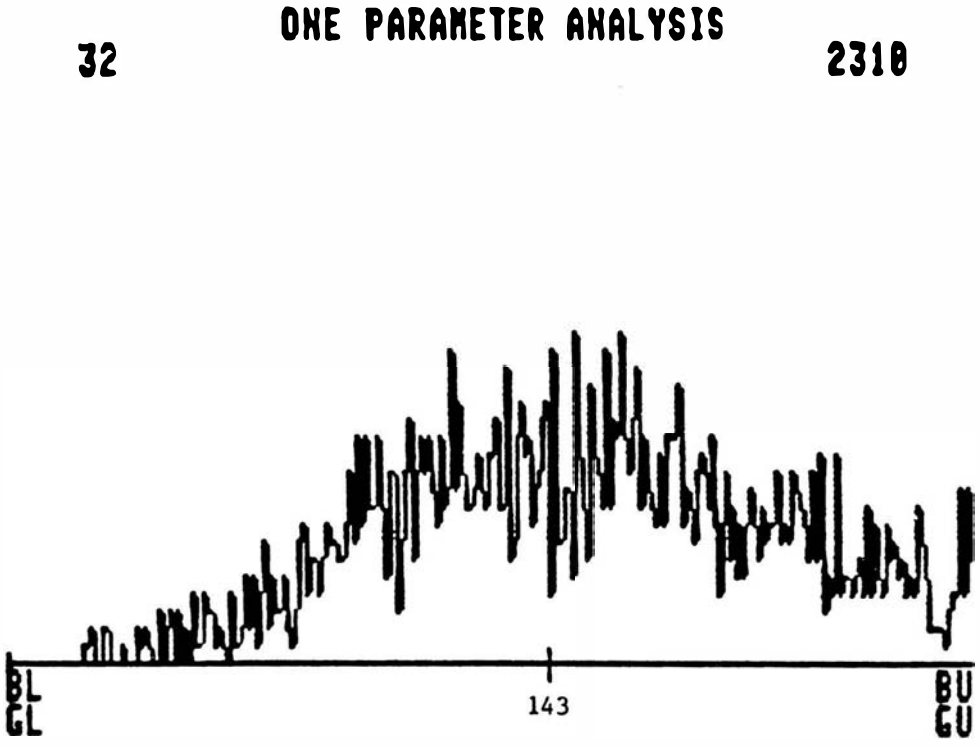
128
4983
BL
GL
138
BU
GU
C18P3-18-40 22/ 8/85 11:02
1P-256 LS

Figure 18. This plot shows the light scatter analysis of HUVEC treated with 0.5 µM DEX. HUVEC were plated in T-25 tissue culture flasks at standard density. The cultures were fed with fully supplemented media containing 0.5% µM DEX dissolved in EtOH on the second and fourth day, with cell harvest on the seventh day. The final concentration of ETOH in the steroid containing media was 0.1%.

Figure 18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2310
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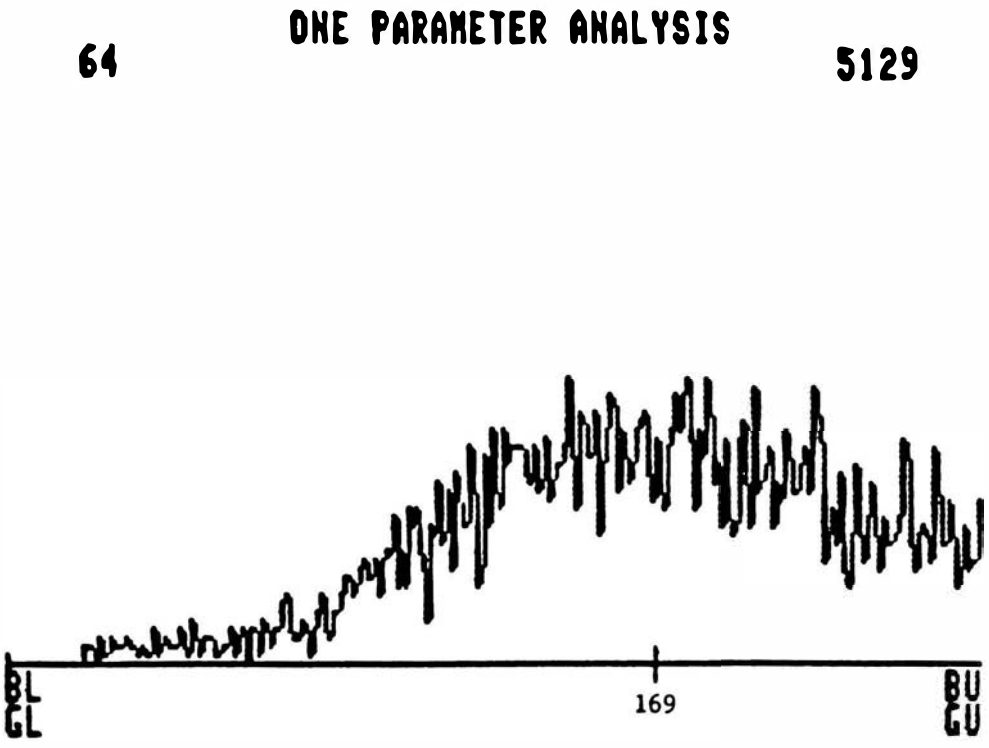
BU
GU
C18P3-17-37 22/ 8/85 10:54
1P-256 LS

Figure 19. This plot demonstrates the light scatter analysis of HUVEC treated with 1 μM DEX. HUVEC were plated at standard density in T-25 tissue culture flasks. The cultures were fed with fully supplemented media containing 1 μM DEX dissolved in EtOH on the second and fourth day after plating, with cell harvest on the seventh day. The final concentration of EtOH in the steroid continuing media was 0.2%.

Figure 19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BL
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169
BU
GU
C182P313-35 22/ 8/85 10:47
1P-256 LS

Table 1. Effect of dexamethasone (DEX) on growth characteristics of HUVEC (second steroid feeding) - Experiment I. This table illustrates the effect of a second steroid feeding upon the cellular and population growth of HUVEC. Second passage HUVEC were plated in T-25 flasks at standard density. On the second and fourth day after plating, cultures were fed with DEX dissolved in DMSO at the doses indicated. The final concentration of the DMSO solvent in the DMSO control and all the treatment groups was 0.1%. Cells were harvested and counted on the seventh day. Values represent the mean cell counts or corresponding mean population doublings of triplicate flask determinations, and are given with the SEM. Although not significant, there was a trend toward cellular and population growth inhibition with all four doses of DEX employed.

Table 1

Effect of Dexamethasone on Growth Characteristics of HUVEC (Second Steroid Feeding) - Experiment I

Treatment	Cell Number ± SEM	Population Doublings ± SEM
0.1% DMSO Control	$9.13 \pm .20 \times 10^5$	$3.25 \pm .03$
1.0 µM DEX	$6.38 \pm .34 \times 10^5$	$2.74 \pm .08$
0.5 µM DEX	$6.86 \pm .80 \times 10^5$	$2.83 \pm .16$
0.1 µM DEX	$7.38 \pm .80 \times 10^5$	$2.93 \pm .16$
0.01 µM DEX	$7.30 \pm 1.18 \times 10^5$	$2.89 \pm .24$

Table 2. Effect of dexamethasone (DEX) on growth characteristics of HUVEC (second steroid feeding) - Experiment II. This table illustrates the effect of a second steroid feeding upon the cellular and population growth of HUVEC. Fourth passage HUVEC were plated in T-25 flasks at standard density. On the second and fourth day after plating, cultures were fed with DEX at the doses indicated. The final concentration of DMSO solvent in the DMSO control and all the treatment groups was 0.1%. Cells were harvested and counted on the seventh day. Values represent the mean cell counts or mean population doublings of triplicate flask determinations, and are given with the SEM. An increase in cellular and population growth with all steroid doses was noted. The increase in population growth was significant for 1.0 µM, 0.5 µM, and 0.01 µM DEX. The much greater growth overall is apparent, compared to Table 1.

Table 2

Effect of Dexamethasone on Growth Characteristics of HUVEC (Second Steroid Feeding) - Experiment II

Treatment	Cell Number ± SEM	Population Doublings ± SEM
0.1% DMSO Control	$1.04 \pm .07 \times 10^6$	$2.90 \pm .03$
1.0 µM DEX	$1.33 \pm .03 \times 10^6$	$*3.26 \pm .04$
0.5 µM DEX	$1.24 \pm .04 \times 10^6$	$*3.16 \pm .04$
0.1 µM DEX	$1.16 \pm .01 \times 10^6$	$3.06 \pm .01$
0.01 µM DEX	$1.26 \pm .07 \times 10^6$	$*3.18 \pm .08$

* Significantly different from DMSO controls ($p < .05$).

Table 3. Effect of dexamethasone (DEX) on growth characteristics of HUVEC (four day counts) - Experiment I. The table illustrates the effect of DEX upon the cellular and population growth in a four day growth assay. The purpose of this experiment was to determine if growth inhibition or enhancement occurred at an earlier time period, an effect that might have been masked in a seven day assay. Second passage HUVEC were plated in T-25 flasks at standard density. On the second day after plating only, cultures were fed with DEX at the doses indicated. The final concentration of the DMSO solvent for the DMSO control and all treatment groups was 0.1%. Cells were harvested and counted on the fourth day, two days after steroid administration. The values represent the mean cell counts or corresponding mean population doublings of triplicate flask determinations, and are given with the SEM. An early cellular and population growth inhibition was observed with the DEX doses employed. The decrease in population growth was significant for 1.0 μM and 0.1 μM DEX.

Table 3

Effect of Dexamethasone on Growth Characteristics of HUVEC (Four Day Counts) - Experiment I

Treatment	Cell Number ± SEM	Population Doublings ± SEM
0.1% DMSO Control	$3.35 \pm .28 \times 10^5$	1.80 ± .13
1.0 μM DEX	$2.48 \pm .11 \times 10^5$	*1.37 ± .06
0.5 μM DEX	$2.68 \pm .26 \times 10^5$	1.48 ± .14
0.1 μM DEX	$2.52 \pm .22 \times 10^5$	*1.39 ± .13
0.01 μM DEX	$2.94 \pm .14 \times 10^5$	1.62 ± .07

* Significantly different from DMSO Controls ($p < .05$).

Table 4. Effect of dexamethasone (DEX) on growth characteristics of HUVEC (four day counts) - Experiment II. The table illustrates the effect of DEX upon the cellular and population growth of HUVEC in a four day growth assay. The purpose of the experiment was to determine if growth inhibition or enhancement occurred at an earlier time period, an effect that might have been masked in a seven day assay. Fourth passage HUVEC were plated in T-25 flasks at standard density. On the second day after plating only, cultures were fed with DEX dissolved in DMSO at the doses indicated. The final concentration of the DMSO solvent in the DMSO control and all treatment groups was 0.1%. Cells were harvested and counted on the fourth day, two days after steroid administration. The values represent the mean cell counts or the corresponding mean population doublings of triplicate flask determinations, given with the SEM. Although cellular and population growth inhibition occurred, it was not significant for the four doses of DEX employed. Again, there was relatively less inhibition of growth with the lowest DEX dose.

Table 4

Effect of Dexamethasone on Growth Characteristics of HUVEC (Four Day Counts) - Experiment II

Treatment	Cell Number ± SEM	Population Doublings ± SEM
0.1% DMSO Control	$6.23 \pm .43 \times 10^5$	$2.16 \pm .11$
1.0 μM DEX	$5.17 \pm .84 \times 10^5$	$1.86 \pm .23$
0.5 μM DEX	$4.77 \pm .31 \times 10^5$	$1.78 \pm .09$
0.1 μM DEX	$4.74 \pm .21 \times 10^5$	$1.77 \pm .06$
0.01 μM DEX	$6.16 \pm .34 \times 10^5$	$2.15 \pm .08$

Table 5. Effects of dexamethasone (DEX) upon endothelial cell cycle kinetics. The table illustrates the effect of dexamethasone on the percent of cells in the various phases of the cell cycle as determined by cytofluorometry. Second passage HUVEC were plated in T-25 flasks at standard density. On the second and fourth day after plating, cultures were fed with DEX dissolved in EtOH at the doses indicated. The final concentration of EtOH solvent in both the EtOH control and 1 μM DEX treatment group was 0.2%, and in the 0.5 μM DEX treatment group, 0.1%. Cells were harvested and processed for flow cytometry on the seventh day. DEX at 0.5 μM caused an increase in the percentage of cells in the S phase, while at a higher dose (1 μM), effected an increase in the percentage of cells in the G_2-M phase.

Table 5

Effects of DEX on Endothelial Cell Cycle Kinetics

Culture Treatment	G_0-G_1	S	G_2-M
Control	49.6%	36.9%	13.5%
Control 0.2% EtOH	44.2%	40.1%	15.7%
0.5 μM DEX	25.2%	56.9%	17.9%
1 μM DEX	41%	34.9%	24.1%

Part II

Male-Female Senescent Patterns in Cultured Endothelium

Table of contents

List of Figures

List of Tables

Introduction

The aim of the present study is to explore the possibility that there are differences regarding the expression of *in vitro* senescence between male and female cultures of human umbilical vein endothelial cells (HUVEC). The objective is to provide a clearer understanding of the senescent process in this culture system. The fact that primary age-associated cellular changes occur in the endothelium *in vivo* engenders relevance to the study of cellular senescence in cultured endothelial cells (EC) (Mueller *et al.*, 1980). The study of the growth characteristics and proliferative life span of EC *in vitro* may be related to diseases such as atherosclerosis and metastatic cancer (Levine and Mueller, 1979). Human EC are characterized by finite life spans and low frequencies of spontaneous transformations (Levine *et al.*, 1983). Altered proliferative or functional capacities in aging endothelium would have very serious consequences for the organism in question. Since the endothelium occupies a very unique and strategic location within the body, it acts as a highly selective barrier for substances entering and leaving the blood. The endothelium is an essential component in wound healing and is the site of synthesis of a wide variety of

metabolically active compounds (Levine et al. 1983). Because of its distribution in practically all tissues, the study of endothelial cellular aging can be said to reflect a study of aging of the body as a whole.

This review is divided into four sections. The first section describes the results of studies which have explored the phenomenon of endothelial cellular senescence in vivo. The second section outlines senescence of EC in vitro, with emphasis on cultured endothelial cells as an appropriate model for in vitro senescence. The third section details male-female differences in the endothelium that may affect endothelial aging or the expression of the senescent cell phenotype in experimental model systems. The fourth and final section, a summary, explores the role of the endothelium in atherogenesis.

Endothelial senescence in vivo

The occurrence of large, multinucleated cells characteristic of aging EC in cultures have in vivo correlates and have been described by a number of authors (Lautsch et al., 1953; Short 1954; McGovern 1955; Scott 1956). Multinucleated giant cells were found in the aorta of aging humans (Lautsch et al., 1953), over venous intimal thickenings in aged humans (Short, 1953), and in

the veins of aging rats (McGovern, 1955). McGovern regarded multinucleated cells as evidence of regeneration following injury; cellular injury may play a role in senescent cell expression *in vivo*. These large cells were uncommon, but could be found, in young human subjects, indicating that they were not solely a phenomenon in aged individuals. It is plausible that senescent cells occur in all persons, their number increasing as a function of age.

Scott (1956) described multinucleated cells overlying organized thrombi in vessel walls of humans. Hicks *et al*. (1983), examining Fischer rats, reported an increase in the cytoplasmic areas of EC, and an increase in the cross sectional areas of the basement membrane. Cell organelles may also undergo changes with aging. Miranov and Miranov (1985) found that the aortic endothelial cell cytoskeleton in rats became more highly structured with aging, an adaptive reaction which increased the intracellular contacts and cell adhesion to the underlying substrate. The functional ramifications of this observation were not outlined. Multiple factors, including anatomical and hemodynamic differences, influence the organization of the endothelial cell cytoskeleton *in situ* (White *et al*. 1983).

Poole et al. (1958) mechanically injured the aorta of young rabbits and found that within three days, large cells, containing up to ten or more nuclei in their cytoplasm, appeared in the edges of the denuded area. They hypothesized that giant cells, with multiple nuclei, represented a syncytium which failed to break up into individual cells, the nuclei remaining in a conglomerate within the center of the cells. Exactly why this happened was not entirely clear. Lautsch et al. (1953) observed that the nuclei tended to be grouped centrally within the endothelial cell cytoplasm, and that two or more groups of nuclei could be seen within the confines of one giant cell. Stehbens (1965), studying endothelial cell repair following mechanical trauma to rabbit veins, found an increased number of multinucleated EC in the region of injury three days after the mechanical insult. In contrast to the above, Reidy et al. (1983) measured regrowth in denuded rat and rabbit arteries and reported that endothelial cellular regeneration stopped before regrowth was complete, and that cell senescence was not responsible.

Aside from the knowledge that senescent cells occur with greater frequency in aged individuals and that they can be found in proximity to mechanically wounded areas of the endothelium in vivo, little is understood regarding

their significance and function. The literature suggests that these cells do play some role in the related processes of senescence and repair.

Senescence in Cultured Endothelial Cells

The phenomenon of age associated cellular changes that takes place in the endothelium *in vivo* makes the study of cellular senescence in cultured EC particularly relevant. Cells in the body extend across a range from rapidly and continuously dividing (epithelium, hemapoietic cells) to fixed, post mitotic, highly differentiated (neurons, muscle fibres). Since the vascular endothelium is an intermediate class of cells which exhibits a slow turnover *in vivo*, cellular senescence in endothelial cells might have unique characteristics and consequences for the organism.

With repeated passage, cultured endothelial cells (EC) undergo morphological and proliferative changes which are similar in many respects to the changes observed in aged or senescent EC *in vivo*. The basic cause of the senescent process *in vitro* is unknown, although Grove and Cristafalo (1977) viewed cellular aging as being due to a transition from a rapidly proliferating state through a series of progressively slower cycling ones, to a

condition in which the cells were arrested or were proliferating so marginally so as to be incapable of repopulating the culture vessel. Whether the decline in proliferating capacity is due to a uniform increase in the generation time of the cells or to an increase in heterogeneity in the culture (resulting in a mixed population in which some cells divide normally, while others are incapable of division) remains unknown. The latter theory, that of a mixed cell population, is plausible on empirical grounds but has not been proven definitively (Cristafalo, 1972). Heterogeneity of the cell population was supported by Schwartz et al. (1981), who felt that the limitations to replicative lifespan manifested in cell culture might have been a result of random distribution of proliferative potential in the culture, rather than a true limitation of the ability of individual cells to have divided.

In general, all non-transformed cell cultures undergo a natural history which includes three phases. The first phase consists of the primary culture up to the first confluence. The second phase is characterized by rather luxuriant cell growth necessitating subcultivation. The third and final phase marks the period of cellular aging, senescence, loss of growth potential, and eventual cell death. (Haymark, 1976). It is of interest that the

transition between Phase II and Phase III may be rather abrupt in EC in comparison to other cell lines - fibroblasts, for example (Grove and Cristafalo, 1977). A limitation which must be kept in mind, however, in evaluating aging, is the fact that all well-documented reports of *in vitro* senescence are based upon paradigms where subculturing is via enzymatic dissociation (trypsin), which introduces a major source of artifact in the experimental process (Ruben and Rafferty, 1978). Cellular aging probably has multiple components, and *in vitro* methodology should reflect, as best as possible, the interaction of cells with hormones and other factors in their environment, a process that occurs *in vivo* (Cristafalo, 1976).

A number of researchers have documented the expression of the senescent phenotype and lowered growth rate with aging in various human and animal endothelial cell cultures. Mueller *et al.* (1980) found a replicative life span of approximately 80 cumulative population doublings (CPD) in BAEC; growth rates decreased and cell size increased as the CPD increased. Rosen *et al.* (1981) extended these studies and suggested that cellular senescence in EC may contribute to the genesis of atherosclerosis and the process of tumor invasion and metastasis.

Weber et al. (1984) studied cultured BAEC and found that senescent cells appeared at approximately the eighth passage. The senescent cells were often multinucleated and two to three times larger than regular EC; they had vacuoles present in the perinuclear regions; and contained giant or irregular mitochondria, abundant RER, and occasional Weibel-Palade bodies. The Weibel-Palade body, a rod shaped cytoplasmic inclusion, is an endothelial-specific organelle and as such can be used as an identifying marker for EC (Gimbrone, 1976). The curious longitudinal fibers observed running parallel to the long axis of the cell often bridged over the nucleus, these fibers were hypothesized to be "stress" fibers not related to cellular motility (Weber et al., 1984).

Pursuant to this, it has been speculated that the giant cells may form as a result of some type of repair process, obtained through cell spreading. The origin of senescent cells was felt by Wright (1972) to be due to mitosis occurring in some instances without cytoplasmic division, giving rise to the giant multinucleated cells. Either process might possibly explain the thin, veiled cytoplasmic cords (see below) frequently seen in senescent cells. Duthu and Smith (1980), supporting the findings of Weber et al. (1984), remarked on the increase in the

number of pinocytotic perinuclear vacuoles in EC in confluent BAEC cultures, a phenomenon even more striking in senescent cells.

Rosen et al. (1981) depicted senescent BAEC as having increased cellular areas and volumes, and increased protein content. Senescent cells, however, qualitatively retained their ability to express Factor VIII antigen, corroborating their earlier study (Mueller et al., 1980). A large proportion of their cells were rapidly dividing until 75% of their life span was completed; also at this point an increase in cell attachment area paralleled a decrease in the number of labeled nuclei in a [^{3}H] thymidine study. However, the cells still could achieve confluent monolayers until 90% of their life span was completed. Bierman and Schwartz (1984) documented increased binding and degradation of low density lipoproteins in BAEC, and suggested that this finding played a role in arterial wall changes that occurred with age in vivo. There was a gradual decrease in glycosaminoglycans and collagen in cultured porcine EC with age (Bihari et al. 1981).

Bowersox and Sorgente (1982), studying the chemotactic response of BAEC to fibronectin and endothelial cell mitogens, such as endothelial cell growth

factor (ECGF) and tumor extracts, demonstrated that cells from later passages (i.e. after 15-16 *in vitro* subculturings), were less responsive to chemotactic stimulation when compared to early passage cells. Johnson & Longenecker (1982) described senescence in BAEC; the onset of Phase III in these cells could be delayed if the cells were maintained under fibroblast growth factor (FGF) dependent conditions. They believed that *in vitro* studies should focus on a cell type which begins its cultured life span expressing its differentiated phenotype and which also is known to be involved with an age-related pathology *in vivo*. HUVEC meet these criteria.

Although most studies of *in vitro* senescence have been performed on BAEC, current perspective has included the exploration of senescence in human endothelium in culture as well. The question of whether cellular density at plating may be a factor in the senescence of human endothelial cell cultures was raised by Glassberg *et al.*(1982) who cultured human iliac artery EC and reported that the cells became senescent and lost viability after 8-10 passages when passed at a 1:3 split ratio. According to Cristafalo (1976), there is a tendency for HUVEC to grow differently and senesce at different rates depending on the split ratio at passage (1:5 vs. 1:10 split ratios, for example). Cells passed at a greater density (i.e., a

lower split ratio) may be affected by contact inhibition and not proliferate as rapidly as cells passed at a somewhat lower density (Maciag et al., 1981). In cell culture, the "split ratio" used in passage procedures could effect the total number of population doublings achieved in a given assay.

Duthu and Smith (1980), in examining culture conditions for BAEC, found that the effect of FGF upon cell proliferation in BAEC was inoculation density dependent. The cell division rate was significantly increased at inocula less than 1×10^4 cells/cm^2, but there was no effect at higher densities. Glassberg et al. (1982), in comparing HUVEC to iliac artery EC, felt that species differences regarding growth and function might be more apparent than differences between arteries and veins, or diversities between adult and newborn vessels. Since it is possible to correlate the replicative ability in HUVEC cultures with cell density at the time of plating (Henriksen et al., 1975), this parameter is also important in examining the growth patterns and senescent expression in HUVEC.

Mayuma (1963), the first author to report the isolation of HUVEC in culture, described many binucleated and multinucleated cells in his primary cultures.

Although not all binucleated EC in culture are senescent cells, the majority of the multinucleated cells do exhibit the characteristics of the senescent phenotype. Fryer (1966), in an early discussion of "atypical cells" in HUVEC, described large, circular, sometimes multinucleated cells, that increased in number with increasing age of the culture. The cells contained abundant fat droplets, and showed a greater affinity for india ink stain than normal EC. Gimbrone et al. (1973) mentioned a morphologically distinct subpopulation of large cells in HUVEC, occasionally multinucleated, which could be separated from certain primary cultures. Maciag et al., (1981) described the occurrence of large, multinucleated senescent cells in HUVEC, adding that they were occasionally found in primary cultures.

This senescent phenomenon can be observed repeatedly in long term culture of HUVEC. It is of interest that similar, large senescent veiled cells have been observed in the edge of intimal lesions and in the aorta of aging humans (Haudenschild et al., 1975). These authors wrote of large multinucleated "veil" like cells seen in their cultured HUVEC, starting after the second passage. In senescent cells, the cytoplasm takes on a curious webbed appearance; cytoplasmic spokes will appear to extend from the nucleus(i) in radial fashion, much like broad spokes

on a wheel. Senescent cells are commonly five to ten times larger than typical EC in culture, contain fewer 60-70 A filament bundles in the cell periphery, and lack specific endothelial granules, or Weibel-Palade bodies (WPB) (Haudenschild et al., 1975). Gimbrone et al. (1973) rendered an account of cells significantly larger than normal HUVEC, lacking WPB, but containing cytoplasm filled with myofibrillar elements and fusiform densities. Another in vivo correlation was provided by Weber et al. (1984), who remarked that while the arteries of children were covered by a homogeneous sheet of small to medium sized EC, adult arteries displayed a polymorphic luminal surface due to the presence of large, giant EC which tended to accumulate in areas of repeated endothelial injury or high cell turnover.

Gospodarowicz et al. (1978), studied HUVEC and corroborated the earlier descriptions of senescent cells in these cultures, noting that the use of FGF and thrombin in the culture media could prevent precocious senescence. They noted that in the umbilical veins, the vascular cells may be pre-programmed to senesce rapidly after birth, although there was no evidence that the expression of senescence in culture differed as a function of the different sources of origin of cells, or among species. In conclusion, Gospodarowicz et al. (1978) warned that

studies performed on cell populations with excessive senescence might lead to misleading conclusions regarding the actual capability of the cells in reference to the function or behavior in question.

Grinspan et al. (1981) further characterized the in vitro senescence of human umbilical vein endothelial cells (HUVEC) in culture. After continued subculture, the proliferation rate of EC slows and harvest cell densities decrease. Morphological changes ensue which are typical of in vitro senescence: cells become larger and have an increased cytoplasmic to nuclear ratio; the attachment area of individual cells increases; and large, multinucleated cells with veil-like cytoplasm appear in the culture. They also raised the question of whether culture life span was limited by the number of cell division (genomic time) or by a fixed amount of chronological "metabolic" time. Since a wide variety of human tissues of fetal, neonatal, or adult origin are incapable of unlimited proliferation, it has been thought that the phenomenon of limited life span must be programmed in some way. In addition, Cristafalo (1972) felt that the aging of fixed post-mitotic cells (i.e. neurons) quite probably proceeds by a different mechanisms compared to that observed in proliferating tissues (i.e. epithelium). What remains obscure is the relationship, if

any, between the observed programmed limit of population doublings for a particular cell line, and the actual events that limit cellular functions during senescence *in vivo*.

Evidence for a pre-programmed process of senescence can be found in HUVEC. Subcultures of HUVEC tended to exhibit increasing pleomorphism of cells, with increased numbers of veil-like cells. Also, the tendency to form confluent monolayers was decreased with cell passage (Maciag *et al.*, 1981). Although the large, multinucleated cells typically appeared in greater numbers in later passages of EC (Haudenschild *et al.*, 1975), the fact that large veil-like cells can occasionally be found in primary HUVEC cultures suggests that senescence of EC is a preprogrammed event.

Knaver and Cunningham (1983) grew HUVEC on a fibronectin matrix, using ECGF to potentiate the growth of the cells. They found that there was a change in the gross morphological appearance of the cells between early and late passages; cells in the latter were larger in size and more irregular in shape. They estimated the incidence of multinucleated "veil" cells as comprising one to two percent of cells in early cultures and 10-12% of the total cells by passage 16. It was suggested that in all culture

systems, there were deficiencies which resulted in the gradual decrease in the differentiated state of the culture.

That the substratum-matrix upon which cells grow in culture can be an integral factor in determining proliferative rates of EC was underscored by Furcht et al. (1986) who found that there were different substratum requirements for microvascular EC and large vessel EC. This differential phenotypic behavior of EC from different sources regarding their response to matrix or basement membrane components exemplifies the fact that many factors are influential in promoting or inhibiting endothelial cell growth in culture. However, the suggestion that basement membrane components may modulate the cellular phenotype of the endothelial cell indicates the possibility of these entities influencing the expression of senescence in some way. Cristafalo (1972) hypothesized that the many and varied environmental effects by which the cell life span could be influenced were superimposed on an existing program for a preset life span, thus perhaps modifying its duration, but in the end, not its ultimate disposition.

Maciag et al. (1981) carried HUVEC cultures through 34 population doublings, but not to the end of their life

span. He was one of few authors to attempt a quantitative assessment of senescent cells in HUVEC. Starting in the eighth passage, giant, multinucleated cells with broad, veil like cytoplasm appeared in culture. Their initial density was one/10^3 cells, but by the fourteenth passage, they increased in number, achieving a density of one/10^2 cells. The authors called for detailed studies of cellular senescence in HUVEC, to see if EC growth rates indeed did decline at later stages, as did the doubling rates of adult (Schwartz, 1978) and fetal (Rosen et al., 1981) bovine aortic endothelial cell cultures.

Kan et al. (1985), using fibroblast conditioned medium, cultured HUVEC through 50 population doublings but did not specifically address the senescence phenomenon. In this study, medium conditioned from human diploid fibroblast cultures markedly enhanced the division of HUVEC at low seeded density. They defined the end of a proliferative life span as that cumulative population doubling at which the EC density did not double beyond the inoculation density (2×10^4 cells/35 mm dish) after two weeks. Rosen et al. (1981) suggested three weeks at the above inoculation density as a criteria for judgment as to when "end of life span" ensues.

In summary, quantitative assessment of *in vitro* senescence in EC has not been comprehensively reported in the literature. Many studies regarding endothelial cultures pool the results from numerous cell passages, although it has recently been demonstrated that there is a significant variability in properties of and growth characteristics during *in vitro* propagation (Goldsmith *et al.*, 1984). This variability should be entertained when exploring properties of subcultured endothelium.

Male-Female Differences in EC

Exploring the male-female differences in growth and senescence of HUVEC is of strong relevance, since no studies have addressed this aspect *in vitro*. Balconi *et al.* (1983) examined factors which affected success rates of primary HUVEC cultures; the sex of the newborn did not significantly influence the successful establishment of primary cultures of HUVEC. Unfortunately, secondary and later passage cultures were not studied.

On a functional level, male HUVEC synthesized more PGI_2 and PGE_2 than female HUVEC when the cells were stimulated with thrombin (Batres and Dupont 1986). The sex steroids 17 beta estradiol and testosterone were found to stimulate PGI_2 secretion in female, but not male,

piglet EC in culture (Seillan et al., 1983). In addition, female, but not male EC could convert testosterone into the estrogens estriol, estrone, and estradiol. The fact that 17 beta estradiol induces stimulation of the production of PGI_2, which inhibits platelet aggregation, could provide a partial explanation for the beneficial role generally attributed to naturally occurring estrogens in preventing cardiovascular diseases (Seillan et al., 1983). Lennon and Poysner (1986), studying the effect of age on the vascular prostaglandin production in male and female rats, found that PGI_2 production decreased in males and in aged rats, and linked this process to the difference in vascular disease incidence in males and females. Cunard et al. (1986) outlined the response of ring pulmonary artery preparations to the synthetic endoperoxide analog U46619, and reported that in denuded vessels, a significantly lower U46619-elicited maximum tension occurred in female vs. male rat preparations.

Nordoy et al. (1978) studied the inhibitory effects of a monolayer of HUVEC and PGI_2 upon platelet aggregation, and found less of an inhibitory effect of a monolayer of HUVEC or PGI_2 upon ADP or collagen induced platelet aggregation in females than males. Karanian et al. (1981) studied whole thoracic aorta preparations in the rabbit and rat and found that the maximum contractile

response to various prostaglandins was greater in male specimens than female specimens; prostaglandin receptors in the thoracic aorta appeared to be gender related. A further link to gender differences in incidence and expression of cardiovascular diseases could be made if additional studies indicated parallel gender differences in the growth and senescence of endothelial cells.

It is believed that exposure of the vascular endothelium to increases in shear stresses, as typically occurs in hypertension, creates an increased demand on the normal cellular mechanisms involved in anchorage of the cells to the substratum. These cellular mechanisms include microfilamints containing both actin and myosin; these "stress fibers" are seen much more frequently in arterial, rather than, venous, endothelium (White et al. 1983). These authors reported that the proportion of EC containing stress fibers in the descending aorta was significantly greater in males vs. females in both normal and spontaneously hypertensive rats. Also, in male rats, the stress fibers were thicker and had more prominent striations.

Male/female differences in senescent phenotypic expression have not been studied previously. Experiments examining gender differences in HUVEC regarding in vitro

senescence will augment the knowledge of the aging process of male and female EC in this culture system. Also, it seems likely that continued experimentation with EC *in vitro* will suggest pertinent questions to be asked regarding *in vivo* pathologies, and serve as a ground for exploring methodology that can be used in answering these questions.

The Role of EC in Atherosclerosis

Age related changes in the endothelium lining the blood vessels are believed to be significant events which are integral in the expression of two major pathologic processes: atherosclerosis and tumor invasion and metastasis (Mueller and Levine 1979; Grinspan *et al.* 1981). It is well known that the incidence of atherosclerosis and thrombosis is higher in males than in females (Batres and Dupont 1986). Atherosclerotic lesions tend to occur more frequently in hypertensive individuals, and in areas of the vascular system subject to hemodynamic stress, such as the aortic bifurcation (Clarkson *et al.*, 1985). Sauer *et al.* (1981) described the age-related intimal alterations, including endothelial cellular changes, in spontaneously hypertensive rats. They demonstrated a proliferation of organelles in EC, most notably in Weibel Palade bodies, in contrast to previous

in vitro findings (Haudenschild *et al.* 1975, Weber *et al.* 1984a). Haudenschild *et al.* (1981) compared intimal changes in normal, spontaneously hypertensive, and deoxycortisone/salt treated rats and found the latter two groups to exhibit an overall increased number of EC, and to contain a greater number of EC with bizarre shapes, nuclear folding, and nuclear bulging toward the lumen. These changes were observed in aged normotensive animals, but to a lessor extent and with slower progression. Scannning electron microscopy of these changes revealed EC with a raised appearance and irregular surfaces.

Ross and Glomset (1976) in a detailed two part report outlined the then current theories of the pathogenesis of atherosclerosis. They described the early lesion of atherosclerosis as being the fatty streak, a focal accumulation of smooth muscle cells (SMC) surrounded by lipid deposits. Macrophage derived foam cells were a major constituent of this early pathological entity. These fatty streaks come to cover a significant percentage of the intimal surface as a person ages. The *fibrous plaque*, a more advanced lesion, consisted of an accumulation of intimal, lipid laden SMC and collagen, elastic fibers, and proteoglycans. This fibrous cap covered a large, deeper deposit of free extracellular lipid and cellular debris. Long standing lesions were

characterized by fibrous caps altered by hemorrhage, calcification, and mural thrombosis.

The role of EC in this process was unclear, but some role indeed was entertained, since the endothelium separates the blood from the endothelial substratum and underlying connective tissue, the latter areas known to be involved in the development of the atherosclerotic pathology. Ross and Glomset (1976), in implicating the vascular endothelium, were among the first authors to propose the endothelial cellular injury hypothesis as a major event leading to atherosclerotic lesion manifestation. Basically, areas in the vessel wall, subject to the mechanical stresses of hypertension and the proposed desquaminative effects of hyperlipidemia, were subject to endothelial barrier disruption. Platelets adhering to the subendothelial connective tissue at the sites of injury released a factor which was capable of promoting the proliferation of SMC. Eventually, a fatty streak developed, and the cascade of events leading to the full blown clinical presence of the atherosclerotic plaque ensued (Ross and Glomset, 1976). EC of the vessel wall were thought to exhibit diminished activity with aging, eventually affecting the endothelial barrier by reduced reparative ability. The injury theory of plaque formation lent support to the additional hypothesis that endothelial

injury occurred more frequently and/or was repaired less efficiently or completely with increasing age (Levine et al. 1983). The older an organism, the much more likely it was that a significant degree of endothelial injury had been sustained.

The theory came to be refined as knowledge concerning the contributing factors increased. Basically, an undefined injury to the endothelium led to desquamination of EC, which exposed the thrombogenic basement membrane to the circulating blood. Platelet adhesion and degranulation were subsequent to release of platelet derived growth factor, mitogenic and chemotactic for SMC. It is of interest that a dying endothelial cell can produce in less than three days as much PDGF- like proteins as a healthy cell can produce over a 19 day period. It would appear, then, that denuded, dying EC would hasten or enhance growth factor release from platelets, thus accelerating the process of proliferation and infiltration of SMC. After intimal proliferation of SMC, the cascade of processes then continues through lipid accumulation and macrophage infiltration, combining to form the well characterized atherosclerotic plaque (DiCorleto and Chisolm 1986).

Levine and Mueller (1979) felt that injury to the endothelium increased vessel permeability, allowing extravasation of potentially atherogenic macromolecular substances into the arterial wall. Even subtle injury might promote an increase in transendothelial pinocytosis, thereby transporting atherogenic substances into the subendothelium. Also, endothelial cell injury apparently occurred more frequently and/or was repaired less efficiently with increasing age. White et al. (1983) proposed that the prominent microfilament bundles (stress fibers) observed in the aortic endothelium of hypertensive rats might play a part in cell contractility, thereby possibly affecting this altered vessel wall permeability.

It was observed by both Lautsch et al. (1953) and Haudenschild et al. (1975) that large senescent cells occured in the edge of intimal lesions of the vascular system and in the aortas of aging humans. Raised levels of cortisol in the plasma correlated with an increased risk of coronary heart diseases (Jarvelainen et al. 1982). It was thought that cortisol acted neutrally or in a protective manner, attenuating endothelial cell effects upon SMC growth. In lesions already induced by other factors, it was believed that cortisol accentuated the atherosclerotic process due to increased formation of

connective tissue macromolecules in the SMC. Thus EC may play an indirect but vastly significant role in the development of this pathological lesion. However, that an intact endothelium was active in regulating proliferation of SMC was proposed by Willems et al. (1982), who described the inhibitory effect of media conditioned by confluent HUVEC monolayers upon smooth muscle cell and endothelial cell [^{3}H] thymidine incorporation. Apparently then, HUVEC possess growth inhibitory activity which inhibits DNA synthesis and proliferation in EC as well as SMC. Their findings also lend support to the theory that substances released by confluent EC may contribute to the density dependent regulation of endothelial growth that has been observed by scientists studying EC in culture.

However, it became obvious that not all features of the atherosclerotic lesion could be explained by the reaction-to-injury hypothesis (Thorgeirsson and Robertson 1978). For example, it would be presumptive to conclude that endothelial cellular replication results simply from the disruption of the normal cell-cell contact. Endothelial cellular mutation leading to altered growth patterns may occur spontaneously or perhaps be genetically influenced- other concepts that should be entertained in exploring factors which may influence endothelial behavior in atherosclerosis. Also, although endothelial cell

replication may be increased in response to hypertension or hyperlipidemia, this does not automatically imply areas where the endothelium has become denuded (Schwartz et al. 1981). A critical period or degree of denudation, and unique or characteristic location of the denudation injury may also be factors involved.

Schwartz et al. (1981) remarked that there was no convincing evidence that excessive growth of the endothelium contributed to the atherosclerotic lesions. That the endothelium partakes in some way in the process was evident in the fact that platelet derived, endothelial cell derived, and macrophage derived growth factors could all stimulate the growth of SMC. Schwartz et al. outlined three ways in which endothelial cellular functions could lead to the proliferation of SMC: either by altered transport, altered platelet interactions, or altered control of the production and release of growth factors. Interestingly, though, it is not known whether the endocytotic process observed in cell culture of EC is the same as the mechanism involved in the transport of LDL across the vessel wall in vivo.

DiCorelto and Chisolm (1986) admitted that although the response to injury hypothesis of atherosclerotic lesion development had a marked influence regarding

atherogenesis, evidence had not been produced convincingly that frank denudation of the endothelium occurs as an early event in atherogenesis. They contended that only in advanced plaques had regions of vessel wall without endothelium been identified morphologically, and that endothelial denudation by itself could not be used to identify an early vascular wall lesion. They hypothesized an attractive theory that called for an active role of EC in atherogenesis. In an activated state, EC could express binding sites for monocytes; secrete monocyte activators; secrete oxygen free radicals which could conceivably modify surrounding LDL; and finally synthesize and secrete a PDGF-like protein or other mitogens and chemoattractants for SMC in the media of the vessel. Since not all mitogens secreted by EC have been identified, it is rather clear that the endothelial injury hypothesis will undergo further modification as research unfolds in this area.

Ross (1986) later modified his approach to the injury hypothesis of atherosclerosis. He felt that cultured endothelium was a useful yet limited model of endothelium in vivo. However, cultured confluent endothelial models have a low but definite rate of turnover, which may approximate the state of "injury" in vivo. Ross proposed that EC in vitro may be in an abnormal or "injured" state, and were continuously

stimulated to form and secrete growth factors, including PDGF. It was further postulated that in its subtle form, injury to the endothelium might cause no morphologic alterations in EC, but might be sufficient to stimulate the cells to form and secrete growth factors. In the *in vivo* lesion, chronic repeated bouts of injury might cause EC to proliferate repeatedly at the edge of intimal lesions, eventually undergoing senescence. Whether these senescent cells release mitogenic substances or other factors more readily or efficiently is not known, but is an interesting speculation in the pathogenesis of atherosclerosis.

Glagov *et al.* (1987) emphasized that a variety of tissue responses occurred within atherosclerotic lesions. They stressed the fact that the endothelial transport system may undergo modification, making transport of LDL and other substances facilitative in atherogenesis development. There was no evidence that there was microinjury of the endothelium at sites of lesion formation; only when the atherosclerotic lesions became large did the overlying endothelium demonstrate intercellular separations or perforations with focal bridging. Also, plaques tended to occur in zones of relatively low flow velocity, where flow fields were complex, and reversed during the cardiac cycle. Perhaps,

they felt, the "low" flow could favor transendothelial transfer of atherogenic substances into the arterial wall, and such interactions in the presence of an otherwise morphologically intact endothelium, rather than one with focal desquamination, could be major factors in lesion development. Finally, Reidy et al. (1987) removed zones of aortic rat endothelium with a thin, flexible nylon catheter, and found no replication of medial SMC and no intimal thickening underlying the mechanical denudation sites. Additional studies along these parameters will illuminate the many factors involved in atherosclerotic plaque progression.

In summary, large, senescent multinucleated EC have been described in both *in vivo* and *in vitro* experimental systems, but their exact significance and role in normal or pathologic processes has not been established. Since these senescent cells are associated with atherosclerotic lesions in humans and animals, and have been found with increasing frequency with aging in both *in situ* and culture conditions, their relevance to aging and systemic changes in the vascular tree needs further clarification and definition. Also, due to the pronounced differences in incidence and severity of atherosclerotic disease in males and females, basic gender differences may play some role.

Materials and Methods

Experimental Methodology

Culture of HUVEC. Harvest of EC from umbilical cord veins was based on the method of Gimbrone et al. (1974). Term umbilical cords were obtained from the Department of Obstetrics and Gynecology at the Medical College of Virginia. Using sterile technique, unclamped umbilical cord segments at least 10 cm. long were cannulated at both ends using one-way stopcocks and the blood flushed by perfusion of 200-400 ml. of phosphate buffered saline solution (PBS- pH 7.2). The lumen of the vein was filled with 0.1% collagenase (Clostridium histolyticum, type I, Sigma Chemical Company, St. Louis, MO) in PBS with calcium and magnesium [$CaCl_2$ (anhyd), 0.10 gm/L, and $MgCl_2 \cdot 6\ H_2O$. 0.10 gm/L], and the stopcocks closed. The cord segment was then incubated in a bath of PBS at 37 degrees C for 15 minutes. The collagenase solution was collected in a conical centrifuge tube with an equal volume of Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. The EC were then pelleted by centrifugation at 200 x g for 5 minutes.

Defined fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) was used. Other media and reagents were from Gibco Laboratories (Grand Island, NY) or Meloy Laboratories (Springfield, VA). The pelleted EC were resuspended in complete Medium 199 (CM 199) containing Earle's Salts, L-glutamine, 100 U/L penicillin, 100 µg/ml streptomycin, 0.25 µg/ml Fungizone, 20% FBS, 90 µg/ml heparin (sodium salt, grade 1, from porcine intestinal mucosa, Sigma Chemical Co., St. Louis, MO), and 20 ug/ml endothelial cell growth factor (ECGF, tissue culture grade, Meloy Laboratories, Springfield, VA or a generous gift from Dr. Thomas Maciag of the American Red Cross, Rockville, MD). Thornton et al. (1983) have described the use of such concentrations of ECGF when heparin is used in the medium, the heparin potentiating the effects of the ECGF. Cultures were grown in gelatin coated T-25 flasks. Gelatin (0.2% wt/vol) promotes the attachment and growth of HUVEC (Gordon et al., 1983; Folkman et al., 1979; Thornton et al., 1983; Walker et al., 1984). Except for periods of time when passing or other experimental manipulations were being performed, cultures were incubated continuously at 37 degrees C in a humidified atmosphere of 95% air, 5% CO_2.

Identification of HUVEC. The endothelial nature of the cultured cells was determined by observing the typical

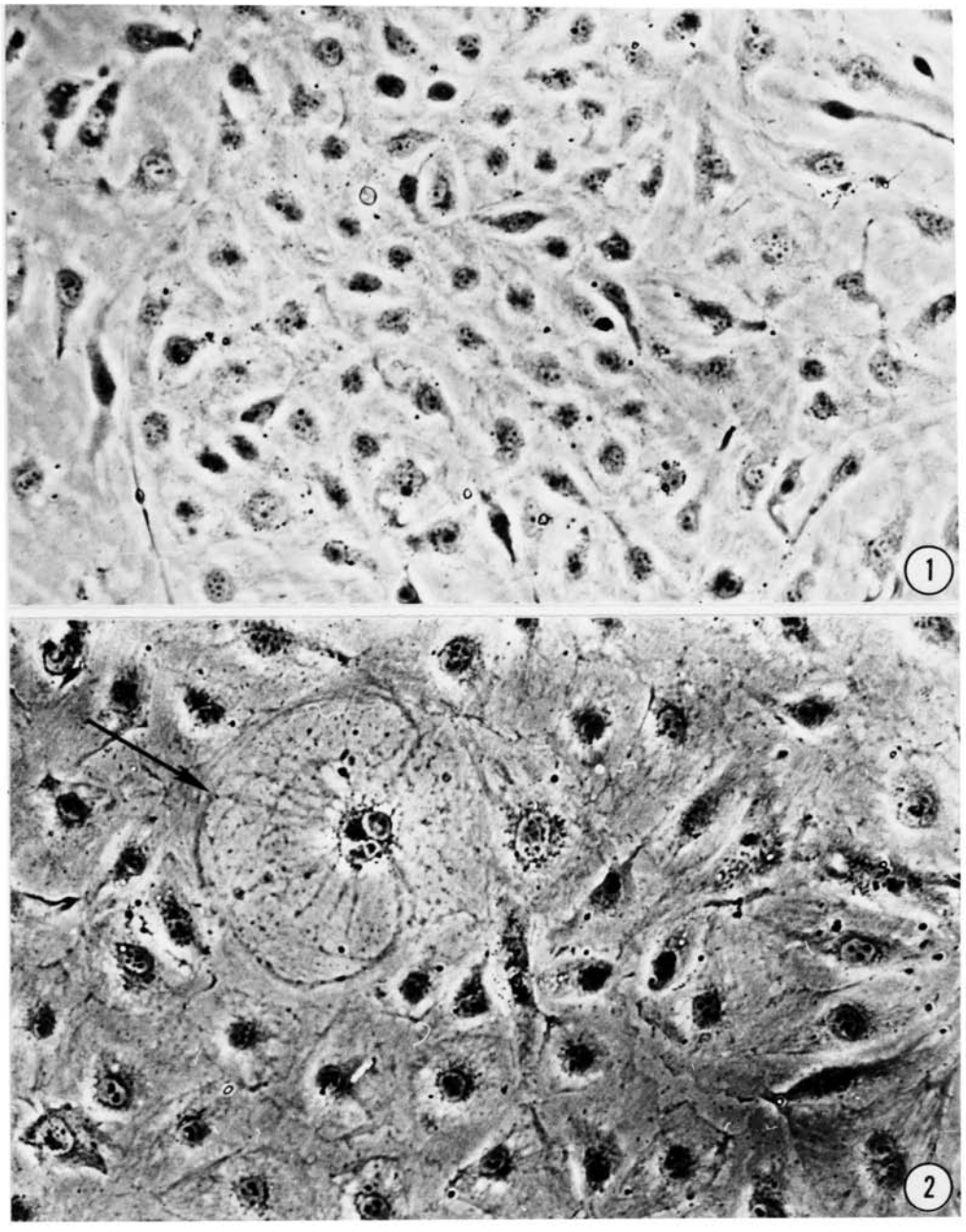
"cobblestone" morphological appearance of the monolayer when viewed under phase contrast microscopy (Nikon Inverted Phase Contrast Microscope, Model M), and by immunofluorescent staining for the factor VIII antigen. Staining for factor VIII antigen was performed on selected confluent cultures.

Identification of Senescent HUVEC. Senescent EC were readily identified by their morphological appearance under phase contrast microscopy. Using a 200 x magnification, senescent cells demonstrated large cytoplasmic areas (larger than non-senescent EC) and a thin, veiled cytoplasm. Senescent cells were often multinucleated, the nuclei clustering centrally. Senescent HUVEC frequently displayed indistinct borders under phase constrast microscopy. If a particular mono-nucleated cell was equivocal concerning size, the presence of veiled cytoplasm was the criterion upon which a cell was judged senescent. Text Figures 1 and 2 demonstrate senescent cells in female and male cultures respectively.

Fixation of EC. At selected times after plating, depending on the experimental paradigm, cultures were fixed using the following procedure. After removal of the medium, the cells were washed twice in fresh PBS (pH 7.2) with calcium and magnesium in order to remove the residual

Text Figure 1. C57 P10. This contrast photomicrograph shows female HUVEC in the tenth passage. Primary cultures of female HUVEC were passed at a 1:10 split ratio for the first three passages. Starting with the fourth passage (P4), the cultures were passed at a 1:5 split ratio. The cells are in a typical swirled configuration, showing darkened, granular cytoplasm, with paler nuclei and prominent nucleoli. Cells still formed a crowded swirled monolayer seven days after the tenth passage. x 260.

Text Figure 2. C58 P10. This phase contrast photomicrograph shows male HUVEC in the tenth passage. Primary cultures of male HUVEC were passed at a 1:10 split ratio for the first three passages. Starting with the fourth passage (P4), the cultures were passed at a 1:5 split ratio. The cells demonstrate changes typical of *in vitro* senescence, with increased cellular size, larger cytoplasmic/nuclear area ratio, a greater accumulation of peri-nuclear cytoplasmic granules, and veil-like cytoplasm. A characteristic multinucleated veiled senescent cell can be seen (arrow) with its large area and "spoke wheel" cytoplasmic cords extending radially from the nuclei to the cell border. At seven days after the tenth passage, the monolayer is less crowded and swirled in appearance when compared to tenth passage female HUVEC. (Text Figure 1). x 260.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serum containing medium. Each flask was washed individually and then immediately fixed, as PBS caused cells to round up and detach if left on the cells after a few minutes. After each flask was washed, 2.5% glutaraldehyde in 0.1M Na cacodylate buffer (pH= 7.3) was added, and the flask was incubated at room temperature for one hour. After incubation, the glutaraldehyde fixative was removed and the flasks were rinsed once with 0.1 M Na cacodylate (pH= 7.3) containing 7% sucrose. The cultures were then stored in the sucrose cacodylate buffer at 4 degrees C until further processing and evaluation were undertaken. Selected fixed flasks were photographed under phase microscopy using 35 mm Pan X film and a Nikon EFM camera system.

Assessment of Male-female senescent patterns. To assess male and female senescent patterns, selected male-female culture pairs from the same desired passage were grown for seven days and fixed as described above. In certain cases, cultures were fixed at four days after passage. Cellular counting to determine the percentage of veiled (senescent) cells was done with the aid of a 25 cm^2 etched glass plate aligned to the bottom of the culture flask. The glass plate divided the culture surface of the flask into 25 separate squares, each measuring one square centimeter. In order to eliminate experimental bias

during flask evaluation, counting was performed using a single blind method. The flasks were coded by someone other than the evaluator after identifying labels were removed from the flask. In this way, the origin, gender, and passage number of the cells were unknown to the evaluator during the counting process. The experimental code was not broken until all flasks from a particular experiment had been evaluated.

Cellular counting was performed with the aid of an ocular grid in combination with a 10x widefield ocular and a 20 x objective lens on a Nikon binocular phase contrast microscope. With this 200x magnification, the area encompassed by the grid was 0.117649 sq. mm. Sixteen grid areas per flask, within 16 of the 25 large (1 cm^2 squares, were evaluated (please see the Senescent Data Assessment Form in the Appendix). Within all 16 grids, all senescent cells were counted (S). In four of the sixteen grids (grids 1,6,11,and 16), all non-senescent cells were counted (N) and multiplied by a factor of four (4N). The total number of cells (T) in the 16 grids was estimated as 4N + S. The percentage of senescent cells was equal to the sum of senescent cells in the 16 grids divided by the total number of cells in the grids, or S/T x 100. From this information, the cellular density of the flask (number of cells per cm^2) was calculated. By multiplying

cellular density by the percentage of senescent cells, the senescent cell density was derived. For each flask at each passage, the data were expressed according to the following three parameters: the percentage of senescent cells, the senescent cellular density, and the total cellular density.

Experimental Designs

Effect of Culture Gender on Senescent Expression and Passage Number (1:5 split ratio). In order to determine the effects of gender and passage number on the percentage of senescent cells and cellular density, three different male-female culture pairs were used. Each male-female culture pair was begun on the same day. The culture pairs were fed with CM-199 every two or three days and passaged on a weekly basis at the split ratios indicated below. Beginning at selected passage numbers, cultures from the male-female culture pairs were fixed on the seventh day after passage and the percentage of senescent cells in the cultures determined as described above. Cellular density, i.e. the number of cells per square centimeter, was also determined. The male-female differences, if present, were also related to the cell passage number, so that variation

in the amount of senescence could be determined as the culture increased in age.

The C 57(F) - C 58(M) culture pair (Pair I) was passed at a 1:10 split ratio through the fourth passage (P1-4). Beginning with P5, the cultures were passed at a 1:5 split ratio. The rationale for the early passage at a higher split ratio lay in the fact that premature confluence and overcrowding ensued if primary and early passage cultures were passed at a 1:5 split ratio. At each passage for Pair I, four or five female and four or five male flasks were plated, and the cultures refed with CM 199 every two or three days. On the seventh day after plating, a male and female flask were selected to continue the cell line and passed at the desired split ratio. The cultures were carried in this manner through the twentieth passage. Beginning with P10 and continuing through P20 inclusive, a male and a female flask from each passage were glutaraldehyde-fixed weekly on the seventh day after plating and stored in sucrose-cacodylate buffer at 4 degrees C and subsequently evaluated for senescent expression. Thus, the three parameters of senescence expression (percentage of senescent cells, senescent cellular density, and total cellular density) for each of 22 flasks (11 female and 11 male) from 11 passages (P10-20 inclusive) comprised the data for C 57(F) and C 58(M).

The primary cultures of the Pair II, C 59(F) and C 60(M) were passed weekly at a 1:10 split ratio through the third passage (P1-P3). Beginning with P4, the cultures were passed weekly at a 1:5 split ratio. The cultures were fed with CM 199 every two or three days. The culture pair was continued in this manner through the nineteenth passage. Beginning with P9 and continuing through P19 inclusive, a male and female culture from each passage were glutaraldehyde fixed weekly on the seventh day after plating, stored in sucrose cacodylate buffer at 4 degrees C and subsequently evaluated for senescent expression. The three parameters of senescent expression for each of 22 flasks (11 female and 11 male) from 11 passages (P9-19 inclusive) comprised the data for C 59(F) - C 60(M).

In order to assess senescent expression in HUVEC at an earlier point in the culture life span, the primary cultures of Pair III, C 63(F) and C 64(M), were passed weekly at a 1:10 split ratio through the third passage (P1-P3). Beginning with P4, the cultures were passed weekly at a 1:5 split ratio. Cultures were fed with CM 199 every two or three days. The culture pair was continued in this manner through the ninth passage. Beginning with P1 and continuing through P9 inclusive, a male and female culture from each passage were

glutaraldehyde fixed weekly on the seventh day after plating, stored in sucrose-cacodylate buffer at 4 degrees C and subsequently evaluated for senescent expression. The three parameters of senescent expression for each of the 18 flasks (nine female and nine male) from nine passages (P1-9 inclusive) comprised the data for Pair III.

Effects of Culture Gender and Passage Number on Senescent Expression (standard density). In order to determine the effects of gender and passage number on senescent expression in HUVEC plated at standard density (1.25×10^5 cells/flask), a male female culture pair (C 67(F) and C 68(M)) was begun on the same day. The primary cultures of this pair were trypsinized and passed at standard density at seven days. At weekly intervals, one male and one female culture per culture pair were used to plate flasks at standard density for each succeeding passage. Passage was repeated until terminal senescence, defined as failure of the cultures to double in population in seven days. Beginning with P2 and continuing through P16 inclusive, a male and a female flask from each passage were glutaraldehyde-fixed on the seventh day after plating, stored in sucrose buffer, and then evaluated for senescent expression. The three parameters of senescent expression for each of the 30 flasks (15 female and 15 male) from 15

passages (P2-16) comprised the data for this phase of the study.

In the above experiments, when the flasks were fixed at seven days after plating, most of the cultures so evaluated tended to be confluent or subconfluent, especially in earlier passages. Since crowding may affect the number of senescent cells in a given culture, it was decided to evaluate male and female cultures at five days after plating. In order to assess senescent expression at this earlier time, the same male-female culture pair (C 67(F) -C 68(M)) was used. Beginning with P2 and continuing through P14, a male and female culture from each passage were glutaraldehyde-fixed weekly on the fifth day after standard density plating, stored in sucrose buffer, and subsequently evaluated for senescent expression. The three parameters of senescent expression for each of the 26 flasks (13 female and 13 male) from 13 passages (P2-14 inclusive) comprised the data for the five day assessment. Enough culture flasks were plated at standard density each week to run both the five and seven day assessments concurrently.

Effect of Plating Density on Senescent Expression. It has been our observation that cultures plated at a 1:10 split ratio actually showed more sustained proliferative ability

than those passed at a 1:5 ratio. In order to maintain the cell turnover, thereby "aging" the culture at a more rapid rate, the primary cultures of a male-female pair (C 69(F) - C 70(M)) were passed weekly at a 1:10 split ratio through the ninth passage (P1-9). Cultures were fed with CM-199 every two or three days. Beginning at P10, the cultures were trypsinized and passed at standard density (1.25×10^5 cells/flask) at weekly intervals. Passage was repeated until the cell number failed to double within seven days. The female cultures were carried through P19, and the male cultures through P17. Beginning with P6 and continuing through P17, a male and female culture from each passage (except P10) were glutaraldehyde-fixed on the seventh day after passage, stored in sucrose buffer, and subsequently evaluated for senescent expression. The three parameters of senescence expression for each of the 22 flasks (11 female and 11 male) from 11 passages (P6-17, exclusive of P10) comprised the data. It should be reiterated that flasks fixed and evaluated from P6-9 were from a 1:10 split ratio passage, while flasks fixed and evaluated from P11-17 were from a standard density passage.

Effect of a 1:4 vs. a 1:16 Split Ratio on Senescent Expression. In order to ascertain if cultures plated at higher density express a greater degree of senescence than

cultures plated at a lower density, the following experiment was done. The primary cultures of a male-female pair (C 75(F) - C 76(M)) were passed weekly at a 1:10 split ratio through the third passage (P1-P3). At P4, three male and three female cultures were plated at four times standard density (5 x 10^5 cells/flask). The cultures were fed with CM 199 every two or three days. Beginning with P5, the cultures were passed weekly at a 1:4 split ratio. For comparison, cultures were plated at a lower density as follows. Using the same male-female pair, three male and three female cultures were plated at P4 at standard density. Beginning with P5, the cultures were passed weekly at a 1:16 split ratio. The cultures were fed with CM 199 every two to three days. Cultures plated at both densities were carried through the sixteenth passage. Two male and two female flasks from each passage were glutaraldehyde-fixed on the seventh day after plating, stored in sucrose buffer, and subsequently evaluated for senescent expression. A total of 96 flasks were assessed: 48 male flasks (24 at a 1:4 split and 24 at a 1:16 split) and 48 female flasks (24 at a 1:4 split and 24 at a 1:16 split). Comparisons regarding senescent expression were thus made for both gender and split ratio.

Results

Effect of Culture Gender and Age on Senescent Expression (1:5 split ratio)

The purpose of these experiments was to determine if culture gender and age had an effect on senescent expression in HUVEC. Three separate male-female culture pairs were used. In these and all experiments described in this study, even numbered cultures were female EC, and odd-numbered cultures were male EC. The primary cultures were passed at a 1:10 split ratio from P1-4 for the C 57-C 58 pair (I), and from P1-3 for the C 59 - C 60 pair (II), and at a 1:5 split ratio for both thereafter. Table 1 illustrates the total cells, the total senescent cells, and the percentage of senescent cells, generated in a cm^2 area over ten passages (P10-19). The percentage of senescent cells is also illustrated by bar graphs in Figure 1. Chi square analysis, using the total number of senescent cells and the total number of non-senescent cells generated in a cm^2 area, was the test statistic used to compare the percentages of senescent cells in this and all subsequent experiments. Likewise, for all experiments, the Wilcoxon Rank Sum Test was the test

statistic employed to compare the total cells generated in a cm^2 area over the passages indicated. In this results section, and the discussion section that follows, total senescent cells and total cells always signify the number of senescent cells or total cells generated in a cm^2 area over the passages indicated.

Table 1 and Figure 1 show that for ten passages (P10-19), in both Pair I and Pair II, male HUVEC showed a significantly greater percentage of senescent cells and significantly fewer total cells than female HUVEC. In Pair I, male flasks generated almost three times as many senescent cells over ten passages as female flasks. The number and percentage of senescent cells for male cultures increased with increasing passage number, yet the total cell number was significantly lower than that for female cultures. In Pair II, these same relationships held true. As in Pair I, male cultures had a significantly greater total senescent cell number and percentage of senescent cells than females, yet significantly fewer total cells. The bar graphs in Figure 1 demonstrate the percentage of senescent cells for the two culture pairs; the significantly greater percentage of senescent cells in the male cultures compared to female cultures should be noted.

Table 2 is a comparison of senescence in the five earlier passages (P10-14) versus the five later passages (P15-19) for the male-female culture pairs described in Table 1. The percentage of senescent cells for each culture and passage series are shown by bar graphs in Figure 2. Table 2 and Figure 2 demonstrate that the male-female differences in percentage of senescent cells are sustained for both culture pairs for both the earlier (P10-14) and later (P15-19) passages. There was a significantly greater number of total cells in the P10-14 passages than the P15-19 passages for female cultures. However, this was not true for male cultures (C58 and C60). Table 2 also shows that when female cultures were compared to male cultures regarding total number of cells, there was a significant difference for the P10-14 passages but not the P15-19 passages. This implies, then that most of the male-female difference regarding cumulative cellular density occurred during the first five passages (P10-14). However, the percentage of senescent cells did not differ significantly between P10-14 and P15-19 for any of the culture pairs, although there was a trend toward an increase in later passages. Again, comparing earlier and later passages, the sum of senescent cells increased while the total number of cells decreased.

In order to assess senescent expression in HUVEC earlier in the culture life span, male-female culture Pair III (passed at a 1:10 split ratio until P4, then at a 1:5 split ratio through P9), was studied in passages P1-9. Table 3 compares total cells, sum of senescent cells, and the percentages of senescent cells for passages P1-4 with those for passages P5-9. Although the percentages of senescent cells were higher in male than in female cultures, these differences were not statistically significant. However, the percentages of senescent cells were significantly greater for later passages (P6-9) compared to earlier passages (P1-4) for both male and female cultures. Note that this was not the case for Pair I or Pair II. Whether the increase in senescent expression in Pair III (C 63 - C 64) during P6-9 over that observed in P1-4 was due to the higher density (1:5 vs. 1:10 split ratio) of plating cannot be ascertained definitively; a later experiment (Table 7) addresses this question more specifically. Since Pair III was not continued beyond P9, it is not possible to determine if the increase in senescence which occurred during P6-9 would have been moderated in later passages (i.e. P10-19), as in the first two culture pairs studied. Table 3 also shows that there was a significantly greater total number of cells in P1-4 vs. P6-9 for female cultures, but not for male cultures.

In summary, when primary cultures of HUVEC were passed initially at 1:10 split ratios followed by a longer series of passages at 1:5 split ratios, there was a significantly greater expression of senescence in male cultures than in female cultures. Concerning the total number of cells generated over an equal number of passages, a significant difference between earlier and later passage groups was found for female cultures but not for male cultures.

Effect of Culture Gender and Passage Number on Senescent Expression (standard density plating)

In the previous experiments, primary cultures that were cobblestone in appearance and confluent were passed at 1:10 split ratios followed by 1:5 split ratios. The purpose of the next set of experiments was to correct for possible differences in confluent cell density between male and female primary cultures. This was done by plating at a standard density (SD- 1.25×10^5 cells/flask) in all passages. As in the previous experiments, two passage series were compared to determine the effect of passage number, as well as gender, on senescent expression. Both five day and seven day assessments were

performed, and the results are depicted in Tables 4 and 5 and in Figure 3.

Table 4 compares total cells, sum of senescent cells, and percentages of senescent cells for three series of four passages each for male and female cultures in a five day assessment. There was no significant difference in senescent expression between female (C 67) and male (C 68) cultures for any of the three passage series compared. Nor were there significant differences in percentages of senescent cells among the three passage series for male or female cultures. Note that in the male cultures, the percentage of senescent cells was greater in earlier passages (P2-5) than later passages (P6-9 or P10-13), a finding which is somewhat unexpected. In the later passages, there was a trend for female cultures to show a greater percentage of senescent cells than male cells, but the difference was not significant.

Regarding total number of cells generated over the passages indicated, the only significant difference between male and female cultures was in the P10-13 group (Table 4). In the SD study, in contrast to earlier findings, the difference in total cells generated during early (P2-5) passages vs. later passages (P6-9, P10-13) was significant for male cells but not for female cells.

In fact, in contrast to male-female culture pairs passed at a 1:5 split ratio, Table 4 shows that the total number of cells increased during passages 6-9 compared to 2-5 for both females and males, although significant only in the latter. This is paradoxical, since one would have expected the total number of cells to decrease in succeeding passages. However, since this was a five day assessment, cultures evaluated at seven days might not show this "reverse" phenomenon. Indeed, this increase in total cell number generated in later passages was not true for the seven day assessment (see Table 5).

Table 5 compares total cells, sum of senescent cells, and the percentages of senescent cells for three series of four passages each for the same male and female cultures described above, this time in the usual seven day assessment. Again it is seen that there was no significant difference in percentages of senescent cells between males and females for any of the three passage series studied. The percentages of senescent cells increased in succeeding passage groups for both male and female cultures. For female cultures, the percentage of senescent cells was significantly greater in the later passage series (P10-13) compared to either the P2-5 or the P6-9 series; a similar trend in the male cultures was not significant.

When the total cells generated in the three passage series were compared, no significant differences were found between male and female cultures for any of the three series. In fact, the three series parallelled each other, with similar decreases in cumulative cellular density as the culture age progressed (Table 5). For both male and female cultures, there were significantly more total cells generated in P2-5 than in P10-13. This finding contrasted with earlier experiments (1:5 split ratio), in which only the female cultures displayed a similar significant difference.

When one compares the five and seven day assessments (Tables 4 and 5, Figure 3), some interesting patterns are noticed. There were no significant differences between five and seven day assessments regarding percentages of senescent cells generated over the three passages series for either female or male cultures. However, Figure 3 reveals a trend toward greater percentages of senescent cells in cultures assessed at five days vs. seven days. This was true for all three passage series in female cultures and for the P2-5 series in male cultures. Since multinucleated veiled (senescent) cells have not been observed to proliferate in cultures of HUVEC, these cells would be passed along at a relatively fixed number,

increasing only slightly as a result of terminal differentiation in each passage. The relatively constant number of senescent cells would then constitute a higher percentage of the total cell population at five days than they would at seven days, when a continued proliferation of non-senescent cells would have increased the total cell number.

In comparing total cells at five versus seven days, the increase at seven days was significant only for earlier passages (P2-5) for both male and female cultures (Tables 4 and 5). In fact, there was a decrease in total cells generated in later passages at seven days for male cultures but not female cultures (Tables 4 and 5).

In summary, when cultures of HUVEC were passed at standard density, there was no significant difference in total cells, sum of senescent cells, or percentages of senescent cells between male and female cultures. This was true for both five day and seven day assessments. There was a trend for the percentage of senescent cells to be greater in cultures assessed at five days than at seven days, but the difference was not significant. There was a significant decrease in the total cells generated between early and later passages for both male and female

cultures, in contrast to earlier experiments where this difference was significant for female cultures only. The total cells generated at five days for later passage male cultures was actually greater than that found at seven days.

Effect of 1:10 Split Ratio Passage Followed by SD Passage

The purpose of the next set of experiments was to compare senescent expression between male and female cultures that had been "aged" in terms of population turnover by repeatedly passing the cultures at a low density (1:10 split ratio). In these experiments, a primary male-female culture pair (P 69 and P 70) was passed at a 1:10 split ratio up to and including P9, then subsequently at standard density. Table 6 compares the total cells, sum of senescent cells, and the percentages of senescent cells for two series of four passages (P6-9, P11-14) and one series of three passages (P15-17) for male and female cultures. There were no significant differences in the percentages of senescent cells between male and female cultures for any of the three passage series studied (Table 6 and Figure 4). However, there was a larger, albeit non-significant, difference between male and female cultures for P6-9, where the cultures were passed at a 1:10 split ratio, than there was between male

and female cultures for P11-14 or P15-17, where the cells were passed at standard density. This is better illustrated by the bar graphs in Figure 4.

As in previous experiments, differences in total cell number were apparent for female, but not male cultures. There was a significant difference in total cells between P6-9 and P11-14 for female, but not male, cultures. As in previous experiments, the percentage of senescent cells increased, while total cells decreased with increasing passage number.

Comparison of 1:4 and 1:16 Split Ratio Passage

Although our earlier experiments with a 1:5 split ratio passage demonstrated a greater expression of senescence in male cultures than in female cultures, this difference was not sustained when initial cell number was corrected for by passage at standard density. The possibility of differences in passage density being a factor in senescent expression was examined in the present experiment, which was designed to determine if cultures passed at a higher density (1:4 split ratio) would show a greater degree of senescence than cultures passed at a lower cell density (1:16 split ratio). Male and female differences were also examined within this experimental

paradigm. Table 7 compares total number of cells, sum of senescent cells, and the percentages of senescent cells generated for male and female cultures for five earlier passages (P5-10) with those of five later passages (P11-16) for the 1:4 split ratio cultures and the 1:16 split ratio cultures.

Table 7 shows that there was not a significant difference between male and female cultures regarding percentage of senescent cells for either the 1:4 split ratio group or the 1:16 split ratio group. When early (P5-10) and later passages (P11-16) were compared, there was a significantly greater percentage of senescent cells in later passages of both male and female cultures when the cultures were passed at a 1:4 split ratio (high density), but not when they were passed at a 1:16 split ratio (low density). Also, the difference in number of senescent cells was greater for the former vs. the latter. Lastly, for both early and late passage series, and for both male and female cultures, there was a trend toward cultures passed at a 1:4 split ratio to show a greater percentage of senescent cells than cultures passed at a 1:16 split ratio. However, this difference was significant only for female cultures at a later passage (Table 7 - C 75, 1:4, P11-16 compared to C 75, 1:16, P11-

16). The percentage of senescent cells is illustrated by bar graphs in Figure 5.

Table 7 demonstrates that there were significantly more total cells generated in earlier (P5-10) than in later (P11-16) passages for both male and female cultures passed at high density, as well as for female cultures passed at low density. In comparing the high density cultures to the low density cultures, there was a significantly greater total number of cells generated for female cultures from later passages (P11-16) at low density (1:16 split ratio) than in later passage female cultures at high density (1:4 split ratio). This difference parallels the significant difference seen in the percentages of senescent cells between these passage series. In viewing Table 7 and Figure 5, there was a trend for both male and female cultures passed at low density to generate a greater number of cells and exhibit fewer senescent cells for comparable passages than their companion cultures passed at a high density, although this difference was significant only for the female cultures mentioned above. From the above, it appears that culture density at passage may be at least as important in determining cellular senescent expression in HUVEC as culture gender.

Discussion

The existence of multinucleated, veiled cells, considered to be senescent, has been known since the earliest successful cultures of endothelium were established from human umbilical veins. Mayuma (1963), the first author to report the successful culture of endothelial cells isolated from human umbilical veins, described senescent cells in his cultures. Since in vitro aging is now a recognized phenomenon in cultures of human EC (Levine and Mueller, 1979), the present study was begun to document quantitatively the occurrence and expression of senescence in cultures from male and female cords, in early and late passages, and at various passage densities. Since our initial observations suggested that there was a difference between male and female cultures of HUVEC regarding the incidence and progression of endothelial cellular senescence, experiments were undertaken to determine quantitatively if cellular gender was a significant factor in the expression of endothelial cellular senescence. Male-female differences in senescent phenotypic expression have not been addressed previously in the literature. The working hypothesis of this study was that possible gender differences regarding expression

of *in vitro* senescence might help to explain the variations in growth and culture life span observed in cultures from different umbilical cords (previously of unknown sex).

The major findings of this paper can be summarized as follows:

1. When primary cultures of HUVEC are passed at a 1:10 split ratio for the first three or four passages, and subsequently passed at a 1:5 split ratio, there was a significant difference in senescent expression between male and female cultures. This difference was expressed for both the P10-14 and the P15-19 passage series, suggesting that gender differences in senescent expression were sustained through the passages studied. Under these passage conditions, there was a significant difference in total number of cells generated over equal numbers of passages between earlier (P10-14) and later passage (P15-19) groups for female, but not male, cultures.

2. When cultures of HUVEC are passed at standard density, there were no significant differences in total cells, total senescent cells, or percentages of senescent cells between male and female cultures. This phenomenon was true in both five day and seven day assessments.

There was a significant decrease in the total cells generated between early and later passages for both male and female cultures, in contrast to earlier experiments where this difference was significant for female cultures only. The total cell generated at five days for later passage male cultures was actually greater than that found at seven days.

3. When cultures of both male and female HUVEC were passed at high density (1:4 split ratio), significantly greater senescence expression occurred in later passages vs. earlier passages, an effect not seen when the cultures were passed at a lower density (1:16 split ratio). This suggested a hastening of the senescent rate when cultures are passed at a higher density, an effect which appears to be gender independent.

4. There was a trend for both male and female cultures passed at a lower density to generate a greater number of cells, fewer senescent cells, and exhibit a lessor percentage of senescent cells over comparative passage series when contrasted with male and female cultures passed at a higher density.

Our results demonstrated that when primary cultures of HUVEC are passed at low density (1:10 split ratio) for

the first several passages and subsequently passed for a longer period at high density (1:5 split ratio), male cultures developed a significantly greater percentage of senescent cells than female cultures. However, when initial cell numbers were standardized by passing the primary cultures at a uniform density, significant differences in senescent expression between male and female cultures did not occur. Further experiments suggested that cultures passed consistently at a low density exhibited a lessor degree of senescent expression than cultures passed at a higher density, and that this effect was apparently gender-independent. This implied that cellular density at passage might be more important than gender in the expression of senescence in HUVEC.

When cultures of HUVEC were passed at standard density, there were no significant differences in senescent expression or total cells generated between male and female cultures (Tables 4 and 5; figure 3) at either the five day or the seven day assessment. At five days, the percentage of senescent cells was greater in earlier passages (P2-5) than later passages (P6-9 or P10-13) in the male cultures, and this finding was accompanied by significantly fewer cells generated at earlier passages than at later passages, which might explain the greater senescent expression in the earlier passage male cultures.

Another factor might be that the number of senescent cells did not increase appreciably during later passages for male cultures assessed at five days (Table 4). It is possible that male cultures exhibited slower growth during later passages (P6-9, P10-13) at seven days; total cells actually decreased in later passage male cultures at seven days compared to five days.

The transition from Phase II to Phase III in the life history of a cell culture, signified by an increase in the number of senescent cells and a decrease in cellular growth, may be rather abrupt in fibroblasts (Grove and Cristafalo, 1977; Haymark, 1976). This appears to be true in the culture system using EC, particularly when cells are passed at standard density. Note Table 5, which shows an abrupt change in the total cells, total senescent cells, and percentages of senescent cells between the P6-9 passage series and the P10-13 passage series for female cultures.

The "aging" of HUVEC by passing primaries at a 1:10 split ratio for nine passages followed by standard density passing did not produce a significant difference between male and female cultures regarding senescent expression or total cells generated for any of the three passage series (Table 6). However, Table 6 shows that male-female

differences that did exist were greater for earlier passages (P6-9) compared to later passages (P11-14). It is interesting that male-female differences in senescent expression tend to decrease when cultures are passed at standard density. This is illustrated in Table 6 which demonstrates that there were fewer senescent cells and a lower percentage of senescent cells for early passage female cultures (P6-9), accompanied by a greater cellular density, compared to early passage male cultures.

The results in Table 6 and Figure 4 further encouraged the author to explore cellular density at passage as a possible factor in senescent expression in male and female HUVEC. Glassberg et al. (1982), who cultured EC from the human iliac artery, found that cells became senescent, losing viability at passages 8-10, when passed at a 1:3 split ratio. However, no comparisons were made with cultures passed under different density conditions. Cristafalo (1976) contended that HUVEC senesce at different rates, depending on the split ratio at passage, although he did not offer quantitative data to support his statement.

It is known that senescent EC are more common in confluent cultures than in less crowded cultures. Since endothelial cellular senescence represents a form of

differentiation (whether it is terminal differentiation is currently controversial), our findings suggest that inhibition of expression of this differentiated phenotype can be done by increasing the split ratio at passage, i.e. passing the cultures at a lower density. This results in less crowding in each passage, giving the cells more room to grow and proliferate, thereby decreasing the chances for the senescent phenotype to be expressed. Passing at a 1:16 split ratio would decrease the chances of the relatively fewer number of senescent cells reaching the next passage. This, in combination with relatively greater growth in the cultures passed at lower density, might result in a lower senescent percentage for the low density passage cultures. On the other hand, since the growth of EC is generally contact inhibited, passing the cultures at a high density (1:4 split ratio) would cause the cells to reach confluence rapidly, stop proliferating, and undergo senescence. Subsequently passing the cultures further at a 1:4 split ratio would increase the likelihood of the senescent cells appearing in the next passage.

An additional factor here might be the period of time spent at confluence prior to culture fixation and evaluation. Endothelial cultures passed at high density (1:4 split ratio) would reach confluence sooner than cultures passed at lower density (1:16 split ratio), the

former conceivably spending a longer time in the confluent state than the latter. This would create a greater tendency for the senescent phenotype to be expressed, hence, the greater percentage of senescent cells in cultures passed at a high density.

Confluent cultures of HUVEC will senesce rapidly if not passed. As total cell number decreases as the culture ages, the percentage of senescent cells increases. Note that this can occur without an appreciable increase in the absolute number of senescent cells from passage to passage. Regarding senescent expression, the culture density at passage may be a stronger factor in senescent expression than the number of population doublings that the culture has undergone by that same passage.

Only a few authors have attempted to estimate quantitatively the incidence of senescent veiled cells in human umbilical vein endothelial cellular cultures. Knaver and Cunningham (1983) estimated the incidence of endothelial cellular senescence to be 1-2% in early cultures, and up to 10-12% at approximately Passage 16, although they did not indicate the cellular plating density or passage split ratio used. Their estimates are comparable to those found in this study, except that there was a much higher percentage (24-28%) of senescent cells

in later passage (P15-17) HUVEC passed at a 1:10 split ratio followed by standard density passage (See Table 6 and Figure 4). Maciag et al. (1981) estimated the density of senescent EC in cultures of HUVEC (1/1000 cells, 0.1% initially increasing to 1/100, 1% by passage 14). Their estimates are somewhat lower than those reported in this research, as the percentage of senescent cells averaged much more than 1% by passage 14 in all passage paradigms that were used.

The heterogeneity of the endothelial cellular culture, mentioned by Schwartz et al. (1981), might be a factor in senescent expression in both male and female cultures. Senescence observed in endothelial cells in vitro might be the result of the random distribution of senescent cells during passage of the culture, rather than abrupt increases in senescent cells derived from normal cells during that particular passage. In a like manner, limitations in the replicative lifespan of cultures of HUVEC might be due to random distribution of proliferative potential in the culture.

According to Gospodarowicz et al. (1978), senescence in HUVEC may be a pre-programmed event. The question of whether human umbilical vein endothelial cells are programmed to die shortly after the birth of the child has

not been answered. Or do these cells die because the umbilical cord is severed, and the EC, dissociated from their normal connection to the placenta, then die. No one has kept an umbilical cord viable *in situ* for much longer than nine months, so this question remains unanswered. The fact that senescent cells can be found in primary cultures of HUVEC (Maciag *et al.*, 1981) suggests that senescent cells were present *in vivo* in situ. Since the umbilical cord forms and degenerates within nine months, senescent cells in primary cultures may be due to the "accelerated aging" in human umbilical vein endothelium, or alternatively, the cells may became senescent or undergo senescent-like changes when they are plated. It is clear that factors which would affect senescent expression in primary and early passage cultures of HUVEC need further study and exploration.

Are multinucleated cells evidence of attempted regeneration following cellular injury? There is the possibility that enzymatic dissociation, as occurs when the cultures are passed, may play a role in senescent expression in HUVEC (Ruben & Rafferty, 1978). *In vitro* senescence is typically evaluated in cultures of EC where subculturing methods are via this enzymatic dissociation (collagenase for primaries, trypsin for secondary and subsequent subculturing). The actual effect of repeatedly

breaking the bonds between cells and their extracellular substrate upon senescence, as occurs during trypsinization and passing, is unknown.

The question of whether senescent cells represent a syncytium, where an endothelial cell divides but fails to break up into individual cells, the nuclei remaining within the center of the senescent cell, remains unanswered. Why this would specifically occur is not known. Future research might employ "wounding" an aged culture, and then exploring the leading edge of the migrating, dividing EC and determining the effect on senescent patterns.

The role of the senescent endothelial cell in the process of tumor invasion and metastasis constitutes a largely unexplored area of research. By nature of its structure, the senescent cell, with its veiled, thread-like cytoplasmic extensions bridging the nucleus and its large area, might be more susceptible to the ability of an attached tumor cell to invaginate the endothelial cell, thus making the senescent cells more susceptible to transendothelial intravasation or extravasation of primary or metastatic tumor cells respectively. However, this is purely speculative at this time.

Rosenthal et al. (1981) suggested that cellular senescence in EC may contribute to the genesis of atherosclerosis. Haudenschild et al. (1975) remarked that veiled, senescent endothelial cells are commonly observed on the edge of intimal lesions in vivo. Weber et al. (1984) suggested that endothelial cellular senescence in vivo may be the result of either high cell turnover or repeated endothelial injury. High cell turnover is a likely possibility when one considers that the incidence of senescent endothelial cells increases with cell passage number. However, repeated endothelial injury, although not sufficient to mechanically denude the vessel wall, may encourage endothelial cellular turnover. If, however, the cells are prevented from proliferating due to lack of room in which to divide, as in confluent cultures of EC in vitro, division may occur without cytoplasmic separation, with senescent cells ensuing. Whether the senescent phenotype is more apt to be expressed in areas of endothelial cellular microinjury is unknown. However, it should be remembered that confluent EC have a low but definite rate of cell turnover, which may approximate the state of "injury" in vivo. In the in vivo situation, chronic repeated bouts of subtle endothelial "injury" might cause the EC to proliferate repeatedly at the edge of intimal lesions, which can result in senescent cells. However, the data from the 1:4 and 1:16 comparison

experiment would refute the concept that simple continuous cell turnover in EC, as would occur to a greater degree in the 1:16 split ratio passage cultures, would increase the number of senescent cells present. In fact, this study has suggested that passage at the 1:16 split ratio reduces senescent expression compared to higher density passage, where proliferation is less. Maciag et al. (1981) reported that endothelial cells passed at a greater density may be affected by contact inhibition, and may not proliferate as rapidly. This might well affect the expression of endothelial cellular senescence.

It can be recalled that in any culture system, there will be deficiencies in the media or procedural components of cellular culture, perhaps contributing to the gradual differentiated state of the culture in question. How much of the senescent process in vitro can be attributed to the culture environment and how much to senescent phenotypic expression of the cells themselves is an unsettled question.

Do the senescent cells secrete or promote activation of growth factors of other substances which enhance the proliferation of smooth muscle cells and/or induce alterations in the endothelial substrata? Or are the senescent cells the result of the transformation induced

in the endothelium by the atherosclerotic lesion itself? Presumably, an expanding mural lesion would stretch the overlying EC, perhaps making them more likely to express their senescent phenotype.

The translation of information obtained from the *in vitro* situation to the events observed *in vivo* is not always easy, and in many cases purely speculative. However, the above questions and additional courses of inquiry concerning senescent expression of HUVEC afford attractive future avenues of study of this remarkable process.

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Appendix

Figure 1. Effect of culture gender on senescent expression in HUVEC passed at a 1:5 split ratio (P10 - P19). The C 57(F) and C 58(M) culture pair and the C 59(F) and C 60(M) culture pair were passed weekly. A male and a female flask from each culture pair were fixed and evaluated weekly. The bar graphs illustrate the percentage of senescent cells, calculated by dividing the sum of of senescent cells/cm^2 generated during P10 - P19 by the total number of cells/cm^2 generated during these passages. F = female; M = male. a significantly different from b and c from d, $p < 0.001$.

Figure 1. Effect of Culture Gender on Senescent Expression in HUVEC Passed at a 1:5 Split Ratio (P10-P19)

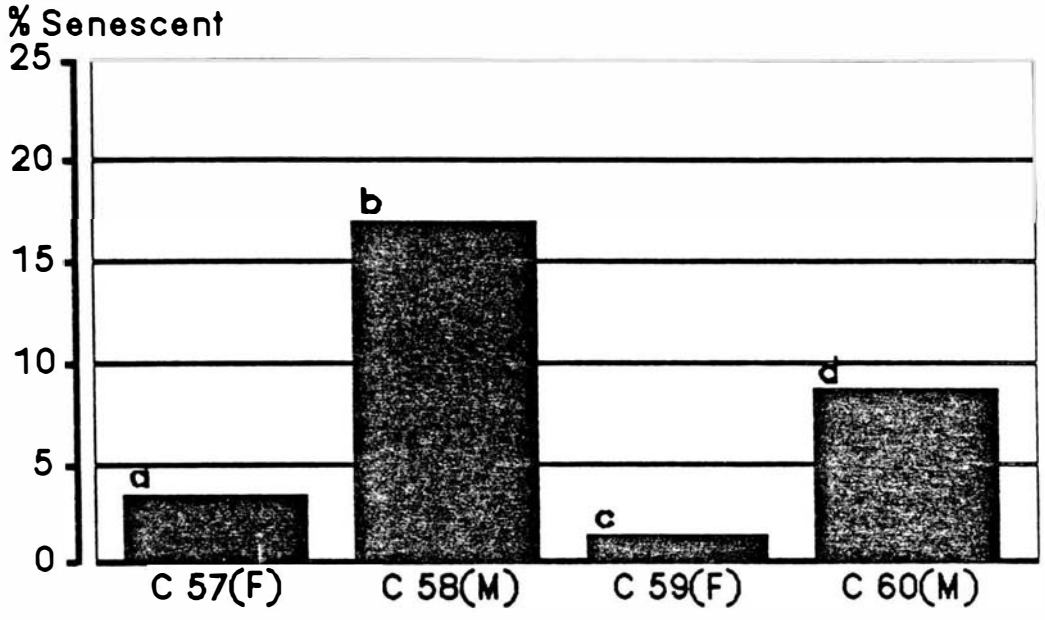


Figure 2. Effect of culture gender and passage number on senescent expression in HUVEC (1:5 split ratio). The same male-female culture pairs described in Table 1 and Figure 1 were used to compare senescent percentages for five earlier passages (P10 - P14) with those of five later passages (P15 - P19). The bar graphs show the percentage of senescent cells exhibited by each culture, calculated by dividing the sum of senescent cells generated during the passages indicated by the total number of cells generated during the same passages. a significantly different from c; b from d; e from g, all $p < 0.001$. f significantly different from h, $p < 0.01$.

Figure 2. Effect of Culture Gender & Passage Number on Senescent Expression in HUVEC (1:5 Split Ratio)

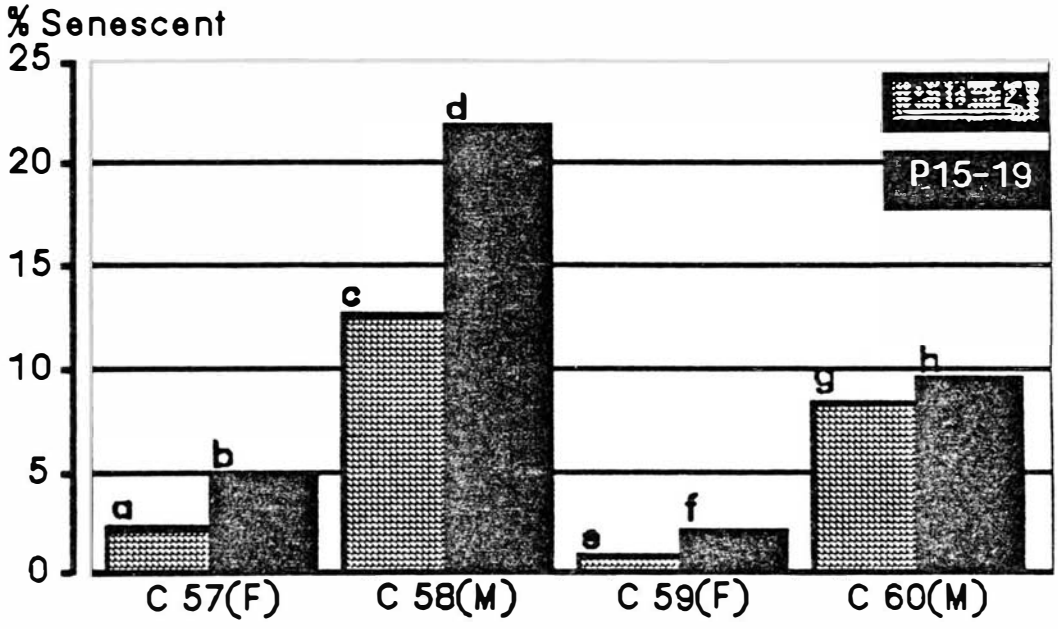
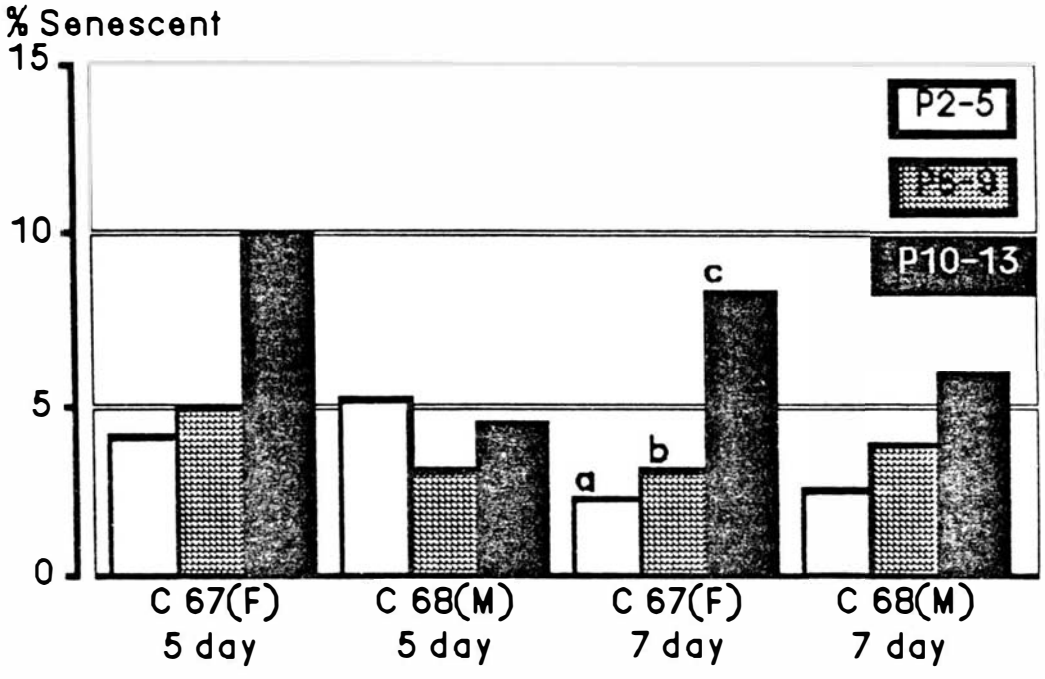


Figure 3. Effect of culture gender and passage number on senescent expression in HUVEC (standard density). Comparison of a five day vs. seven day assessment. The primary cultures of C 67(F) and C 68(M) were passed weekly at standard density (1.25×10^5 cells/T 25 flask) through P14. From P2 through P14, a male and female flask were fixed and assessed each week at both five days and seven days after plating. The bar graphs compare the percentages of senescent cells between male and female cultures for three series of four passages each: P2-5, P6-9, and P10-13. a significantly different from c, $p < 0.01$; b significantly different from c, $p < 0.05$.

Figure 3. Effect of Culture Gender & Passage Number on Senescent Expression in HUVEC (Standard Density)



Comparison of 5-Day vs 7-Day Assessment

Figure 4. Effect of 1:10 splits followed by SD plating on senescent expression in male and female HUVEC. The primary cultures of C 69(F) and C 70(M) were passed weekly as described in Table 6. A male and female flask were fixed weekly from P6 through P9, and from P11 through P17 (At P10, all available cells were used to plate subsequent flasks at standard density. The bar graphs compare the percentages of senescent cells between male and female cultures for two groups of four passages each (P6-9, P11-14) and one group of three passages (P15-17). Senescent expression did not differ significantly between male and female cultures in this experiment.

Figure 4. Effect of 1:10 Splits Followed by SD Plating on Senescent Expression in Male & Female HUVEC

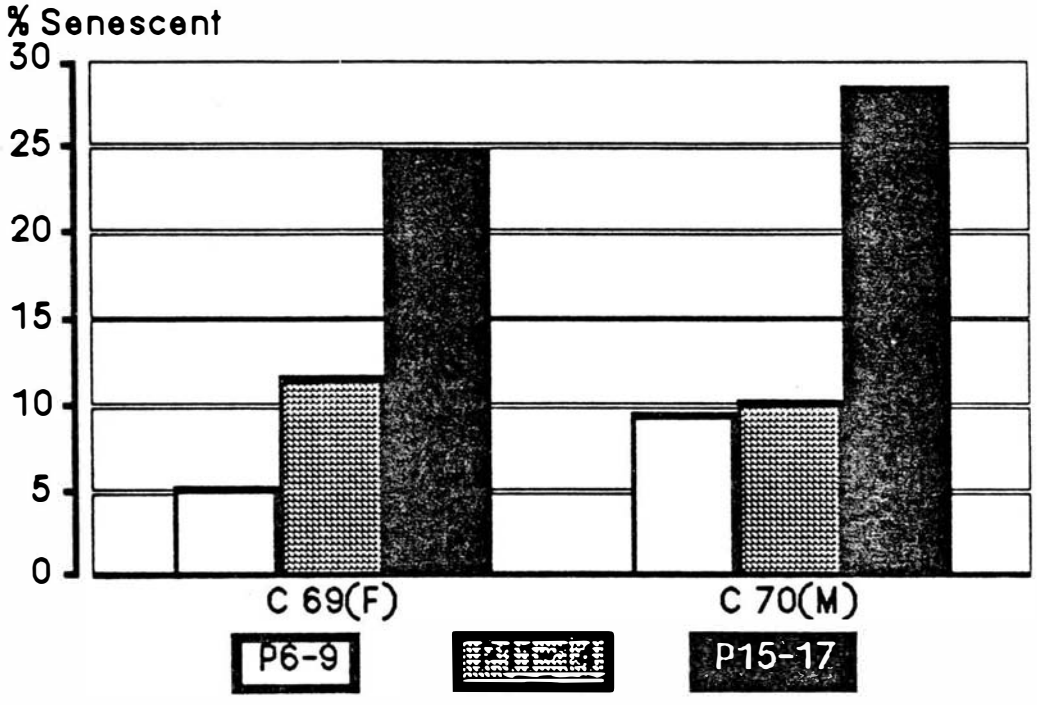
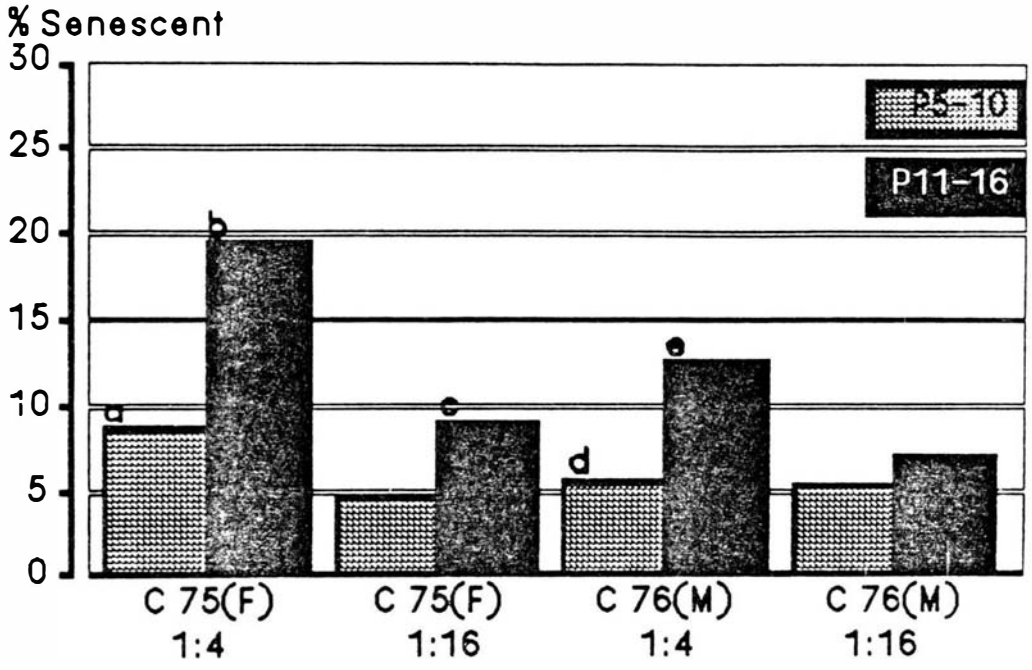


Figure 5. Effect of 1:4 vs. 1:16 splits on senescent expression in early vs. late passage HUVEC. Comparison of male and female cultures. The primary cultures of C 75(F) and C 76(M) were passed weekly as described in Table 7. At each passage from P4 through P16 inclusive, two male and two female flasks from each split ratio were fixed and evaluated. Senescent expression parameters as outlined in this figure are the result of the mean of two such flask determinations for each culture and passage number. The bar graphs compare the percentages of senescent cells between male and female cultures for five earlier passages (P5-10) with those of five later passages (P11-16) for the 1:4 and the 1:16 split ratio groups. a significantly different from b, $p < 0.01$; d significantly different from e, and b significantly different from c, $p < 0.05$.

Figure 5. Effect of 1:4 vs 1:16 Splits on Senescent Expression in Early vs. Late Passage HUVEC



Comparison of Male & Female Cultures

Table 1. Effect of culture gender on senescent expression in HUVEC passed at a 1:5 split ratio (P10 - P19). The primary cultures of C 57(F) and C 58(M) were passed weekly at a 1:10 split ratio through P4, and then at a 1:5 split ratio beginning with P5 and extending through P 20. The primary cultures of C 59(F) and C 60(M) were passed weekly at a 1:10 split ratio though P3, and then at a 1:5 split ratio beginning with P4 and extending through P19. A male and female flask were fixed weekly from P10 to P19 inclusively. The sums of total cells/cm^2 and total senescent cells/cm^2 generated from P10 - P19 (ten passages inclusive) are illustrated in the table. The percentages of senescent cells, calculated by dividing the sum of senescent cells/cm^2 by the sum of total cells/cm^2 for each culture, is illustrated in the last column. F= female; M= male.

Table 1

Effect of Culture Gender on Senescent Expression in HUVEC Passed at a 1:5 Split Ratio (P 10 - P 19)

Culture	Total Cells*	Senescent Cells*	Percent
C 57(F)	$^{a}3.99 \times 10^{5}$	13594	$^{e}3.41$
C 58(M)	$^{b}2.06 \times 10^{5}$	34812	$^{f}16.90$
C 59(F)	$^{c}5.51 \times 10^{5}$	7792	$^{g}1.40$
C 60(M)	$^{d}2.25 \times 10^{5}$	19696	$^{h}8.75$

*generated over 10 passages in a cm^2 area.
a significantly different from b, c from d, and a from c, $p < 0.05$; e significantly different from f, and g from h, $p < 0.001$.

Table 2. Effect of culture gender and passage number on senescent expression in HUVEC (1:5 split ratio). Using the same male-female culture pairs described in Table 1, Table 2 compares both the total cell number and the sum of senescent cells generated in five earlier passages (P10-14) with those in five later passages (P15-19). The percentages of senescent cells for each culture and passage group, calculated as described in Table 1, are compared in the last column.

Table 2

Effect of Culture Gender and Passage Number on Senescent Expression in HUVEC (1:5 Split Ratio)

Culture	Total Cells*	Senescent Cells*	Percent
C 57(F)			
P 10-14	$^{a}2.32 \times 10^{5}$	5450	$^{i}2.35$
P 15-19	$^{b}1.64 \times 10^{5}$	8144	$^{j}4.97$
C 58(M)			
P 10-14	$^{c}1.10 \times 10^{5}$	13716	$^{k}12.47$
P 15-19	$^{d}9.70 \times 10^{4}$	21096	$^{l}21.74$
C 59(F)			
P 10-14	$^{e}3.07 \times 10^{5}$	2810	$^{m}.92$
P 15-19	$^{f}2.44 \times 10^{5}$	4982	$^{n}2.04$
C 60(M)			
P 10-14	$^{g}1.08 \times 10^{5}$	8948	$^{o}8.29$
P 15-19	$^{h}1.12 \times 10^{5}$	10748	$^{q}9.59$

*generated over the indicated number of passages in a cm^2 area.
a significantly different from b, a from c, e from f, and e from g, $p < 0.01$; i significantly different from k, j from l, and m from o, $p < 0.001$; n significantly different from q, $p < 0.01$.

Table 3. Effect of culture gender and passage number on senescent expression in HUVEC - 1:10 followed by 1:5 split ratio. The primary cultures of C 63(F) and C 64(M) were passed weekly at a 1:10 split ratio through P3, and then at a 1:5 split ratio from P4-9. A male and female flask were fixed weekly from P1-9. The table compares the total cell number and sum of senescent cells generated for four earlier passages (P1-4) with those of four later passages (P6-9). The percentages of senescent cells for each culture and passage group are compared in the last column.

Table 3

Effect of Culture Gender and Passage Number on Senescent Expression in HUVEC- 1:10 Followed by 1:5 Split Ratio (P 1 - P 9)

Culture	Total Cells*	Senescent Cells*	Percent
C 63(F)			
P 1-4	$^{a}3.89 \times 10^{5}$	1829	$^{c}.47$
P 6-9	$^{b}1.77 \times 10^{5}$	7875	$^{d}4.45$
C 64(M)			
P 1-4	3.28×10^{5}	4274	$^{e}1.30$
P 6-9	1.08×10^{5}	10337	$^{f}9.57$

*generated over the indicated number of passages in a cm^2 area.
a significantly different from b, $p < 0.05$; c significantly different from d, $p < 0.01$; e significantly different from f, $p < 0.001$.

Table 4. Effect of culture gender and passage number on senescent expression in HUVEC - five day assessment (standard density). The primary cultures of C 67(F) and C 68(M) were passed weekly at standard density (1.25 x 10^5 cells/ T 25 flask) through P14. Flasks from each culture were fixed and assessed at five days after plating. The table compares the total cell number and sum of senescent cells generated for three series of four passages each: P2-5, P6-9, and P10-13. The percentages of senescent cells for each culture and passage series are compared in the last column.

Table 4

Effect of Culture Gender & Passage Number on Senescent Expression in HUVEC - Five Day Assessment (Standard Density)

Culture	Total Cells*	Senescent Cells*	Percent
C 67(F)			
P 2-5	1.56×10^5	6476	4.15
P 6-9	2.10×10^5	10321	4.91
P 10-13	$^{a}1.29 \times 10^5$	12822	9.94
C 68(M)			
P 2-5	$^{b}1.52 \times 10^5$	7791	5.12
P 6-9	$^{c}2.36 \times 10^5$	7505	3.18
P 10-13	$^{d}2.02 \times 10^5$	9078	4.49

*generated over the indicated number of passages in a cm^2 area.
a significantly different from d, b from c, and b from d, $p < 0.05$.

Table 5. Effect of culture gender and passage number on senescent expression in HUVEC - seven day assessment (standard density). The primary cultures of C 67(F) and C 68(M) were passed weekly at standard density through P14. A male and female flask were fixed weekly from P2 through P13. The table compares the total cell number and sum of senescent cells generated for P2-5, P6-9, and P10-13 as in Table 4. The percentages of senescent cells for each culture and passage series are compared in the last column.

Table 5

Effect of Culture Gender & Passage Number on Senescent Expression in HUVEC - Seven Day Assessment (Standard Density)

Culture	Total Cells*	Senescent Cells*	Percent
C 67(F)			
P 2-5	$^{a}2.85 \times 10^{5}$	6752	$^{e}2.37$
P 6-9	2.76×10^{5}	8058	$^{f}3.11$
P 10-13	$^{b}1.62 \times 10^{5}$	13416	$^{g}8.28$
P 68(M)			
P 2-5	$^{c}2.57 \times 10^{5}$	6043	2.50
P 6-9	2.25×10^{5}	8762	3.89
P 10-13	$^{d}1.86 \times 10^{5}$	10888	5.85

*generated over the indicated number of passages in a cm^2 area.
a significantly different from b, c from d, and f from g, $p < 0.05$; e significantly different from g, $p < 0.01$.

Table 6. Effect of 1:10 splits followed by standard density (SD) plating on senescent expression in male and female HUVEC. The primary cultures of C 69(F) and C70(M) were passed weekly at a 1:10 split ratio through P9, and then at standard density beginning at P10 and extending through P17. A male and female flask were fixed weekly from P6-P9 and from P11-P17. The table compares the total cell number and sum of senescent cells generated for two series of four passages each (P6-9, P11-14) and one series of three passages (P15-17). The percentages of senescent cells for each culture and passage series are compared in the last column.

Table 6

Effect of 1:10 Splits Followed by Standard Density Plating on Senescent Expression in Male and Female HUVEC

Culture	Total Cells*	Senescent Cells*	Percent
C 69(F)			
P 6-9	$^{a}1.63 \times 10^{5}$	8316	5.10
P 11-14	$^{b}1.02 \times 10^{5}$	11691	11.46
P 15-17	4.50×10^{4}	11086	24.64
C 70(M)			
P 6-9	1.19×10^{5}	11076	9.31
P 11-14	1.10×10^{5}	11254	10.23
P 15-17	4.90×10^{4}	13794	28.15

*generated over the indicated number of passages in a cm^2 area.
a significantly different from b, $p < 0.05$.

Table 7. Effect of 1:4 or 1:16 split ratio passage on senescent expression in early vs. late passage HUVEC. Comparison of male and female cultures. The primary cultures of C 75(F) and C76(M) were passed weekly at a 1:10 split ratio through P3. At P4, both male and female cultures were either plated at standard density or four times standard density (5×10^5 cells/flask). Beginning with P5 and extending through P16, the flasks plated at four times SD were passed weekly at a 1:4 split ratio, and the flasks plated at SD were passed at a 1:16 split ratio. Beginning with P4 and continuing through P16 inclusively, two male and two female flasks from each passage and from each split ratio were fixed and evaluated. The raw data consisted of the mean of two determinations for both male and female cultures at each passage. The table compares both the total cell number and sum of senescent cells generated for five earlier passages (P5-10) with those of five later passages (P11-16) for the 1:4 split ratio group and the 1:16 split ratio group. The percentages of senescent cells for each male-female culture pair, split ratio, and passage series, calculated as described in Table 1, are compared in the last column.

Table 7

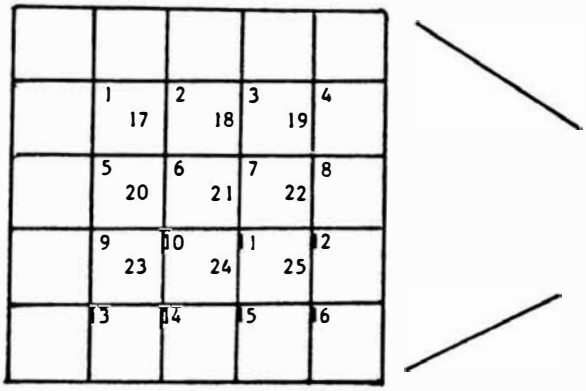
Effect of 1:4 or 1:16 Split Ratio Passage on Senescent Expression in Early vs. Late Passage HUVEC Comparison of Male and Female Cultures

Culture	Total Cells*	Senescent Cells*	Percent
C 75(F) 1:4			
P 5-10	$^{a}2.35 \times 10^{5}$	20792	$^{g}8.80$
P 11-16	$^{b}1.38 \times 10^{5}$	26710	$^{h}19.35$
C 76(M) 1:4			
P 5-10	$^{c}2.33 \times 10^{5}$	13181	$^{i}5.66$
P 11-16	$^{d}1.40 \times 10^{5}$	17454	$^{j}12.47$
C 75(F) 1:16			
P 5-10	$^{e}3.12 \times 10^{5}$	14673	4.70
P 11-16	$^{f}1.88 \times 10^{5}$	16921	$^{k}9.00$
C 76(M) 1:16			
P 5-10	2.47×10^{5}	12988	5.26
P 11-16	1.56×10^{5}	11093	7.11

*generated over the indicated number of passages in a cm^2 area.
a significantly different from b, c from d, g from h, and i from j, $p < 0.01$; e significantly different from f, b from f, and h from k, $p < 0.05$.

Senescent Data Assessment Form

Code No._____ Flask No._____ Male_____ Female_____



AREA	NON-SENESCENT	SENESCENT	AREA	NON SENESCENT	SENESCENT
1	_____	_____	7		_____
2		_____	8		_____
3		_____	9		_____
4		_____	10		_____
5		_____	11	_____	_____
6	_____	_____	12		_____
AREA	NON-SENESCENT	SENESCENT	AREA	NON SENESCENT	SENESCENT
13		_____	19	_____	_____
14		_____	20		_____
15		_____	21	_____	_____
16	_____	_____	22		_____
17		_____	23	_____	_____
18		_____	24		_____
			25		_____

Senescent Data Assessment Form

Calculation of Percentage of Senescent Cells

Code No._____ Flask No._____ Male____ Female____

Sum of numbers of non-senescent cells in areas 1,6,11&16 = N = _____

Calculated number of non-senescent cells in areas 1-16 =4N = _____

Sum of numbers of senescent cells in areas 1-16 = S = _____

Total number of cells in areas 1-16 = 4N + S = T = _____

Percentage of senescent cells =S/T= _____ %

Calculation of Cellular Density

Same flask as above

At 200X, the area of each sample grid = 0.117649 mm^2

Mean of total cell numbers in areas 1,6,11&16 = _____

Number of cells per cm^2 = $\frac{100\ mm^2}{0.117649 mm^2}$ X Mean

= 849.986 X Mean

= _____

Vita