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Factors affecting the growth and differentiated function of adrenal cortical cells in vitro

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FACTORS AFFECTING THE GROWTH AND DIFFERENTIATED FUNCTION OF ADRENAL CORTICAL CELLS IN VITRO

by

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DISSERTATION

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To my wife, Karen, my Mother

and Father, and all my Family

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ABSTRACT

This thesis is a composite of three individual studies concerning the control of proliferation and differentiation of adrenal cortical cells in vitro. Chapters 1 and 2 discuss extracellular variables that influence the growth and differentiation of adrenal cortical cells in vivo and in vitro. Chapters 3-5 are individual studies which present the novel information of this thesis.

In Chapter 3, the influence of fibroblast growth factor (FGF) and epidermal growth factor (EGF) on the proliferation of cultured human fetal adrenal cells is examined. Exposure of either fetal or definitive zone cells to FGF or EGF markedly enhanced their proliferation. Half-maximal stimulation of cell proliferation for both zones occurred at a concentration of 3×10^{-11} M for EGF and 8×10^{-9} M for FGF. In addition, ^{125}I-labeled EGF binding to both zones demonstrated high affinity ($K_D=10^{-9}$M). Human fetal adrenal cells maintained on a bovine corneal ECM had a significantly higher growth rate than cells maintained on plastic alone. The results demonstrate 1) the mitogenic role of EGF and FGF for human fetal adrenal cells, and 2) that the substrate upon which fetal adrenal cells are maintained has a profound influence on their proliferation.

In Chapter 4 a serum-free medium (SFM) for bovine adrenal cortex (BAC) cells is described. BAC cells maintained on ECM-coated dishes proliferated rapidly when serum was replaced by HDL (25 μg protein/ml), insulin (10 ng/ml), and FGF (100 ng/ml). A requirement for transferrin (1 ng/ml) became apparent only when cells were grown for at least four generations in the absence of serum. Early passage (P_1-P_3) BAC cells cultured in SFM responded to ACTH with increased 11-deoxycortisol secretion; this

effect was not observed in later passage cells (P_7-P_{15}). The cells' ability to utilize LDL-derived cholesterol and to respond to db cAMP by increased steroid release was preserved in cells cultured for over 60 generations in the SFM.

The life-span of BAC cells grown in the SFM on fibronectin versus ECM-coated dishes was compared. Cells seeded in serum-containing medium and grown in SFM had a life span of 34 versus 60 generations when maintained on fibronectin- or ECM-coated dishes, respectively. Cells seeded in the complete absence of serum in the SFM on ECM- or fibronectin-coated dishes could be passaged for 26 or 13 generations, respectively. FGF was an absolute requirement for cells cultured on fibronectin, but not ECM-coated dishes. The observations made demonstrated the influence of the ECM not only in promoting cell growth and differentiation, but also on the lifespan of cultured cells.

In Chapter 5 the effect of insulin on LDL metabolism in confluent cultures of BAC cells maintained in SFM on ECM-coated dish is analyzed. Cells were exposed to F12 medium supplemented with HDL (1 μg protein/m) and transferrin (1 μg/ml). Cholera toxin (CT) (10 ng/ml) and insulin (100 ng/ml) were added singly or together to the medium for 24 hours. At the end of this exposure time ^{125}I-LDL (10 μg protein/ml) was added for 4 hours and its internalization and degradation were correlated with the release of 11-deoxycortisol (11-DOC). Preincubation with CT but not insulin increased LDL internalization and degradation by 10- and 5-fold respectively, and 11-DOC release by 2.7 fold. When insulin was added with CT, LDL internalization and degradation increased by 45- and 22-fold respectively. This correlated with a

9.6 fold increase in 11-DOC released into the medium. The effect of insulin was dependent on time and concentration ratio and could be observed after 4 hours of preincubation with concentrations as low as 1.0 ng/ml. Chloroquine (100 nM) inhibited ^{125}I-LDL degradation by more than 95%, with 11-DOC secretion decreasing 80% in cells preexposed to CT and insulin. These results suggest an important role for insulin when BAC cells are stimulated to produce steroids by induction of receptor-mediated endocytosis and catabolism of LDL.

Chapter I. General Introduction

This thesis is concerned with the application of in vitro cell culture techniques to identify the factors and mechanisms relevant to in vivo cell growth and differentiation. While this thesis examines cells from the adrenal cortex, it is likely that the methods used and results can be extrapolated to other mesodermally derived tissues.

In this first chapter, extracellular variables that influence the proliferation of cells in vitro are considered. This includes the substrate upon which the cells will eventually migrate, differentiate, and divide. Humoral factors such as those present in serum or plasma and which are required for the establishment of primary cells in culture will be discussed. Finally, the rationales for cell culture under serum-free condition will be discussed.

I. Extracellular variables that influence the proliferation of cells in vitro.

A. Substrates

Evidence that the substrate upon which cells are maintained is important for their proliferation is plentiful. Attempts have been made to recreate both natural and artificial substrates which increase attachment, migration, proliferation, and differentiation. For example, a variety of synthetic and natural polymers that have a net-positive charge have been shown to stimulate cell division when used to coat the culture surface (1). Among these are histones, protamine, polylysine, polyhistidine, and DEAE-dextran. These basic polymers easily coat tissue culture dishes since the dishes themselves have been treated (sulfonation) so as to have a net-negative charge. While the

mechanism of action of these basic polymers is not fully understood, one mechanism might be to help anchor certain acidic proteins or other components found in plasma or secreted by the cells as extracellular matrix. Fibronectin, an acidic protein, found in plasma, on cell surfaces, and in the extracellular matrix of most mesodermally derived cells, might fit such a role. This protein has a cell-specific attachment domain which has recently been determined (2). It also has domains which have been shown to bind glycosaminoglycans and collagen specifically (3). These compounds have been shown to be ubiquitous components of basement membranes (that part of the extracellular matrix in closest association to the basal surface of cells) and extracellular matrices. The negatively-charged tissue culture dish with positively-charged bound polymers could also bind negatively (acidic)-charged proteins or ions such as calcium, which in turn can bind cells as well as other components of basement membranes. This may be required to initiate the sequence of events leading to the formation of an extracellular matrix (ECM) or basement membrane in vitro. The precise components of ECM as well as their molecular arrangements may have profound effects on the ability of a given cell type to migrate, proliferate, and differentiate in vitro. An alternative to "coating" or adding matrix materials to serve as a proper substrate is to let cells which normally produce an ECM in vivo, perform this task in vitro. This approach has been used in the case of Corneal endothelial cells which are known to produce an ECM containing most of the components common to extracellular matrices (4).

Although the exact nature and composition of ECMs produced in vivo by various tissues have yet to be elucidated, their composition for the most part

includes collagens, proteoglycans, and glycoproteins (5,6). Collagens are one of the major components of the extracellular matrix and the most abundant proteins in our bodies. They are a family of closely related proteins, each from a different gene product. Most tissue types except blood-borne cells and neuroectoderm produces collagen, but various tissues produce only certain of the five collagen isotypes (6). Three types of interstitial collagen (I, II, and III), which are components of the extracellular matrix, are present in varying ratios in various tissues. Two types of basement membrane collagens (IV and V) have been localized by biochemical and immunochemical analysis in vivo (5,6). Besides serving a structural role, collagen was first postulated to induce differentiation of muscle cells in vitro by Konisberg and Hauschka (7). It has subsequently been shown to replace the requirement of the corneal epithelium for underlying matrix in order for the proper differentiation and response to epidermal growth factor (8,9).

Glycosaminoglycans (GAGs) are sugar polymers of high molecular weight composed of repeating dimers of amino sugars, which are covalently linked (except for hyaluronic acid) to protein as proteoglycans (PG). Like collagen, PGs are secreted by most tissues, although various tissues and varying amounts of different PGs. The major GAGs include hyaluronic acid, heparin, heparin-sulfate, keratin, keratin-sulfate, dermatin, dermatin-sulfate, chondroitin, and chondroitin-sulfate. Their molecular structures as PGs differ in extracellular matrices from various tissues as does their association with various collagen types (6). PGs are thought to fill the spaces around collagen fibrils providing a hydrated, viscous gel while the fibrils define tissue shape and tensile strength (6).

Glycoproteins of extracellular matrices are considered structural and differ from proteoglycans by their higher proportion of protein and the type of polysaccharide side-chains (5,6). Fibronectin, laminin, and chondronectin are such associated proteins considered to be involved in cell-substrate adhesion. Fibronectin has been most widely studied. Fibronectin is found early in development as is collagen and laminin. It is neither found at the cell surface nor is it secreted by tumor cells. Fibronectin has cell attachment and PGs attachment regions and also binds to collagen and fibrin (2,3,6). It is found in basement membranes and may be linked to actin across the plasma membrane of cells via another hydrophobic protein (6). The effects described for fibronectin on cell shape and migration may therefore be mediated by cytoskeletal changes. Laminin is a very large, insoluble glycoprotein also found in basement membranes and clearly involved in epithelial attachment to collagen (5). Chondronectin may be a protein that is similar in function to fibronectin, but specific to chondrocytes (5,6).

The composition of the ECM produced by corneal endothelial cells <u>in vitro</u> has, in part, been analyzed. Type III collagen is the major component found although the basement membrane collagens (types IV and V) and interstitial collagens (types I and II) are present (9). Fibronectin and laminin are present in large quantities and co-distribute with collagen type IV (9). Although proteoglycans are present (in particular heparan sulfate PGs) the precise types, quantities, and ratios of each are currently under study. Staining with alizarin red indicates that a large amount of calcium is associated to dishes coated with extracellular matrix.

In vitro, the reconstitution of an ECM from its separate elements may result in an incomplete ECM due to unidentified components, incorrect ratios of known components, or an improper arrangement of components. Therefore, the ECM synthesized and deposited by corneal endothelial cells, whose components and structure are very similar to known in vivo ECMs and basement membranes, offers a major advantage compared to other artificial substrates. Its use, in combination with the proper physiochemical environment and complement of nutritional and hormonal factors could recreate more closely an in vivo environment. The bovine corneal ECM-coated dishes will be utilized extensively for the experiments herein designed to elucidate regulatory factors involved in the growth and differentiation of adrenal cortex cells. These substrates have already been shown to be necessary for the analysis of the requirements of cells in vitro for serum or plasma factors (10).

B. Plasma Versus Serum

This discussion will focus on the requirements of cells in vitro for plasma or more commonly used serum-supplemented media. Other supplements such as lymph (used as early as 1887 to culture frog erythrocytes), chicken embryo extract, and colostrum, have also been used. The latter probably provide hormonal factors and nutrients similar to those found in plasma and serum.

Plasma or serum supports survival and growth of cells in culture. They act by providing hormones, nutrients, and attachment factors to which cells are normally exposed to in vivo (11). Supplementation of media with serum or

plasma can also promote in part cell survival and growth by one or more of the following(12):

(a) by neutralizing trypsin;

(b) by promoting repair of cellular damage done during subculturing;

(c) by binding or neutralizing toxic substances in the culture medium;

(d) by binding an essential nutrient that is toxic when present in excessive amounts and releasing it slowly in controlled amounts as it is needed for cellular growth;

(e) by binding and protecting a labile essential nutrient and releasing it slowly as it is needed;

(f) by stimulating active transport to permit utilization of nutrients present in suboptimum amounts in the defined portion of the medium;

(g) by supplying "carrier" proteins facilitating the cellular entry of low molecular weight substances;

(h) by providing enzymes needed to convert components of the medium to forms that the cells can utilize;

(i) by modifying physiochemical properties of the culture medium, such as viscosity, colloid osmolality, or rate of diffusion;

(j) by solubilizing essential nutrients that do not dissolve readily in aqueous media; and

(k) by coating and modifying culture surfaces.

Culture of most cells in vitro requires the presence of serum; plasma for some cell types is less effective or even ineffective in promoting cell proliferation (13). Consequently, investigators have spent much effort to

identify the various factors in serum that stimulate cell growth in vitro. An important step in the search for serum growth factors was the finding that one of the most potent mitogenic factors present in serum was derived from the platelets. This finding first described by Balk (14) was based on studies of the growth of chick embryo fibroblasts in medium supplemented with plasma or serum. Chicken fibroblasts did not proliferate in plasma-containing medium, but when cells were exposed to serum they proliferated rapidly. It was therefore concluded that serum contained growth promoting activity that was absent in plasma (15). These studies were followed by reports that platelets were the source of a potent mitogen present in serum but not in plasma. Whereas plasma was unable to support the growth of aortic smooth muscle cells or of BALB/c 3T3 cells, serum made from the same pool of blood stimulated their proliferation (16-18). Addition of a platelet mitogen (platelet derived growth factor, PDGF) to cells maintained in plasma restored its ability to support cell proliferation (16-18). The conclusion was drawn that one of the principal mitogens in whole blood serum was therefore derived from platelets (16-18).

Most in vitro studies to date have used cells maintained on plastic rather than on an ECM which, as discussed above, is the natural substrate upon which cells migrate, proliferate, and differentiate in vivo. This difference in the substrate upon which the cells were maintained might have prevented their response to physiological factors present in plasma, thereby creating the difference in mitogenic activity between plasma and serum. In recent studies (19) the proliferative behavior of four different cell types maintained on plastic or the ECM produced by corneal endothelial cells were compared. The

results indicated that bovine adrenal cortical cells, granulosa cells, and vascular or corneal endothelial cells maintained on plastic and exposed to optimal serum concentration did not proliferate unless they were exposed to the potent in vitro mitogen fibroblast growth factor (FGF) (19). In contrast, when the cells were maintained on a corneal ECM they proliferated actively and FGF was not required in order for the cultures to become confluent (19). The conclusion was made that the close contact of the cells with the ECM must have made them responsive to factors present in serum and that adherence to the plastic prevented such a response. That this was likely to be the case was inferred from observations that the growth rate of cells maintained on ECM was a direct function of the serum concentration to which they were exposed. Therefore, with regard to proliferation, those cells that exhibited a total dependence on FGF, but not on serum, when maintained on plastic exhibited a total dependence on serum and no longer required FGF when maintained on the ECM. The important change of substrate from plastic to ECM restored the sensitivity of the cells to mitogens present in serum, which could have been the same as those present in plasma. This is best seen with vascular smooth muscle cells maintained on ECM, which proliferate as well in response to plasma or serum derived from the same batch of whole blood (20). It is therefore likely that the serum factors to which these cells responded when maintained on the ECM are the same as those present in plasma. A similar growth response to plasma factor can also be observed for many other cell types including those derived from the adrenal cortex.

Since no cell type in vivo exists for extended periods in the presence of serum, the ability to culture cells in plasma instead of serum-supplemented

media can be considered an important step toward mimicking an in vivo environment in vitro. However, the components of plasma responsible for the survival, growth, and differentiation of cells in vitro, must be resolved if we are to understand their mechanisms of action. The ability to culture cells in the presence of well defined plasma factors should allow controlled, defined conditions, where a specific component or components of plasma can be analyzed for their effects on growth or differentiated functions.

II. The rationales for serum-free cell culture.

One of the paradoxes arising from an in vitro situation as compared to that in vivo has been that, although hormones are potent mitogens in vivo, they have no effect or the opposite effect (antimitotic) on their target cells in vitro. For example, prolactin, growth hormone, follicle stimulating hormone, and estrogens do not stimulate replication of cells in vitro, although in vivo they can induce hyperplasia of their target organs (21,22). ACTH, the trophic hormone for adrenal cortex cells in vivo, is antimitotic in vitro (23-25). (This particular hormone will be discussed in greater detail in a subsequent section of this thesis.) This paradoxical difference between in vivo and in vitro control of cell proliferation is even more evident at the level of the mechanisms involved in mediating cellular proliferation. In vivo cellular proliferation triggered by tropic hormones results in an increased cellular cyclic AMP content in the target cells (26). In vitro, however with cells whose growth is anchorage dependent, exposure to agents such as ACTH (which increases the cellular cyclic AMP content of adrenal cells) or direct exposure of cells to cyclic AMP leads to inhibition of cell growth (23-25). In contrast, in the case

of cells such as lymphocytes, which have no need for an ECM and whose growth is not anchorage dependent, cyclic AMP as well as hormones capable of activating adenylate cyclase are mitogenic (27). One is therefore led to wonder what the main difference is that is responsible for these discrepancies between the in vivo and in vitro conditions.

A common explanation for the lack of activity of hormones in vitro has been that their action in vivo is indirect and that they generate a second generation of factors (growth factors) that are directly responsible for the control of proliferation of target cells. Equally likely is that when cells are maintained in vitro on a proper substratum, they are capable of responding to mitogenic factors in plasma which are prevented from acting when cells are maintained on plastic. Another possibility is that plasma factors could directly (or indirectly) be inhibitory, or "mask" the effects of hormones or growth factors to be tested for activity.

The ability to culture cells in the absence of serum or plasma is therefore critical for understanding cellular function. In the words of Ham, "as long as we are dependent on fetal bovine serum or comparable form of 'witchcraft' to obtain adequate cellular growth, we have no true understanding of our experimental systems...everytime we have to add undefined supplements to a culture medium to obtain good growth we acknowledge our ignorance about cellular growth requirement." It is indeed frustrating not to know precisely what the cells we are working with require for growth or differentiated function. Serum or plasma may contain substance that directly influence the cellular processes under study. Growth that is dependent on undefined supplements is likely to be suboptimal as reflected by vastly varying

growth rates of cells exposed to various batches of serum or plasma. Finally, the information and insights that serum-free cell culture can bring not only to regulation of cell growth and differentiated function, but to human nutrition could be valuable. Depletion studies on human diploid cells in culture established a requirement for selenium in human cell nutrition (28). Depletion studies on human beings are obviously impossible although certain diseases (i.e., scurvey, ricketts, anemia) are directly related to such nutritional deficiencies.

The initial experiments presented in this thesis therefore examined components, specific to plasma, or growth factors which allowed survival and growth of adrenal cortex cells in the absence of serum. Certain components were examined together and separately, or with serum, on growth rate and long-term growth, i.e., continuous passage. The differentiated function (steroidogenesis) was analyzed in cells cultured under various conditions in the presence or absence of serum or media with specific plasma components and maintained or not on substrates resembling conditions in vivo. Finally, certain molecular functions, as possible mechanisms of either growth or differentiated function, were examined in plasma free medium supplemented with defined plasma components. All the in vitro studies presented in this thesis were carried out in either medium 199 or F12 media which best support the growth of adrenal cortex cells in vitro.

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Chapter II. Experimental Model: The Adrenal Cortex

The cells of the adrenal cortex are under stringent hormonal control and produce glucocorticoids as well as mineralocorticoids which control in part carbohydrate metabolism and electrolyte concentrations. Clinical evidence, and in vivo and in vitro studies, dating back to 1855, have contributed to our knowledge of this endocrine organ. Because the experimental model for the studies presented in this thesis utilized adrenal cortical cells in vitro, and the focus of these studies was to identify and study factors which regulate their growth and differentiation, a brief history of the adrenal cortex as a "life-sustaining" organ will be presented. This will be followed by a description of its ontogeny and cellular physiology. Finally, in vivo studies concerning its humoral regulation will be reviewed followed by in vitro studies and discussion of their relevance to the in vivo situation.

I. In Vivo Considerations

A. Historical

In 1855 Thomas Addison described a pathology whose symptoms were related to "adrenal gland insufficiency." His early studies on the role of the adrenal gland inaugurated a succession of studies which have led to a better comprehension of the role of this gland in vivo (1). The symptoms of Addison's disease include: weakness and fatigue; abnormal pigmentation; loss of weight and dehydration; hypotension and small heart size; anorexia, nausea, vomiting, diarrhea, and nervousness (2). Certainly the extensive symptomology of this

disease, attributed by Addison to a defective function of the adrenal gland, greatly aroused the interest of scientists and physicians of the time.

In 1856, Brown-Sequard performed the classical adrenalectomy experiments which he claimed showed that the adrenal gland was essential to life (3). However, controversy surrounded this claim because some animals survived bilateral adrenalectomy while others did not survive unilateral adrenalectomy. The presence or absence of stress post surgically was realized to be the major determinant of this discrepancy. By 1943, the controversy had been resolved and there was agreement that the adrenal cortex, but not the adrenal medulla, was life-sustaining in most animals (1). The control of both electrolyte concentration and carbohydrate metabolism by the adrenal gland were described at this time. Furthermore, steroids crystallized from adrenal extracts were found to have both types of activities leading to a "unitarian theory" of adrenal cortical hormonal action (4,5).

When better purification techniques were developed to isolate steroids, corticosterone and cortisol were determined to be the major carbohydrate regulating glucocorticoids while aldosterone was found to be the major mineralocorticoid (6-9). By 1952, the life-sustaining hormones of the adrenal cortex had been isolated and their biological responses characterized dispelling the unitarian theory of adrenal cortical hormonal action. The production and roles of the adrenal androgens were little studied at this time.

B. Ontogeny

The human fetal adrenal cortex begins to develop around the third to fourth week of gestation. Neuroectodermal cells migrating from the neural

crest form the medulla at six to eight weeks (10,11). At about this same time, the cortex of the fetal adrenal separates into two zones: a large inner zone (fetal zone) and a small outer zone (definitive zone). The fetal zone produces increasing amounts of androgens throughout its eight-month life; a proportion of which are converted to estrogens by the placenta (12). The definitive zone remains relatively inactive until just prior to parturition when it starts producing large amounts of cortisol. In the neonate the fetal zone atrophies and involutes declining to 50% of its prebirth size (13). This phenomenon suggests a role of the placenta in the maintenance of the cells of this zone.

Following birth, the definitive zone begins to grow towards the medulla and differentiates into 3 different zones; the glomerulosa, fasciculata, and reticularis. Both glomerulosa and fasciculata zones are controlled by trophic hormones that stimulate the release of mineralocorticoids and glucocorticoids. Evidence for existence of a trophic hormone that controls the release of androgens from the reticularis zone is being looked for.

After the development and differentiation of the three zones, no known changes in adrenal anatomy or physiology occur until adrenarche. Adrenarche precedes puberty by about one to two years in man. At this time blood levels of androgen rise and a corresponding hypertrophy of the zona reticularis is observed (14). Although a postulated pituitary hormone, AASH (adrenal androgen stimulating hormone), is believed to mediate this phenomenon, it has not yet been isolated (15). Such a hormone, like angiotensin II, might be synthesized and processed by extrapituitary tissues. The morphological, biochemical, and developmental events that occur in the ontogeny of this gland suggest it is under a complex control by various trophic agents.

C. Cellular Physiology

In man the adrenal cortex exists morphologically as three distinct zones. Just underneath the fibrous capsule is the glomerulosa, so named because the cells appear in clusters or whirls forming glomeruli. These cells respond to angiotensin II, elevated potassium ion, and ACTH, by specifically producing the mineralocorticoid aldosterone. The absence of the enzyme 17 hydroxylase in these cells results in the exclusive synthesis of aldosterone (or its precursors) since 17 hydroxylase is needed for cortisol and androgen synthesis. The cells of the glomerulosa are small and comprise 10% or less of the cortex. In most studies of regenerating adrenal glands the mitotic activity is found to be highest in this zone (20).

The middle zone or fasciculata is so named because it appears as columns or fascicles. Its mass comprises approximately 70% of the cortex. The cells of this zone are heavily lipid-laden and are the major glucocorticoid producers. These cells respond to ACTH or agents that raise intracellular cAMP concentrations by mobilizing stored cholesterol in the production of cortisol. These cells have recently been shown to utilize low-density lipoprotein (LDL) as a major cholesterol source and substrate for steroid biosynthesis (16-17).

The most internal zone of the cortex is the reticularis, so named because it appears as a reticulem or net of cells which abuts the medulla. Early short-term in vitro studies of separate zona fasciculata and zona reticularis tissues by Griffiths and coworkers (18) led them to conclude that the functional activities of these tissues were essentially identical in terms of their patterns of steroidogenesis. The only evidence for a specific difference between human

fasciculata and reticularis cells is the biochemical observation of high steroid sulfokinase activity in the reticularis zone (19). Sulfation of the androgen DHEA is observed in the internal fetal zone of the fetal adrenal. During adrenarche, elevated DHEA corresponds with an hypertrophy of the reticularis zone (15). It appears, however, that under a constant environment (i.e., in monolayer culture) the cells from both zones eventually behave in an identical fashion. That is, they respond to the same trophic hormone (i.e., ACTH) with identical steroid profiles, and their sulfokinase activity approaches identical levels (20). The specific production of androgens by the reticularis zone is still unresolved.

Blood enters through the cortex in an extensive capillary network just underneath the capsule and flows toward the medulla through each zone. Necrosis of adrenal cortical tissue is seen mainly in the inner reticularis zone in man and other animals (20). While in vitro the absence of ACTH in monolayer culture does not result in cortical cell death, its absence in vivo results in rapid fatty degeneration and necrosis in the cortex (20). The in vitro data therefore suggest that requirements other than ACTH are needed for adrenal cortical cell viability in vivo.

The roles of steroids in human physiology are many. Glucocorticoids secreted by the fetal adrenal gland late in gestation play an important role in effecting homeostatic adjustments necessary for extrauterine life. These hormones affect the maturation of a variety of enzyme systems, including enzymes in the lung, liver, pancreas, gastrointestinal tract, and adrenal medulla (21,22). For example, in the adrenal medulla of both the fetus and the adult, cortisol induces the enzyme phenyl-ethanolamine-N-methyltransferase

(PNMT) which converts norepinephrine into epinephrine. In the lung, it induces the surfactant dipalmytylphosphatidyl choline important in the prevention of hyaline membrane disease in premature infants and also in maintaining the lowered surface tension necessary for lung inflation (23-25). Glucocorticoids are both anabolic and catabolic agents depending on their target organ. In the liver, cortisol induces glycogenesis and gluconeogenesis. It is responsible for protein catabolism in muscle which results in the free amino acids needed for gluconeogenesis. Chronic exposure to cortisol results in Cushings syndrome, whose symptoms can be observed in patients who have had organ transplants and are given cortisol-like compounds as immunosuppressive therapy (another function of glucocorticoids). Cortisol also has profound cardiovascular effects. It can enhance the production of angiotensinogen by the liver (24), but more importantly sensitizes the arterioles to the pressor effects of norepinephrine and related drugs. Finally, a tendency to atherosclerosis follows a rise in circulating blood lipids and cholesterol levels due to excess cortisol (24).

Aldosterone is the most potent endogenous mineralocorticoid and leads to sodium retention and concurrent potassium loss in the urine. Its primary action is exerted at the level of the ascending limbs of the distal convoluted tubules in the kidneys. It also acts on transport mechanisms in sweat glands, salivary glands, intestinal mucosa, and in exchanges between extracellular and intracellular fluid throughout the body (26). Excess aldosterone, which occurs in Conn's syndrome, is characterized by hypertension, low potassium, polyuria, and metabolic acidosis. Lack of aldosterone leads to sodium loss, a rise in potassium, dehydration and circulatory collapse. While aldosterone is the

major mineralocorticoid in vivo, deoxycorticosterone (DOC), an intermediate in aldosterone biosynthesis, has significant mineralocorticoid activity and can be elevated as a result of hyperglucocorticoid conditions (26).

Androgens have gross anabolic-like actions. They enhance the synthesis of proteins from amino acids and thereby counteract the catabolic action of glucocorticoids. The list of androgenic effects include: hirsutism; increased muscle mass; enhancement or assumption of a male-body build; deepening of the voice; increase in acne; and increase in the size of the clitoris or the phallus (20,23,24). If overproduction of adrenal androgens occurs during fetal life, prior to sexual differentiation of the external genitalia (twelfth week of life), it can cause the formation of ambiguous genitalia which result in variable degrees of pseudohermaphroditism (20). Their antiglucocorticoid effects may be important in the fetus where anabolic actions lead to increased muscle mass and birth weight, and therefore successful parturition. They also serve as the precursor to estriol, the estrogen of pregnancy, produced by the placenta. Liggins and coworkers (27) have developed a scheme which they hypothesize leads to parturition in the sheep. They claim the increasing concentrations of androgens produced by the fetal adrenal are converted to estriol by the placenta which leads to an increase in the ratio of estrogens to progesterone in the fetus. This increase in estrogen prior to parturition, along with increased cortisol produced both in the fetal and the maternal adrenal leads to an increase in prostaglandin synthesis. The increased prostaglandins result in the ensuant initiation of parturition. Jaffe and coworkers (28) have recently described the same set of observations in the rhesus monkey and have proposed a similar sequence of events.

Quantitatively the most important androgens secreted by the adrenal cortex are DHEA-S, DHEA, and androstenedione. These steroids are the precursor of the biologically more potent androgens circulating in human blood, i.e., testosterone and dihydrotestosterone. With the exception of congenital adrenal hyperplasia, the adrenal is seldom the cause of significant virilization in the male. However, adrenal virilization (the symptoms of androgen excess) can rapidly affect the female phenotype with manifestations of hirsutism, amenorrhea and male fat distribution (20,23,24).

Together the three classes of steroids produced by the adrenal gland are important homeostatic regulators. The regulation of sodium and potassium by mineralocorticoids insures the electrochemical gradients operative for nerve conduction, heart contraction, and transport of nutrients in most cells of our body. The regulation of blood sugar in periods of fasting by glucocorticoids assures that the brain will be served with its main palatable substrate, glucose. Androgens assure the relocation of consumed substrates so as to increase muscle mass and strength and serve as precursors to regulators of sex and libido. The adrenal cortex by virtue of its hormonal repertoire, does indeed fulfill a "life sustaining" role that pervades every aspect of our physiology.

Humoral Control

In both the human fetus and the adult abundant clinical evidence supports the role of the pituitary hormone, ACTH, as the major trophic hormone of the adrenal cortex. The hypothesis that ACTH is the direct and trophic stimulus for growth as well as function of the adrenal cortex is based on the inverse relationship between ACTH and growth and function. Elevated ACTH is associated with increased steroid production, hypertrophy, and hyperplasia of the cortex while its absence is associated with low steroid levels and cortical atrophy. In the anencephalic fetus, where plasma ACTH levels are low, the adrenal gland is severely atrophied and the fetal zone is either absent or poorly developed (29). Likewise, in adult animals, hypophysectomy leads to low steroid levels and marked cortical atrophy which can be corrected by ACTH administration. ACTH treatment of intact animals causes an increase in adrenal RNA content, followed by an increase in DNA content (30,31). ACTH is also capable of stimulating the release of all three classes of steroid hormones, i.e., mineralocorticoids, glucocorticoids and androgens (20,23,24).

The hypothesis that ACTH is responsible for adrenal cortical cell proliferation was challenged by Cater and Stack-Dunne in 1953. They showed that although ACTH induced the secretion of steroids and maintained adrenal responsiveness, it was relatively ineffective in stimulating mitotic activity in the adrenals of hypophysectomized rats (31,33,34). The question was therefore raised as to whether the trophic action of ACTH involved only the hypertrophy of the adrenal cortical cells or included the hyperplastic effect as well.

Numerous studies have sought to define the role of ACTH or identify a trophic hormone in addition to ACTH, that participates in the regulation of growth and steroidogenesis. Dallman et al. (30,31) designed a series of in vivo experiments to compare the effects of unilateral adrenalectomy and ACTH administration (both known to increase adrenal wet and dry weights), that could be observed separately as well as together. The results clearly indicated that unilateral adrenalectomy results in a rapid increase in RNA and DNA content (cell proliferation) in the contralateral adrenal, whereas treatment with ACTH results in a rapid increase in RNA content (cell hypertrophy) that precedes the increase in DNA. In fact, treatment of rats with ACTH inhibited the proliferative response of the remaining adrenal after unilateral adrenalectomy.

The possibility that compensatory adrenal growth may in part be mediated neurally was suggested as early as 1959 (35,36). Both afferent and efferent nervous effects could be demonstrated. For example, ^{3}H leucine uptake into the ventro-medial hypothalamus and unilateral changes in nuclear volume was shown to follow unilateral adrenalectomy but not sham-operation (35-37). Unilateral hypothalamic lesions were shown to decrease nuclear volume of cells in the zona fasciculata of the adrenal on the side opposite to the lesion (35,38). Engeland and Dallman (39,40) in a series of lesion and adrenal manipulation experiments, have presented data which suggest strongly that compensatory adrenal growth is a neurally mediated "reflex" and hypothesize that such reflexes might exist for other internal organs such as the liver and kidney. They propose that the reflex "is comprised of afferent nerves from one adrenal gland, interneurons in, or passing through the

hypothalamus, and efferent nerves from the hypothalamus to the contralateral glands" (35). Their data for afferent and efferent control of contralateral hypertrophy after unilateral adrenalectomy can be summarized as follows: prior treatment of the adrenal to be removed with an anesthetic but not with saline, prevents growth of the remaining adrenal until the effect of the anesthetic is worn off (approximately 12 hours); unilateral hypothalamic lesions or disconnections inhibit compensatory adrenal growth if they are on the same side, but not on the side opposite to subsequent adrenalectomy; hemisection of the spinal cord inhibits compensatory adrenal growth if it is contralateral but not ipsilateral to the side of subsequent adrenalectomy; and finally, manipulation of one adrenal results in growth of the contralateral but not the manipulated gland before 12 hrs (35,39-42).

The in vivo results therefore suggest two types of humoral control of the adrenal cortex: hormonal, as shown by ACTH, and neuro-humoral involving afferent and efferent nerves. A third type of humoral control not yet demonstrated in vivo will be discussed in the following section. In vitro plasma, and other growth factors are capable of stimulating mitosis and growth of adrenal cortex cells.

II. In Vitro Considerations

A. Growth of Adrenal Cortex Cells In Vitro

The maintenance of adrenal tissue outside the body was first described by Carrel and Burrows in 1910 (43). Their organ culture system was utilized by Schaberg in 1957 to demonstrate morphological effects of ACTH. His work suggested a differentiation of glomerulosa cells into fasciculata cells in

response to ACTH and fragments of the anterior lobe of rat pituitary (44). O'Hare and Neville (45) were the first to demonstrate homogeneous dispersed cell cultures from adrenal cortical tissue that could be maintained for long periods of time in a functional state. However, under the conditions they employed these cells did not proliferate. Hornsby et al. (46) studied rat glomerulosa cells and observed that a high potassium concentration was necessary for maintenance of the enzymes required for aldosterone synthesis. Under long-term ACTH or cAMP treatment, the cells became identical in their functional and structural characteristics to zona fasciculata-reticularis cells as characterized by O'Hare and Neville (45).

Masui and Garren (47), using as target cells a functional mouse adrenal cortical tumor cell line, were first to demonstrate in vitro an antimitotic effect of ACTH mediated through cAMP. Ramachandran and Suyama (48) reported a similar result using normal rat adrenal cortical cells. Furthermore, they developed an ACTH analog which was capable of eliciting the same maximal rate of steroidogenesis as ACTH but produced only a small fraction of the cAMP generated by ACTH both in isolated adrenal cortical cells and in the adrenal glands of hypophysectomized rats. They found, however, that both the ACTH analog and cAMP-stimulated steroidogenesis and inhibited DNA synthesis (32,48). They also demonstrated that while insulin had no effect on ACTH induced steroidogenesis, it did induce DNA synthesis. When added together, however, ACTH inhibition of DNA synthesis overrode this insulin effect (32,48).

Gospodarowicz et al. demonstrated the antimitotic effect of ACTH in monolayer cultures of bovine adrenal cortical cells. In addition, they

demonstrated a potent mitogenic effect of fibroblast growth factor (FGF) (49). This polypeptide is isolated from brain or pituitary and is mitogenic in vitro for a vast array of mesodermally derived cells (50). ACTH was still found to inhibit the proliferation of the bovine adrenal cortical cells in response to FGF (49).

Unlike insulin and FGF, which have been shown to increase DNA synthesis and cell proliferation in a number of cell types in vitro, Gill et al. (51) demonstrated the specific effect of angiotensin II on DNA synthesis and cell growth in bovine adrenal cortex cells. They tested 6 cell types including bovine granulosa, aortic endothelial, smooth muscle, lung fibroblast, BALB/c 3T3 and rabbit ear chondrocytes and found that only the adrenal cortical cell responded. They further showed that precursors of angiotensin II, and angiotensin III demonstrated equivalent mitogenic activity but that angiotensinogen and angiotensin I were required in a 10-fold higher concentration. In thymidine incorporation studies, they showed a synergistic effect of insulin with angiotensin II which equalled the maximally mitogenic effect of that produced by FGF. Since angiotensin II had a direct effect on steroid output by these cells it was one of the first hormones demonstrated to induce growth and differentiated function in its target cell. ACTH was again able to inhibit the angiotensin II induced DNA synthesis (51).

Cell culture studies of human adult adrenal cortical cells have not proved of much help in understanding the mechanism of cortical hyperplasia. The cells simply will not grow or will dedifferentiate under conditions currently employed. Human fetal adrenal cells, however, have been useful in elucidating factors involved in cortical hyperplasia in vitro. Results presented

in this thesis will demonstrate that in addition to FGF, EGF was mitogenic for both fetal and definitive zone derived cells and that both cell types have specific EGF receptors as measured by radioligand binding. The effect of ACTH on the specific steroid secretions of each zone is consistent to that which is observed in vivo. ACTH is antimitotic to these cells in vitro.

Therefore, insulin and insulin-like derivatives, FGF, angiotensin II, and EGF are factors which appear to regulate the growth of adrenal cortex cells in vitro. The action of each factor on proliferation is overridden by the antimitotic effect of ACTH. While glucocorticoids secreted by adrenal cortical cells in response to ACTH can inhibit cell proliferation in supraphysiological doses, this does not appear to be the explanation of the ACTH antimitotic effect in vitro, since suppression of steroidogenesis with aminoglutethimide does not prevent ACTH or cAMP from inhibiting growth (20). It has been shown, however, that for bovine adrenal cortical cells in vitro, prolonged exposure to ACTH causes desensitization and escape from this antimitotic effect (52). Therefore, the in vitro inhibition of cell proliferation by ACTH followed by desensitization to ACTH and proliferation after prolonged exposure, corresponds remarkably well to that which has been described in vivo (30,31).

B. Steroidogenic Function of Adrenal Cortex Cells In Vitro

Steroidogenic responses in vitro, while comparable in early passage cultures to their in vivo counterparts, can deviate with time in culture. Bovine adrenal cortical cells, for example, synthesize and secrete steroids in response to ACTH, cAMP derivatives, prostaglandin E_1 prostacyclin, and

cholera toxin (52). Primary cultures initially synthesize cortisol, but under standard culture conditions, whether or not cells proliferate, 11 β hydroxylase activity is rapidly lost and cortisol production ceases (52). Quantitatively, 11 deoxycortisol is the major steroid produced once 11 β hydroxylase activity becomes deficient, although six other steroids are secreted (53). The reason for the loss of 11 β hydroxylase activity in these cells in culture may be that standard culture conditions are incompatible with the expression of this enzyme. 11 β hydroxylase activity has been found to be lowered by a combination of two factors: (a) oxygen tension, and (b) any steroid which is either a substrate for 11 β hydroxylase or its product (54). Its activity therefore has been shown to be restored in vitro by the use of antioxidants and lowered O_2 concentration (54).

In addition to an altered steroid biosynthetic pathway bovine adrenal cortical cells lose their responsiveness to ACTH, but not angiotensin II or prostaglandin E_1 with time in culture (55). During long-term growth, the maximal ACTH-stimulated cAMP production rate falls about 7% per population doubling while PGE stimulated cAMP production rate remains constant (56). The results of Hornsby and Gill (56-57) suggest that the number of functional ACTH receptors decline over time, and that cell density and growth rate are primary determinants (57). Hornsby and Gill conclude that for maintenance of differentiated function of adrenal cortical and probably differentiated features generally in cell culture, cell density and growth rate, as well as oxygen concentration and antioxidizing conditions are of primary importance (52,54,56,57).

The humoral factors which have been elucidated in studies of growth or steroidogenesis of adrenal cortical cells in vitro have all been carried out in what has been referred to as "standard culture conditions." This refers to cells which are cultured on plastic tissue culture dishes in serum-supplemented media in a 5-10% CO_2, 21% O_2, humidified incubator. Although these conditions are clearly not physiological, adrenal cortical cells have shown a remarkable ability to adapt to the given environment and proliferate as well as carry out some of their differentiated functions. While serum is required to culture adrenal cortical cells, or most cells for that matter, its use is of concern since cells, unless at the site of a wound, are not exposed to it in vivo. Most in vitro studies carried out in serum-supplemented media must therefore be subject to this criticism.

Variations in cell morphology and in the expression of adrenal cortical cells' specific properties in response to different batches of serum have been described (58). In particular, the different morphological and functional forms of rat adrenal cortical cells when cultured in horse serum or fetal calf serum have been studied (58,59). Growth as either proliferating, fibroblast-like cells with limited but definite evidence of adrenal cortical differentiation, or as more highly differentiated stationary epithelial cells was obtained through the use of either fetal calf or horse serum, respectively. Substitution of fetal calf serum for horse serum enhanced cell division and motility and reduced steroidogenic capacity (58-60). The horse serum provided a more suitable environment for differentiation presumably because it lacked the growth promoting properties of fetal calf serum or because it contained factors required for differentiation. Furthermore, the two cell-types (epithelioid or

fibroblastic) were interconvertible. Exposure of the epithelioid cell type to fetal calf serum caused modulation to a myofibroblastic form, while exposure of this latter cell type to ACTH caused a reversion to the epithelioid form (60). In her most recent work (61) E. A. Turley presents evidence that the fetal calf serum enhancement of proliferation, motility, and inhibition of steroidogenesis is modulated through the production of extracellular matrix material. The data suggest that ACTH causes disappearance or inhibition of synthesis of this ECM and promotes the functional expression of the adrenal cortical parenchymal phenotype (61). Whether the fetal calf serum contains components of ECM or factors which either stimulate the cells to produce ECM or utilize it from the fetal calf serum is difficult to assess because of the multitude of components found in such serum. While the results are indeed convincing, especially in elucidating the morphological distinctions between the growth or differentiating state of the cells, the individual components responsible for these states, with the exception of ACTH, are still not recognized.

The results of Slavinski et al. showing adrenal cortical cell characteristics in the fibroblast-like cultures grown in fetal calf serum or in the epithelial-like cultures grown in horse serum is valuable information. First, the work points out the inability to classify cells, on the basis of morphological criteria, when cultured under "standard in vitro conditions"; second, it points out the variability of cells in vitro to serum supplements and the need to determine the proper in situ physical and humoral conditions for comparative cell function in vitro; lastly, it specifically implicates factors present in serum which effect gross morphological or functional differences

and therefore the need to identify, isolate, and characterize such factors by such criteria.

It becomes clear that there is a need to culture cells in the absence of such supplemented media. This should allow for a greater understanding of the control of proliferation or differentiation of cells since their responses to individual factors can then be assessed. Implied also is the ability to study the mechanism of action of a given factor in the absence of hundreds of individual components of serum which may alter or interfere with its specific action.

C. Working Hypothesis

If cells can be cultured in vitro in a consistent, controlled, defined environment, specific factors regulating either their growth or differentiation might be elucidated. Furthermore, their mechanisms of action might then be studied. The factors which trigger either cell proliferation or differentiation may have mechanisms of action common to all cells.

The initial focus of the experiments presented in this thesis was to define a growth medium for cells of the adrenal cortex by utilizing ECM coated dishes, specific growth factors and components of plasma. Once adrenal cortical cell growth requirements were established, factors regulating differentiated function (steroidogenesis) were studied. Finally, early events in the course of either proliferation or differentiation were studied in response to the addition of specific factors.

The effect of various substrata, serum, or individual plasma components was tested on the growth and differentiated function of adrenal cortex cells. The results are presented in a chronological manner. Primary cultures of

adrenal cortical cells were established on plastic, extracellular matrix-coated, or fibronectin-coated tissue culture dishes. Serum and the exogenous growth factors EGF and FGF, were studied for their effects on proliferation when adrenal cells are grown on either ECM-coated or plastic tissue culture dishes. Individual components of plasma were studied for their ability to promote cell proliferation of cells maintained on the various substrata. When these components were identified and the optimal growth promoting conditions established, the long-term growth and steroidogenic function of cells cultured in the serum-free defined media were analyzed. Additionally, plasma components such as lipoproteins and hormones were assessed for their ability to stimulate steroid production. The steroidogenic function of cells cultured in the absence of serum was analyzed throughout their in vitro life span. Finally, insulin, which was found necessary for optimal proliferation and steroid production, was analyzed for its ability to stimulate the binding, internalization, and degradation of low-density lipoprotein by adrenal cortical cells.

Chapter III will consider the growth and steroidogenic functions of human fetal adrenal cortex cells. While studies of adult human adrenal cortical cells were carried out the results of these studies will not be presented. Adult human adrenal cells were highly variable in proliferation and they dedifferentiated when maintained in vitro using currently available media. Chapter IV will begin a series of studies, continued in Chapter V on bovine adrenal cortical cells maintained in a serum-free defined environment as discussed above. These cells are attainable on a daily basis. The majority of studies have therefore been conducted on bovine tissue which also appears

to adapt more easily to currently available tissue culture media and *in vitro* environment.

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Chapter 3

Factors Involved in Supporting the Proliferation of Human Fetal Adrenal Cells In Vitro

I. Introduction

Most of the previous studies concerning the human fetal adrenal gland have focused on its steroidogenic capacity (1, 2). By using a variety of techniques and conditions, it has been established that cortisol is the principal steroid produced by the definitive zone, while dehydroepiandrosterone sulfate (DHAS) is the principal steroid produced by the fetal zone (3, 4). However, the factors influencing the proliferation of the two zones are poorly understood. A class of mitogenic polypeptides, termed growth factors, has been shown to play an important role in the proliferation of steroid-secreting cells (5). Such growth factors may mediate the effects of classical growth-promoting hormones, e.g. ACTH and growth hormone, since in most cases these proteins stimulate cell proliferation in vivo but not in vitro (6-8).

The aim of this study was to investigate the influence of epidermal growth factor (EGF) and fibroblast growth factor (FGF) on the proliferation of cultured human definitive and fetal zone cells. EGF, which was first isolated from submaxillary glands of adult male mice (9), is a potent mitogen for many cell types in vitro, including human diploid fibroblasts (10). EGF has subsequently been isolated from human urine and amniotic fluid, primarily in the form of urogastrone (11, 12). FGF has been purified from bovine pituitary and brain (13). It is a potent mitogen for a wide variety of mesoderm-derived cells, including bovine adult and human fetal adrenal cells (14, 15). In addition, FGF has been shown to stimulate proliferation of human umbilical vein endothelial cells in vitro (16).

Our results indicate that EGF and FGF are mitogenic for human definitive and fetal zone cells in vitro. To characterize further the interaction of these mitogens with the target cell, ^{125}I-EGF binding to fetal and definitive zone cells was investigated.

In addition to growth factors, the substrate on which cells are cultured may play a role in the differentiated function and proliferation of those cells. The observations that FGF induces certain cell types to produce their own ECM and that cells which proliferate best under the influence of FGF produce an ECM (17) led to the use of ECM as a substrate for assessing in vitro cell proliferation. In the present study, the proliferative behavior of definitive and fetal zone cells maintained on plastic was compared with cells maintained on an ECM produced by corneal endothelial cells in vitro and characteristic of the basement membrane in vivo.

II. MATERIALS AND METHODS

Materials

FGF was purifed from bovine brains as described previously (18) and EGF (M.W. 6045) was purified as described by Savage and Cohen (19). Medium 199 supplemented with Earle's balanced salt solution (EBSS) was obtained from the Cell Culture Facility, UCSF Medical Center. Fetal calf serum was obtained from Irvine Serum Co. (Irvine, Ca.). Tissue culture dishes were from Falcon Plastics. Dextran (MW 40,000) was from Sigma, Gentamycin was from Schering, and Fungizone was from Squibb. Collagenase (34 u/mg) was obtained from Millipore Corp. and DNAase (bovine pancreas) was from Sigma. ACTH 1 — 39 was a generous gift from Dr. J. Ramachandran, Hormone Research Laboratory, UCSF Medical Center.

Cell dispersion

Human fetal adrenal glands from four fetuses of 13 to 20 weeks' gestation were obtained following elective dilatation and evacuation. The definitive zone was dissected by gently removing the capsule of the gland to which the definitive zone was adherent. This procedure yielded a relatively pure preparation (90-95%) of definitive zone cells. The remaining fetal zone tissue contained approximately 10% definitive zone cells adherent to its surface. To increase the homogeneity of the fetal zone cell preparations, the central portion of the fetal zone was biopsied with a needle. The resultant fetal zone cells were then used for cell dispersion. Minced fetal adrenal tissue was incubated in medium 199 with collagenase (3 mg/ml) in the presence of DNAase (0.002%) for 1 hr at 37°C (95% O_2/5% CO_2). The cells were passed through cheesecloth, centrifuged at 500 xg for 5 min, and resuspended in medium 199 (with EBSS) supplemented with 10% fetal calf serum. It was found that dispersed fetal zone cells incubated in suspension for two hours in the presence of ACTH 1-39 produced significant quantities of DHAS without producing any cortisol (unpublished data). This would indicate a relatively homogeneous preparation of fetal zone cells. For each experiment, only separated cells from the same gland were used.

Steroid measurements

DHAS was measured directly from the medium by radioimmunoassay. The antibody was developed at UCSF in the Dept. of OB/GYN (D. Koritnik, R. Laherty, D. Rotten, R.B. Jaffe, unpublished data). Since the antibody does not distinguish DHAS from DHA, the relative proportion of each steroid was determined. After celite chromatography (20), it was found that DHA accounted for 10% or less of the total DHAS/DHA produced by the human fetal adrenal cells in culture. The measurements are therefore reported as

DHAS, since the conjugated steroid accounts for 90% of the total DHAS/DHA produced.

Cell growth measurements

Separated definitive and fetal zone cells were plated individually at an initial density of 5-10 X 10^3 cells per 35 mm dish in 2 ml of medium 199 (with EBSS) supplemented with 10% fetal calf serum. Sixteen hours later, the media were renewed with fresh medium 199 (with EBSS) supplemented with 10% fetal calf serum. FGF (100 ng/ml) or EGF (20 ng/ml) were added every other day. Cultures were trypsinized after 8 days, and cells were counted with a Coulter counter. Cell counts were compared with those values obtained with the use of a hemocytometer and no statistically significant differences were noted.

For the study of serum dependency, the cells were plated as described above. Sixteen hours later the media were renewed with fresh medium 199 (with EBSS) containing 1% or 10% fetal calf serum. The cultures were then maintained in the presence or absence of either EGF or FGF as described above.

Iodination of EGF

The iodination of EGF was carried out as described by Carpenter and Cohen (21), using a molar ratio of protein to $Na^{125}I$ of approximately 1:1. EGF (5 µg) was dissolved in 25 µl of 0.05 M phosphate buffer, pH 7.5, and added to 25 µl of 2 M phosphate buffer, pH 7.5, containing 2 mCi of carrier-free $Na^{125}I$. Chloramine-T (100 µg in 10 µl) was then added for 1 min at 4°C. The reaction was stopped by the adddition of sodium metabisulfite (200 µg in 25 µl). The labeled protein was separated from unreacted $Na^{125}I$ by gel filtration through Sephadex G-10 with a phosphate-buffered saline (0.9% NaCl) solution containing 0.1% albumin. The labeled EGF was stored frozen in the presence of 0.1% albumin. The specific activity of the EGF was 370,000 to 460,000 dpm/ng.

Measurement of binding of EGF by fetal adrenal cells

Five X 10^4 cells were plated per 35 mm dish and were used when confluent (6 days later) for binding studies. Cell monolayers were washed twice with 2 ml aliquots of Dulbecco's modified Eagle's medium containing 0.2% albumin. The cells were incubated at 37°C for 30 min in 1 ml of binding medium containing ^{125}I-EGF at the required concentration. After incubation, unbound radioactivity was removed by washing the cells eight times with cold (4°C) phosphate-buffered saline containing 0.1% albumin. The radioactivity present on the cell surface but not internalized was removed by treatment with acetic acid using the procedure described by Haigler et al (22). Cultures were treated with 1 ml of acetic acid (0.2 M, pH 2.5) containing 0.5 M NaCl for 6 min at 4°C. The radioactivity within the 1 ml aliquot was measured with a Beckman 310 gamma counter. Nonspecific binding was determined in the presence of 2 μg/ml of unlabeled EGF and amounted to less than 10% of the total activity measured.

Preparation of plates coated with an ECM

Bovine corneal endothelial cells were plated at an initial density of 10^4 cells per 35 mm dish and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum, 5% (vol/vol) calf serum, 5% (wt/vol) Dextran T-40, Gentamycin (50 μg/ml), and Fungizone (2.5 μg/ml). FGF (100 ng/ml) was added every other day. Once the cultures became confluent (ordinarily within six days), the media were renewed and the cultures were further incubated for an additional six days. The cultures then were washed with phosphate-buffered saline and exposed for 30 min to 0.5% Triton X-100 in the same buffer. Once the nuclei and the ECM became visible, the cultures were washed three times with phosphate-buffered saline.

Statistical analysis

Results are expressed as the mean of triplicate cultures ($\pm$ SD) unless otherwise stated. Statistical significance of differences between groups was calculated using analysis of variance (ANOVA). Scatchard analysis was performed using a best-fit computer program.

III. RESULTS

Effects of EGF and FGF on the growth of cultured fetal adrenal cells

To assess the mitogenic roles of EGF and FGF, separated fetal and definitive zone cells were plated at low density in the presence or absence of EGF or FGF. Within nine days the definitive zone cells became confluent in the presence of FGF. While the final density of fetal zone cells was lower than that of the definitive zone cells, EGF and FGF clearly were mitogenic for both zones (Fig. 1).

To assess the steroidogenic function of the adrenal cells, the media were changed on day nine and the cells were incubated in the presence or absence of 10 ng/ml of ACTH 1-39 for eight hours (Fig. 2). Increases in cell number in the presence of EGF or FGF compared with controls were associated with proportional increases in DHAS production in the basal or ACTH stimulated states. Net fetal zone cell DHAS production was approximately 9 times that of definitive zone cells. The relative proportion of DHAS produced by each cell type was similar to that obtained from short-term incubations of dispersed fetal and definitive zone cells. This would indicate that the relative DHAS production for both cell types was maintained under primary cell culture conditions. In addition, EGF demonstrated a slight steroidogenic effect on both cell types.

The morphology of the cultured adrenal cells on day 9 was examined under phase contrast microscopy. After 8 hours of ACTH stimulation, 60% or

more of the fetal and definitive zone cells demonstrated a definite retraction response (Fig. 3). Both definitive and fetal zone cells dmonstrated areas of differentiated cells with abundant granular cytoplasma and variation in the size of the nuclei (Fig. 3). Retraction response to ACTH was most prominent in these areas. In other areas where the cells were less differentiated and more closely packed together, the retraction response to ACTH was less prominent or absent. When the definitive and fetal zone cells were maintained in medium 199 (with D-valine but not L-valine) and 10% dialysed fetal calf serum to reduce any potential fibroblast growth, the same morphologic pattern was noted for both fetal and definitive zone cells, with 60% or more of the cells demonstrating a definite retraction response to ACTH on day 9. No definite retraction response to ACTH was noted for definitive or fetal zone cells maintained on ECM in spite of demonstrable steroid production. Therefore, by 8 to 9 days in culture, the majority of cells from both zones were of adrenal origin, as confirmed by the DHAS production studies and retraction response to ACTH. The less differentiated cells probably represent a morphologic transformation of adrenal cells in culture, but the possibility of some fibroblsat contamination cannot be excluded.

To study the influence of serum concentration on proliferation, low density cell cultures maintained on plastic were exposed to either high (10%) or low (1%) concentrations of fetal calf serum (Fig. 4). Cells maintained on plastic in low serum conditions hardly proliferated in the absence of mitogens. In the presence of EGF or FGF, the increase in cell number over that in the control was dependent on the serum concentration.

When low density adrenal cell cultures were exposed to increasing concentrations of FGF or EGF, both the definitive zone cells (Fig. 5) and fetal zone cells (Fig. 6) demonstrated similar responses to EGF and FGF. Maximal

growth response to EGF was observed at a concentration of 0.5 to 1.0 ng/ml, while the maximal response to FGF occurred at a concentration of 100 ng/ml.

^{125}I-EGF binding studies

^{125}I-labeled EGF binding to fetal and definitive zone cells demonstrated high affinity (K_D 10^{-9} M) for both cell types. For definitive zone cells (Fig. 7), maximal binding was observed at a 7 X 10^{-9} M concentration of EGF. Maximal binding of ^{125}I-labeled EGF to fetal zone cells (Fig. 7) occurred at a similar concentration of EGF. The binding capacity for the fetal zone cells was 28 picomol/10^6 cells, while the binding capacity for the definitive zone cells was 23 picomol/10^6 cells. The number of binding studies is too small to determine if there are any statistically significant differences in the K_D or binding capacity between definitive and fetal zone cells.

Growth of cultured cells on ECM

In contrast to cells maintained on plastic, low density fetal and definitive zone cell cultures maintained on ECM reached a significantly higher cell density. For both cell types, the final density of cells maintained on ECM without growth factors was similar to the final density of cells maintained on plastic in the presence of FGF. Figure 9 demonstrates the effect of substrate on the proliferation of definitive zone cells in the presence or absence of FGF or EGF. Proliferation of definitive zone cells maintained on plastic in the presence of FGF was comparable to that of cells maintained on an ECM in the absence of growth factors. This was also the case for fetal zone cells (not shown). These results emphasize the marked influence of the ECM on proliferation. When the effect of an ECM on the proliferation of both cell types was compared (Fig. 10), fetal zone cells (but not zone definitive cells) demonstrated a significant increase in proliferation when EGF or FGF was present compared to cells maintained on ECM without growth factors.

IV. DISCUSSION

Factors controlling the proliferation of cells of the human fetal adrenal gland are poorly understood. The gland grows rapidly between 10 and 20 weeks of gestation and by the end of the first trimester has attained a size equal to or greater than that of the fetal kidney. At 10 weeks' gestation the gland weighs under 50 mg, and by 20 weeks the weight increases to one gram (23). The bulk of the gland (80%) consists of fetal zone cells which demonstrate the typical characteristics of steroid-secreting cells. This inner, fetal zone is surrounded by the definitive, or adult zone, which is composed of small basophilic cells that resemble those of the adult zona glomerulosa (24). Adrenal glands from anencephalic and apituitary fetuses demonstrate normal development of the gland up to 15 weeks' gestation with subsequent atrophy of the fetal zone in later gestation (25). Although ACTH can be detected in the human fetal pituitary by the tenth week of gestation (26), pituitary hormones do not appear to be essential for the development of the fetal zone for up to 15 weeks' gestation. In late gestation, ACTH may play a more obligatory role, since atrophy of the fetal adrenal and decreased adrenal steroid secretion are noted after treatment with glucocorticoids (27).

Although ACTH may be involved with target cell proliferation and differentiation in vivo, its action may be mediated by other factors. That other factors may be mediating the effects of classical growth-promoting hormones (i.e. estrogen, ACTH and growth hormone) is suggested by the observation that they do not stimulate growth in vitro of the same target cells which they appear to stimulate in vivo (6-8). Furthermore, some of the growth-promoting hormones in vivo actually may inhibit target cell proliferation in vitro, as is the case for ACTH (28).

Because the classical trophic hormones do not have mitogenic activity in vitro and others, such as ACTH, are antimitotic, it is likely that growth factors play a prominent role in the control of cellular proliferation. Our results indicate that EGF and FGF are potent mitogenic agents for both fetal and definitive zone cells in vitro. The dose of EGF producing a half-maximal response for adrenal cell proliferation was 3×10^{-11}M (Fig. 6), while saturation of EGF binding sites occurred at a concentration of 4×10^{-9} M (Figs. 7 & 8). The finding that only a small percentage of occupied EGF binding sites is necessary to effect a maximal proliferative response suggests the presence of spare receptors.

Although EGF and FGF clearly are mitogenic for fetal adrenal cells in vitro, the manner in which they exert their effect is not fully understood. Studies on the control of cellular proliferation by FGF have shown that the cells which proliferate best in tissue culture retain their ability to produce an ECM, whereas cells that proliferate poorly, or not at all, have lost that ability (17). In vivo the ECM serves as the natural substrate on which cells will migrate, proliferate and differentiate (29). Recent studies, including our own, have shown that the ECM can greatly affect the proliferation of certain cells in vitro (30, 31). The emerging view of the ECM is one of an active participant in differentiated cell growth rather than merely as a passive support network. The regulation of the ECM by growth factors represents an important mechanism by which growth factors influence cell proliferation.

EGF and FGF may influence cell proliferation by other means in addition to the regulation of an ECM. In the case of fetal zone cells, the addition of EGF or FGF to cells maintained on an ECM had a significantly greater effect on cell proliferation compared to cells maintained on an ECM in the absence of growth factors. Whatever the mechanism(s) of action, the effects of EGF

and FGF probably are permissive, since their influence on cell proliferation is dependent upon the serum concentration in which they are maintained.

While EGF and FGF are mitogenic for human fetal adrenal cells in vitro, it is possible that these mitogens play a role in development by exerting their influence on a number of key stages of organogenesis. Since FGF is mitogenic for vascular endothelial cells, it is possible that such mitogens could regulate the overall growth of a tissue by affecting capillary density. When active growth of an organ is preceded by capillary invasion during embryonic development, capillary density may be an important aspect of growth control (15). Regulation of the ECM by growth factors also could play a role in embryogenesis. That extracellular matrices promote and stabilize the differentiation of the epithelia associated with them has been confirmed by several studies (29, 32, 33). It is now becoming evident that the ECM can have a marked influence on both cell proliferation and differentiation. The influence of ECM on fetal adrenal cell proliferation emphasizes this point. While the influence of growth factors on cell proliferation is probably not limited to the regulation of an ECM, further studies are necessary to elucidate their mechanism(s) of action. Growth factors may therefore be involved in embryogenesis by influencing capillary density and regulating the extracellular matrix, which can influence cell proliferation and morphogenesis profoundly.

It is of interest to note that EGF is a potent mitogen for human fetal adrenal cells in vitro but not for bovine adrenal cells or the mouse Y-1 adrenal cortical tumor cell line (34). The difference in response is not due to different culture conditions, since under similar conditions the difference in response to EGF is maintained. The difference in response to EGF may indicate a sensitivity to EGF during the fetal stage of development that may be important for embryogenesis. However, the difference in response may also

be species specific. Until adult human adrenal tissue is examined for responsiveness to EGF, the question of species specificity remains unanswered.

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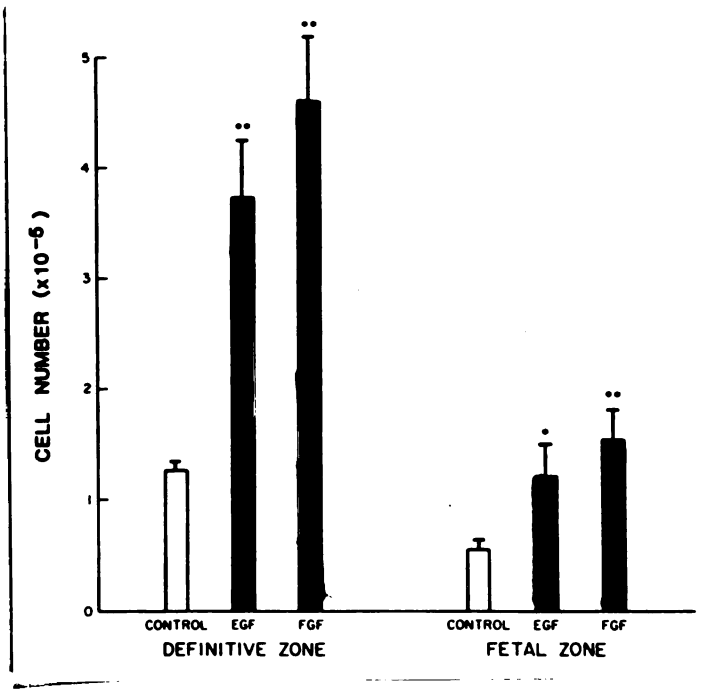

Fig. 1. Effects of FGF and EGF on the proliferation of fetal and definitive zone cells. Adrenal cells were plated at 10^4 cells/3.5 cm plastic dish. They then were maintained in the presence of 10% fetal calf serum with or without EGF (20 ng/ml) or FGF (100 ng/ml). On day 9, cells were trypsinized and counted. The results are expressed as the mean $\pm$ S.D. of triplicate plates (* P 0.01, ** P 0.001).

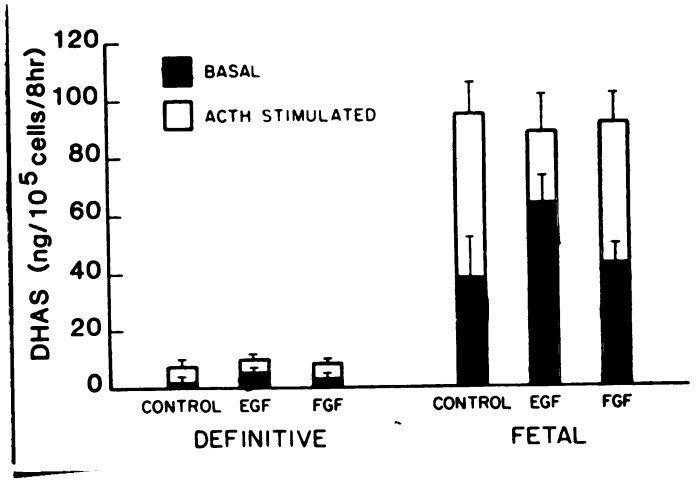

Fig. 2. Effects of EGF and FGF on ACTH-stimulated dehydroepiandrosterone sulfate (DHAS) production. Separated fetal adrenal cells were incubated in the presence of 10% fetal calf serum with or without EGF or FGF for nine days. After fresh medium containing 10% fetal calf serum (± FGF or EGF) was added, the fetal adrenal cells were incubated with (open bar) or without (closed bar) ACTH 1-39. After 8 hours, DHAS concentration was quantified and the cells were counted. Error bars represent the mean ± S.D. for triplicate plates.

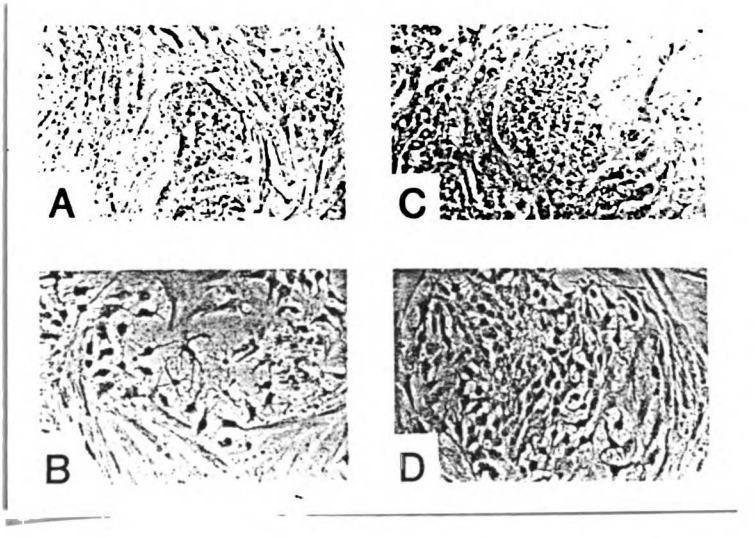

Fig. 3. Morphological appearance of fetal and definitive zone cells in the absence and presence of ACTH. Primary cultures of separated human fetal adrenal cells were maintained in the presence of FGF (100 ng/ml) for 9 days as described in the text. The fetal (C, D) and definitive (A, B) zone cells were then maintained in the presence (B, D) or absence (A, C) of ACTH (10 ng/ml) (phase contrast optics, X 150).

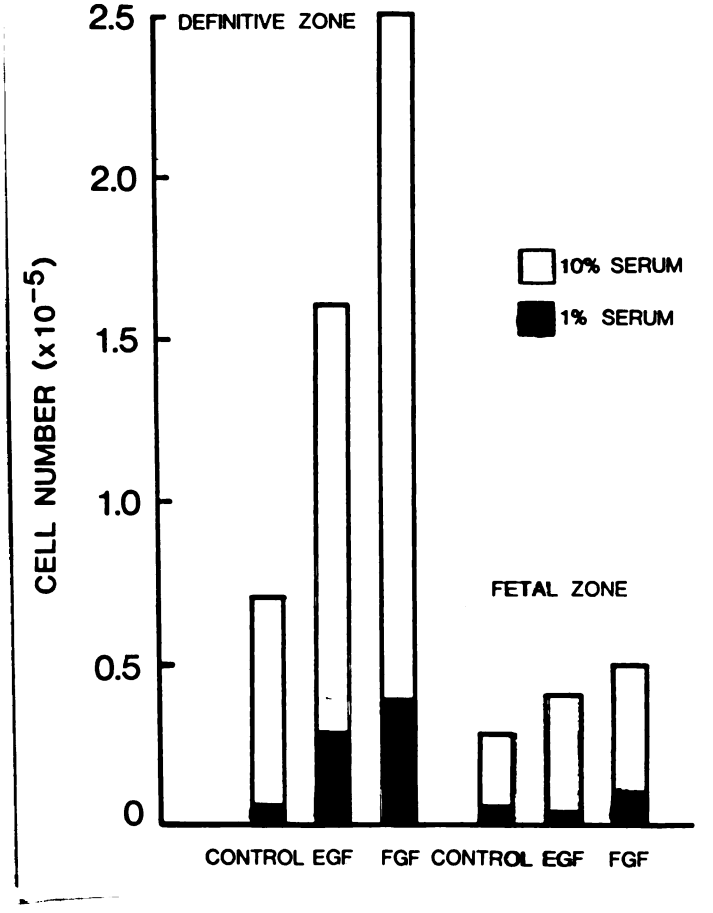

Fig. 4. Relationship between FGF, EGF, and serum concentration on culture growth. Separated fetal adrenal cells were plated at 6000 cells/3.5 cm dish in the presence of 1% (open bar) or 10% (closed bar) fetal calf serum. EGF (20 ng/ml) or FGF (100 ng/ml) were added every other day. On day 9, the cells were trypsinized and counted. The results are expressed as the mean of duplicate plates; S.D. did not exceed 10% of the mean.

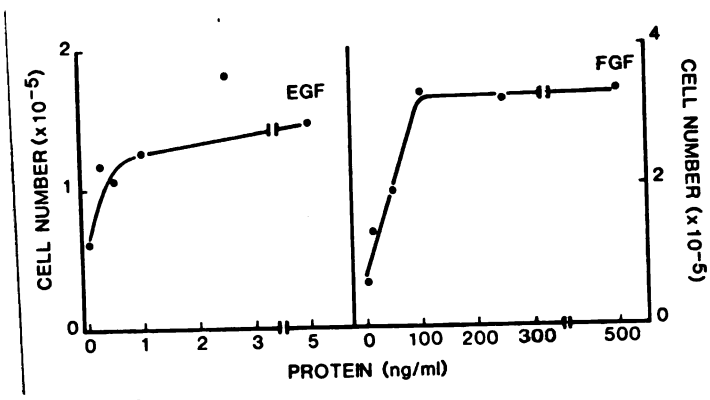

Fig. 5. Effects of increasing concentrations of FGF and EGF on the proliferation of definitive zone cells. Cells were plated at 5000 cells/3.5 cm dish in the presence of 10% fetal calf serum and increasing concentrations of EGF or FGF. On day 9, the cells were trypsinized and counted. Each point represents the mean of duplicate plates. Note difference in scales.

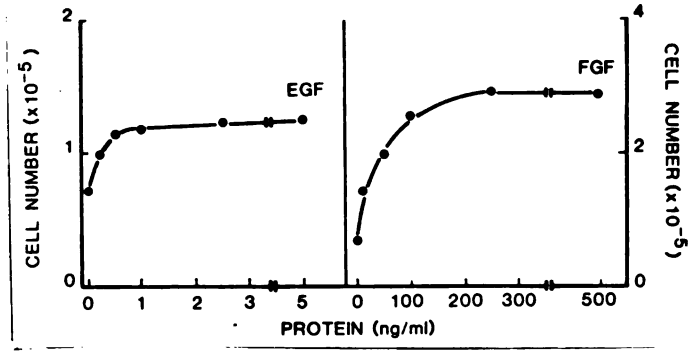

Fig. 6. Effect of increasing concentrations of FGF and EGF on the proliferation of fetal zone cells. Cells were plated at 10^4 cells/3.5 cm dish in the presence of 10% fetal calf serum. Increasing concentrations of FGF or EGF were added every other day. At day 9, the cells were trypsinized and counted. Each point represents the mean of duplicate plates. Note difference in scales.

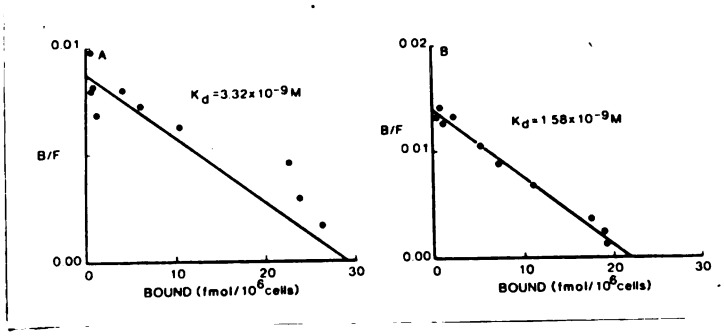

Fig. 7. Saturation analysis of ^{125}I-EGF binding to cultured definitive and fetal zone cells. Five X 10^4 cells were seeded per 3.5 cm dish. Confluent cultures were obtained in the presence of 10% fetal calf serum until confluent (6 days). ^{125}I-EGF bound to the cell surface was determined as described in the text. Each point for the saturation analysis (A) and Scatchard plot (B) represents the mean of duplicate plates.

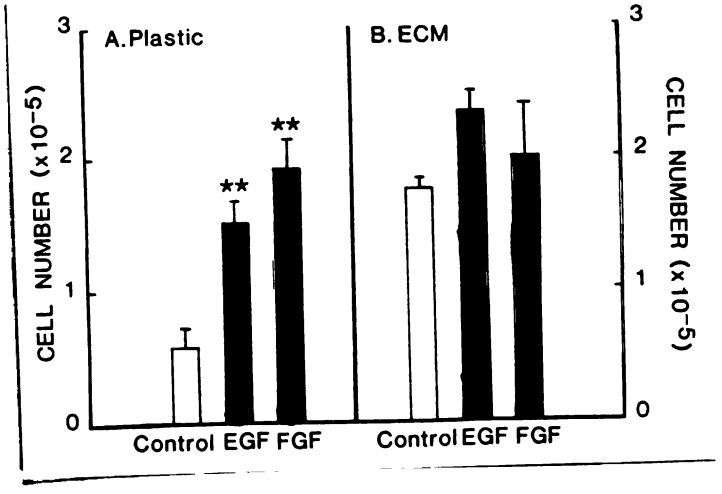

Fig. 8. Influence of substrate, FGF, and EGF on the proliferation of definitive zone cells. Adrenal cells were plated at 10^4 cells/3.5 cm on plastic plates without (A) or coated with (B) an ECM in the presence of 10% fetal calf serum. EGF (20 ng/ml) or FGF (100 ng/ml) was added every other day. At day 9, the cells were trypsinized and counted. The results are expressed as the mean ± S.D. of triplicate plates (** P 0.001).

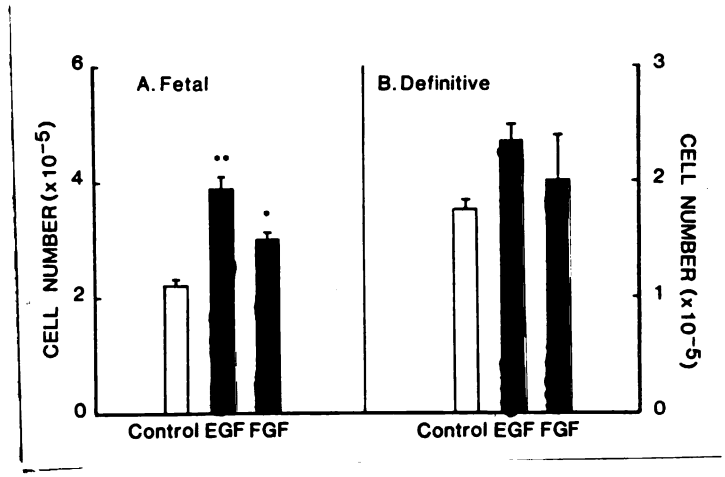

Fig. 9. Effects of ECM, FGF, and EGF on the proliferation of fetal adrenal cells. Fetal zone cells (A) were plated at 10,000 cells/3.5 cm plate coated with an ECM, and definitive zone cells (B) were plated at 5000 cells/3.5 cm plate coated with an ECM. EGF (20 ng/ml) or FGF (100 ng/ml) was added every other day in the presence of 10% fetal calf serum. On day 9, the cells were trypsinized and counted. The results are expressed as the mean $\pm$ S.D. of triplicate plates (* P 0.01; ** P 0.001).

Chapter 4

Factors Involved in Supporting the Growth and Steroidogenic Functions of Bovine Adrenal Cortical Cells Maintained on Extracellular Matrix and Exposed to a Defined Medium

I. Introduction

Previous studies done on the control of proliferation of cultured adrenal cortex cells have shown that fibroblast growth factor (FGF) is a mitogen for that cell type, while ACTH is growth inhibitory (15, 29, 31, 32). Use of FGF has allowed the development of clonal strains of bovine adrenal cortex cells which depend on FGF not only for their proliferation but also for their life span in culture (15, 24, 35). This mitogenic effect of FGF has been shown to be a strict function of the serum concentration to which cultures are exposed, thereby suggesting that as yet unidentified factors in serum work in concert with FGF to promote optimal cell growth and long-term survival (15).

In later studies, it was shown that the requirement for FGF exhibited by cultured adrenal cortex cells was a function of the substrate upon which cells were maintained. While cultures maintained on plastic required both FGF and serum in order to proliferate at an optimal rate, cultures maintained on an extracellular matrix (ECM) required only serum (8).

In an effort to elucidate the nature of serum factor(s) involved in the control of proliferation of bovine adrenal cortex cells, we have in the present study analyzed the effect of the substratum (plastic versus fibronectin- or ECM-coated dishes) and that of plasma factor(s), such as transferrin, HDL, LDL, and insulin, or growth factors such as FGF, in controlling the proliferative rate, life span, and steroidogenic response of bovine adrenal cortex cells maintained under serum-free conditions.

MATERIALS AND METHODS

Materials

Fibroblast growth factor (FGF) was purified from bovine brains as previously described (7). Brain FGF yields a single band on polyacrylamide gel electrophoresis at pH 4.5 and on an isoelectric focusing column (total volume 110 ml, pH range 3.4 to 11.0). The activity focused within a single peak having an isoelectric point of 9.6. When analyzed by SDS slab gel polyacrylamide electrophoresis, a single band (13,000 MW) was observed when gels were stained with silver nitrate (17). Fibronectin was purified from bovine plasma as described by Engvall, Ruoslahti, and Miller (5). When analyzed by slab gel polyacrylamide gel electrophoresis under reduced conditions, the purified bovine plasma fibronectin ran as a doublet with a molecular weight in the range of 220,000.

Crystalline bovine serum albumin was obtained from Schwarz-Mann (Orangeburg, N.J.). Insulin, transferrin, and dibutyryl cyclic AMP (db cAMP) were obtained from Sigma Chemical Co. (St. Louis, Mo.). ACTH 1-24 (Organon, West Orange, NJ) was a gift from the Dept. of Pediatric Endocrinology (Univ. of Calif., San Francisco). Dulbecco's modified Eagle's medium (DME) and Ham's F-12 medium were obtained from Grand Island Biological Co (Grand Island, N.Y.). Calf serum and fetal calf serum were obtained from Irvine Serum Co (Irvine, Ca.). Horse serum was obtained from Colorado Serum Co. (Denver, Colo.).Tissue culture dishes were from Falcon Plastics, Gentamicin from Schering Co. (Kenilworth, N.J.), and Fungizone from Squibb (Princeton, N.J.).

Preparation of low density lipoprotein (LDL), high density lipoprotein (HDL), and high density lipoprotein subfractions (HDL_2, HDL_3)

Human LDL (1.019 d 1.063 g/cm^3), HDL (1.07 d 1.21 g/cm^3), HDL_2 (1.07 d 1.125g/cm^3), and HDL_3 (1.130 d 1.21 g/cm^3) were obtained from

human plasma by differential ultracentrifugal flotation (22). Bovine HDL (1.07 d 1.21 g/cm^3) was obtained from bovine plasma by the same technique (25). In order to remove contaminating plasma proteins and lipoprotein fractions of higher density, the LDL, HDL, and HDL subfractions were washed by flotation through a solution corresponding to their respective upper densities. Protein concentrations were determined as described by Lowry et al (28) and modified by Maxwell et al (30). Each lipoprotein fraction migrated as an homogeneous band on agarose electrophoretic gel.

The purified HDL, HDL subfractions, and LDL preparations were analyzed by double immunodiffusion to determine the degree of cross-contamination of the HDL preparation by LDL and vice-versa (34, 38). When HDL or LDL preparations were analyzed by double immunodiffusion, 0.2 µg of HDL protein gave a single precipitin line against rabbit anti-human $_1$ apoprotein (anti-HDL) (Behring Diagnostics, American Hoechst Co., Somerville, N.J.). In the case of LDL, no precipitin line could be observed even at a 500-fold higher protein concentration (100 µg protein). Likewise, LDL (0.2 µg protein) gave a single precipitin line when tested against rabbit anti-human -apoprotein (anti-LDL) (Cappel Laboratories, Cochranville, Pa.), whereas HDL at 500-fold higher protein concentration did not give a precipitin line. These results demonstrate that HDL preparations contained less than 0.2% LDL, if any at all, and vice versa (Tauber et al, 1980, 1981). To eliminate the possibility of a contamination by plasma proteins, the purity of the LDL and HDL preparations was analyzed by slab gel electrophoresis (10 to 18% and 5 to 18%, respectively, exponential polyacrylamide gel gradient containing 0.1% sodium dodecyl sulfate) with or without prior delipidation with tetramethyl urea (32, 33). When the electrophoretic patterns of HDL and LDL preparations were compared to that of plasma or LPDS, no obvious contamination by plasma proteins (human serum albumin concentration was 0.1%) was observable.

Cell culture conditions

Cultures of bovine corneal endothelial cells were established from steer eyes as already described (7, 9). Stock cultures were maintained on tissue culture dishes in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum, 5% calf serum, 50 ug/ml Gentamicin, and 2.5 ug/ml Fungizone. Prior to being used, all media were passed through a Millipore filter (0.2 μ). FGF (100 ng/ml) was added every other day until the cells were nearly confluent.

Fibronectin-coated dishes were prepared as described by Kramer, Gonzalez, and Nicolson (27). Plastic dishes coated with an extracellular matrix (ECM) produced by corneal endothelial cells were prepared using detergent treatment (0.5% Triton in PBS), as already described (8, 13, 39). The possibility that the growth-promoting effect of the extracellular matrix (ECM) produced by cultured bovine corneal endothelial (BCE) cells could be due to: 1) adsorbed cellular factors released during the cell lysis process leading to the denudation of the ECM; 2) adsorbed serum or plasma factors; or 3) adsorbed exogenous growth factors has been examined (16). Exposure of confluent BCE cultures to 2 M urea in medium supplemented with 0.5% calf serum denudes the ECM without cell lysis (16, 19). The ECM prepared by this procedure supports cell growth just as well as ECM prepared by denudation involving cell lysis. Thus, it is unlikely that the growth-promoting properties of ECM are due to adsorbed cellular factors. When the ECM produced by BCE cells grown in defined medium supplemented with high density lipoprotein, transferrin, and insulin (6) was compared to the ECMs produced by cells grown in the presence of serum- or plasma-supplemented medium, all were found to be equally potent in stimulating cell growth (16). It is therefore unlikely that the growth-promoting ability of the ECM is due to adsorbed plasma or serum components.

Treatment of ECM with dithiothreitol (0.1 M) or 10% -mercaptoethanol did not affect its ability to support cell growth (10, 12, 16, 21). Because both of these treatments are known to destroy the activity of platelet-derived growth factor (34), it is unlikely that the growth-promoting effect of the ECM is due to traces of this factor which could be adsorbed onto it. Likewise, heat treatment (70°C, 10 min) of ECM-coated dishes did not affect the growth-promoting ability of the ECM (10, 16), although this treatment does completely inactivate the growth-promoting effect of FGF (7, 16, 21). It is therefore unlikely that, as suggested by Smith et al (36), the growth-promoting ability of the ECM is due to traces of these factors adsorbed onto the ECM.

Primary cultures of bovine adrenal cortex cells were prepared as previously described (15) from dispersed adrenal cortical cells stored frozen in F-12 medium supplemented with 20% serum and 5% dimethylsulfoxide. Stock cultures of bovine adrenal cortex cells were passaged weekly at a split ratio of 1 to 45 on either plastic dishes or ECM-coated dishes. When stock cultures were passaged on plastic dishes, cultures were grown in F-12 medium supplemented with 5% horse serum, 5% fetal calf serum, 25 µg/ml Gentamycin, and 0.25 µg/ml Fungizone. FGF (100 ng/ml) was added every other day until cultures became confluent. When stock cultures were passaged on ECM-coated dishes, they were exposed to the same supplemented medium but without FGF.

Cell seeding

Cell monolayers from stock plates were dissociated by exposure (2-3 min, 24°C) to a solution containing 0.9% NaCl, 0.01 M sodium phosphate (pH 7.4), 0.05% trypsin, and 0.02% EDTA (STV solution, Difco). When cells rounded up, they were resuspended in F-12 medium supplemented with HDL

(500 µg protein/ml). The cell suspension was then spun down and the cell pellet resuspended in F-12 medium alone. When seeding was performed in the presence of serum, the cell pellet was resuspended in F-12 medium containing 5% horse serum, 5% fetal calf serum. An aliquot of the cell suspension was then counted in a Coulter counter and cells were seeded onto 35 mm plastic or ECM-coated dishes at the inital cell densities described in the figure legends (usually 2 or 4 X 10^4 cells/dish).

Cell growth measurement and culture lifetime determination

For cell growth measurements and culture lifetime determinations, cells were seeded, as described above, at an initial density of 2 X 10^4 or 4 X 10^4 cells, respectively, per 35 mm plastic tissue culture dish. Dishes were coated or not with either fibronectin or an ECM. When cultures were to be maintained in serum-free medium, seeding of the cultures was done in the presence of F-12 medium supplemented with 5% horse serum and 5% fetal calf serum or in F-12 medium supplemented or not with transferrin. When seeding was done in the presence of serum, the dishes were washed twice 6 or 15 hours after seeding with F-12 medium. Two ml of F-12 medium supplemented with various concentrations of HDL, LDL, FGF, and insulin present either alone or in various combinations were then added to the dishes. FGF was added every other day and insulin once at the concentrations indicated in the figure legends. Lipoprotein (either HDL or LDL) and transferrin were added only once (day 0) at the indicated concentrations. Duplicate or triplicate plates were trypsinized and counted with a Coulter counter at appropriate times. Cell number for each duplicate or triplicate never varied by more than 10%. The morphological appearance of the cultures was analyzed by phase contrast microscopy, and pictures were taken once the cultures became confluent. Culture lifetime determinations were performed as already described (12, 14).

All experiments were performed at least 3 times, with the exception of the culture lifetime determinations, and the results were qualitatively and quantitatively similar on each occasion. In all cases, the values used for the determination of growth-rate, final cell densities, or release of steroids were within 10% of the average.

Steroid measurement

11 deoxycortisol was measured by radioimmunoassay after ether extraction from the medium. The antibody was purchased from Dr. G. Abraham, (Division of Reproductive Biology, Harbor General Hospital, Torrance, CA). Radioimmunnoassays were performed as described (1) on duplicate aliquots from duplicate dishes. Differences between duplicate aliquots and dishes were less than 10%. Assay linear dose response was between 5 and 10,000 pg per tube, and samples were diluted to fall on the linear part of the standard curve. The media samples after ether extraction were assayed without prior chromatography. Each figure represents the data of at least one of three separate experiments.

RESULTS

A. Effect of substrate on the proliferative response of adrenal cortex cells exposed to a synthetic medium supplemented with HDL, transferrin, insulin, and FGF added either singly or in combination

Early-passage (P_1) adrenal cortex cells previously grown on plastic and exposed to serum-supplemented medium and FGF did not proliferate when seeded in the presence of serum on ECM-coated dishes and exposed 15 hours later to F-12 medium supplemented or not with transferrin (1 µg/ml), insulin (100 ng/ml), or FGF (100 ng/ml) added either singly or in combination (Fig. 1A). In contrast, when similar cultures were exposed to F12 medium supplemented with either HDL (25 µg protein/ml) or HDL and insulin (100

ng/ml), cells did proliferate and within 8 days reached cell densities respectively 6- and 11-fold higher than that of cultures exposed to F-12 medium (control cultures). Addition of transferrin (1 μg/ml) to such cultures did not further improve their ability to proliferate. Active cell proliferation was also observed when cultures were exposed to both HDL and FGF (100 ng/ml), and cell density after 8 days was 16-fold higher than that of control cultures. The adddition of insulin to cultures already exposed to HDL and FGF further improved the ability of cells to proliferate, resulting in a final cell density after 8 days in culture that was 19-fold that of control cultures. Addition of transferrin (1 μg/ml) to cultures exposed to either HDL-FGF or HDL-FGF-insulin did not have any marked effect on the ability of the cells to proliferate. The final cell densities of cultures grown in serum-free medium supplemented with HDL and FGF or HDL-FGF-insulin were 19% and 47% higher, respectively, than that of cultures grown in the presence of 10% serum.

When cells were seeded on plastic instead of ECM, little, if any proliferation was observed when cultures were exposed to F-12 medium supplemented with HDL alone, HDL and transferrin, or insulin alone (Fig. 1B). Although cells did grow when exposed to media supplemented with HDL and FGF or HDL-FGF and insulin, the extent of their proliferation was much less than that observed with cultures maintained on ECM-coated dishes and exposed to similar conditions. The ability of adrenal cortex cells to proliferate when maintained on plastic and exposed either to HDL-FGF or HDL-FGF-insulin was higher than that of similar cultures exposed to serum alone and lower than that of cultures exposed to serum and FGF (Fig. 1B).

Coating of dishes with fibronectin (Fig. 1C) improved the proliferative response of cells exposed to HDL-FGF and insulin. Nevertheless, the final cell

density of such cultures after 8 days was only 42% that of cultures maintained on ECM and exposed to similar conditions.

These results indicate that, in order for adrenal cortex cells to respond optimally to the plasma factors HDL, insulin, and transferrin, the substrate upon which they rest is an important consideration, since the best proliferative response is observed when cells are maintained on ECM-coated dishes.

B. Requirement for transferrin of early- versus late-passage adrenal cortex cells in order to proliferate actively when exposed to a synthetic medium supplemented with either FGF and insulin or HDL-FGF and insulin

Adrenal cortex cells seeded in the presence of serum do not require transferrin in order to grow when maintained on ECM-coated dishes and exposed to media supplemented with either HDL and FGF or HDL-FGF and insulin. This could either reflect the adsorption of serum transferrin onto the ECM when cells are seeded in the presence of serum or the fact that adrenal cortex cells do not require transferrin in order to proliferate actively.

In order to decide between these alternatives, cells previously grown on plastic and exposed to F-12 medium supplemented with serum and FGF or grown on ECM and exposed to F-12 medium supplemented with either serum alone or HDL-FGF and insulin were seeded in the total absence of serum on ECM-coated dishes. Their growth when exposed to FGF-insulin or HDL-FGF-insulin was then compared as a function of whether or not transferrin was present in the medium.

Cells originating from cultures previously maintained on plastic and exposed to serum and FGF did not proliferate when exposed to F-12 medium supplemented with FGF and insulin. Active proliferation was only seen when HDL was present together with FGF and insulin. The addition of transferrin (1 μg/ml) did not affect their final cell density (Fig. 2A). Similar observations

were made in the case of cells originating from cultures previously maintained on ECM and exposed to serum (Fig. 2B). In contrast, cells originating from cultures previously maintained on ECM and grown for 4 population doublings in the presence of transferrin-HDL-FGF and insulin did require the presence of transferrin in order to grow at an optimal rate. As shown in Fig. 2C, cultures exposed to FGF and insulin did not proliferate actively in the absence of transferrin. In its presence a 7-fold increase in the density of the cultures could be observed over a period of 8 days. Likewise, when cultures were exposed to HDL-FGF and insulin, cultures maintained in the absence of transferrin proliferated at a suboptimal rate, as indicated by the final cell density, which was half that of cultures exposed to transferrin (Fig. 2C).

These results demonstrate that a transferrin requirement becomes apparent only once cultures have been grown for a few population doublings in total absence of serum. They also demonstrate that, although cells previously grown in the presence of serum do not initially respond to FGF and insulin, when exposed to serum-free conditions (Fig. 1A) they do become sensitive to these factors once they have been passaged for a few generations in total absence of serum.

C. Effect of increasing concentrations of transferrin, insulin, and FGF on the proliferation of adrenal cortex cells maintained on ECM-coated dishes and exposed to serum-free conditions

Since transferrin is required in order for HDL, insulin, and FGF to exert their growth-promoting effect on late-passage adrenal cortex cells grown on ECM and exposed to serum-free medium supplemented with these factors, we have analyzed the effect of increasing transferrin concentrations on the proliferation of adrenal cortex cells exposed to optimal concentrations of either HDL-insulin-FGF (Fig. 3A) or FGF-insulin (Fig. 3B). In both cases,

optimal cell proliferation was observed at a transferrin concentration of 1 µg/ml. When the effect of increasing concentrations of insulin (Fig. 3C) was analyzed with cultures exposed to HDL-transferrin, insulin was mitogenic at a concentration as low as 0.5 ng/ml, saturation was observed at 5 ng/ml, and above 100 ng/ml slight toxicity became evident. When the effect of increasing concentrations of FGF (Fig. 3D) was analyzed in cultures exposed to HDL-insulin-transferrin, FGF was mitogenic at 5 ng/ml, saturation was observed at 50 ng/ml, and above 100 ng/ml slight toxicity became apparent, as reflected by the lower final cell density of cultures exposed to an FGF concentration of 250 ng/ml.

On the basis of these studies, the concentration of transferrin which induced optimal cell proliferation is therefore 1 µg/ml, that of insulin is 10 ng/ml, while that of FGF is 100 ng/ml.

D. Effect of increasing concentrations of HDL, HDL subfractions, and LDL on the proliferation of adrenal cortex cells maintained on ECM-coated dishes and exposed to serum-free conditions

When the mitogenic effect of increasing concentrations of bovine HDL was tested in cultures exposed to serum-free medium, HDL was mitogenic at concentrations ranging from 5 to 50 µg protein/ml, and optimal cell proliferation was observed at 25 to 50 µg protein/ml (Fig. 4A). Above 50 µg protein/ml, bovine HDL was cytotoxic. A similar mitogenic effect of human HDL was also observed when tested within the same range of concentrations as bovine HDL on cultures exposed to FGF-insulin and transferrin (Fig. 4B). When the mitogenic activity of human HDL was compared to that of its subfractions, HDL_2 and HDL_3, HDL_3 was more mitogenic than HDL at similar concentrations, while HDL_2 was less so. Maximal mitogenic effects of HDL and its subfactions were in all cases observed at 50 µg protein/ml. Above that

concentration, both HDL and its subfractions were cytotoxic, HDL_2 having the strongest cytotoxicity and HDL_3 the least (Fig. 4B). LDL, in contrast to HDL, was only weakly mitogenic at concentrations below 1 µg protein/ml. Above 1 µg protein/ml LDL was cytotoxic (unpublished data).

E. Growth-rate and final cell density of low density bovine adrenal cortex cells maintained on ECM-coated dishes and exposed either to serum or to serum-free medium supplemented with optimal concentrations of transferrin, HDL, insulin, and FGF present in various combinations

When the ability to proliferate of low density (20 cells/mm^2) bovine adrenal cortex cells exposed to F12 medium was analyzed as a function of medium supplements and of time in culture, the average doubling time of cultures exposed to HDL and transferrin was 39 hours, while that of cultures exposed to HDL-FGF-insulin and transferrin was 24 hours (Fig. 5A). The final cell densities of cultures exposed to the various factors did differ. While cultures exposed to HDL and transferrin had the lowest cell density (120 cells/mm^2), the highest (480 cells/mm^2) was observed with cultures exposed to HDL-FGF-insulin and transferrin. Cultures exposed to HDL-insulin-transferrin or HDL-FGF-transferrin had intermediate final cell densities that were not significantly different (440 cells/mm^2 versus 430 cells/mm^2 respectively). The final cell density of cultures exposed to HDL-FGF-insulin and transferrin was similar to that of cultures exposed to 10% serum, but their average doubling time during their logarithmic growth phase was longer (24 hours) than that of cultures exposed to serum (16 to 18 hours).

When the growth-rate and final cell density of cultures exposed to similar conditions but seeded at a higher density (40 cells/mm^2) were compared, similar results were obtained, except that the final cell density of cultures exposed to HDL-FGF-insulin-transferrin was higher (800 versus 480 cells/mm^2) (Fig. 5B).

F. Life span of adrenal cortex cell cultures passaged repeatedly at low cell density and grown in the absence of serum and in the presence of HDL, FGF, insulin, and transferrin

Cultures seeded at each passage in the presence of medium supplemented with serum and exposed either 6 or 15 hours later to serum-free medium supplemented with HDL, FGF, insulin, and transferrin can undergo 60 population doublings (Fig. 6A). If FGF is omitted, the cultures will still grow to 85% of this value (Fig. 6A). The morphological appearance of such cultures with FGF after 10, 36, or 58 population doublings (3, 10, and 17 passages, respectively) is shown in Fig. 6D-F. The cells cultured without FGF were morphologically identical. Regardless of the passage number the cell morphology was similar, and cells formed a monolayer. The only difference between early-and late-passage cells was in the larger average cell size of late-passage cultures. When cultures maintained under similar conditions were passaged on fibronectin-coated dishes, they had a slower growth-rate and proliferated for only 34 generations (Fig. 6B). If FGF was not present, the cells simply would not proliferate (Fig. 6B). The survival time in culture of cells grown on fibronectin-coated dishes in media supplemented with HDL, insulin, transferrin, and FGF was nearly identical to that of cells cultured on ECM, however. That is, regardless of the growth-rate and number of generations the cells became senescent after approximately 150 days in culture. The morphological appearance of such cultures after 7 population doublings (3 passages) is shown in Fig. 6G. The cells, which were already greatly enlarged, had an atypical morphological appearance and did not form a compact monolayer of closely apposed cells.

When cultures passaged on fibronectin- or ECM-coated dishes were seeded in total absence of serum, they had a shorter life span and underwent

13 or 26 doublings, respectively, before senescing (Fig. 6C). Their survival time was nearly identical, however, and cells ceased to grow when maintained on either ECM or fibronectin after approximately 55 days in culture. The morphological appearance of cells maintained under the identical conditions on either fibronectin- or ECM-coated dishes are shown in Fig. 6H & I respectively. Cells in Fig. 6H are from passsage 7 and in Fig. 6I from passage 24. As was observed with cultures passaged in the presence of serum on ECM- or fibronectin-coated dishes and exposed 6 or 15 hours later to F12 medium supplemented with HDL-insulin-transferrin-FGF, cultures passaged repeatedly in total absence of serum retained the ability to form a cell monolayer at confluence on ECM but not on fibronectin.

G. Effect of growth conditions on 11 Deoxycortisol production by early-passage adrenal cortex cells maintained on ECM-coated dishes and grown in the presence of F-12 medium supplemented with HDL, insulin, and FGF added either singly or in combination

The endocrine status of adrenal cortex cell cultures maintained on ECM and exposed to a defined medium supplemented with various factors was studied by analyzing the total amounts of 11 Deoxycortisol released into the medium during their active growth stage (4 days in culture) (Table I). Cultures seeded at a cell density of 40 cells per mm^2 and exposed to 1% serum proliferated actively over a period of 4 days, reaching a density of 494 cells per mm^2 (Fig. 5B). Such cultures did release 11 Deoxycortisol into their medium (171 ng per 10^6 cells). ACTH addition (1×10^{-8} M) on day 0 and day 2 resulted in a slight decrease in cell density after 4 days in culture (460 cells/mm^2) and in a significant increase in 11 Deoxycortisol released (326 ng per 10^6 cells). The effect of various combinations of HDL, insulin, and FGF on cell proliferation versus their ability to release 11 Deoxycortisol into the

medium was compared. Cultures exposed to F-12 medium supplemented with HDL alone had, after 4 days in culture, the lowest final cell density (139 cells per mm^2), while cultures exposed to a combination of HDL-insulin-FGF had the highest (292 cells per mm^2). Cultures exposed to HDL-FGF or HDL-insulin had an intermediate cell density (153 and 277 cells per mm^2, respectively). The rate of release of 11 Deoxycortisol was similar for all culture conditions and ranged from 147 ng per 10^6 cells for cultures exposed to HDL alone to 169 ng per 10^6 cells for cultures exposed to HDL-insulin. When cultures were grown under similar conditions but exposed to ACTH, a slight decrease in final cell density after 4 days in culture could be observed for all culture conditions. The rate of 11 Deoxycortisol production was increased significantly, ranging from 264 ng per 10^6 cells in cultures exposed to HDL alone to 493 ng per 10^6 cells in cultures exposed to HDL-insulin-FGF (Table I).

H. Effect of increasing concentrations of HDL and LDL on the steroidogenic response of early passage (P_3) bovine adrenal cortex cells grown in defined medium

The effect of increasing concentrations of HDL or LDL on the steroidogenic response of bovine adrenal cortex cell cultures grown to confluence in medium supplemented with transferrin, HDL, insulin, and FGF is shown in Fig. 7. Confluent cultures exposed to F-12 medium supplemented with transferrin (1 µg/ml) and increasing concentrations of HDL (ranging from 5 to 250 µg protein/ml) had a basal steroidogenic release rate which was not affected by the presence of HDL. In contrast, when cells were exposed to increasing LDL concentrations (ranging from 5 to 250 µg protein/ml), their ability to release steroids increased steadily as a function of LDL concentrations to which they cells were exposed. At a concentration of 250 µg protein/ml, there was a 5.2-fold increase in 11 Deoxycortisol released into the medium, as compared to cultures not exposed to LDL (Fig. 7).

Exposure of confluent cultures maintained in defined medium to ACTH (10^{-8} M) alone resulted in a 2-fold increase in steroid release, while exposure to both ACTH and db cAMP (1 mM) resulted in a further 10-fold increase in steroid release over control cultures. When such cultures were exposed to increasing concentrations of either LDL or HDL, increased steroid release as a function of lipoprotein concentration was observed in both cases. This increase was most noticeable in cultures exposed to LDL rather than to HDL, however.

The present results therefore indicate that in cells not exposed to ACTH or db cAMP LDL, but not HDL, can contribute significantly to steroidogenesis. In contrast, when cells are triggered to produce maximal amounts of steroid by exposing them to ACTH alone or to ACTH and db cAMP, both lipoproteins can further support steroid synthesis. The ability of LDL versus that of HDL to support steroidogenesis under such conditions differed in two significant ways, however. First, LDL's ability to increase steroid release is 10-fold greater than HDL on a concentration basis. Second, an HDL concentration of 100 µg protein/ml is saturating, while an LDL concentration as high as 250 µg protein/ml is not.

I. Steroidogenic response of confluent cultures of adrenal cortex cells maintained on ECM-coated dishes and grown in F-12 medium supplemented with transferrin, HDL, insulin, or FGF when exposed to lipoproteins as a function of the culture passage number and as a function of exposure to ACTH, db cAMP, or ACTH and db cAMP

The steroidogenic response as a function of passage number (P_3, P_7, and P_{14}; 9, 24, and 51 population doublings respectively) of cells grown in the presence of transferrin, HDL, insulin, and FGF is shown in Table II. Confluent cultures in their third passage when exposed to HDL released little 11

Deoxycortisol into their medium (40 ng per 10^6 cells per 24 hours). When exposed to LDL, the rate of release increased to 127 ng per 10^6 cells per 24 hours. In media supplemented with either HDL or LDL addition of ACTH (10^{-8} M) produced a small (1.7- to 2-fold) but significant increase in steroid release. Addition of db cAMP (10^{-3} M) resulted in a 7- to 8-fold increase in 11 Deoxycortisol release. This could be further increased by the addition of ACTH to cultures already exposed to db cAMP.

Confluent cultures in their seventh and fourteenth passage and exposed to either HDL and LDL no longer responded to ACTH with an increased release of steroids. Addition of db cAMP was stimulatory, however. Although the rate of 11 Deoxycortisol release declined precipitously between passages 3 and 7 (781 ng per 10^6 cells per 24 hours for P_3 cultures exposed to LDL and db cAMP versus 270 ng per 10^6 cells per 24 hours for P_7 cultures exposed to similar conditions), it did not decline appreciably between passage 7 and 14 (270 ng per 10^6 cells per 24 hours for P_7 cultures exposed to LDL and db cAMP versus 218 ng per 10^6 cells per 24 hours for P_{14} cultures exposed to similar conditions). Similar declines in the rate of steroid production were observed between these different passages for cultures exposed to the various other experimental conditions (see Table II).

DISCUSSION

Previous studies have shown that low density bovine adrenal cortex cell cultures maintained on plastic and exposed to serum-supplemented media require FGF in order to proliferate at an optimal rate and to be passaged repeatedly (15, 24, 35). This mitogenic effect of FGF is a strict function of the plasma or serum concentration to which cells are exposed (15). This suggests that plasma factor(s) are involved in the control of proliferation of bovine adrenal cortex cells. In contrast to cultures maintained on plastic,

cultures maintained on ECM-coated dishes no longer require FGF in order to proliferate readily, even when exposed to low serum or plasma concentrations (8). This suggests that the close contact of the cells with the ECM could have a permissive effect on cell proliferation and make cells more sensitive to plasma factors. The ECM, which provides cells with attachment factors such as laminin and fibronectin, as well as with factors which have a permissive effect on cell growth (10, 11), can therefore be used as a convenient substrate upon which to maintain adrenal cortex cells and in order to elucidate the nature of plasma factors that control their proliferation. To that end, the approach of Gordon Sato and his colleagues (2, 3), who have shown that the serum or plasma requirement for growth of a number of cell lines can be satisfied by the addition of specific hormones and growth factors to synthetic media, was used.

The present results demonstrate that low density adrenal cortex cells seeded on ECM-coated dishes in the presence of serum can actively proliferate when exposed 15 hours later to serum-free medium supplemented with HDL, insulin, and FGF. An absolute requirement for transferrin becomes obvious only when cells are seeded and grown in total absence of serum for a few passages. Use of such medium not only supports rapid cell growth but also the long-term culturing of adrenal cortex cells in total absence of serum. The life span of such cultures will depend, however, on the seeding conditions. While cells seeded in the presence of serum and then exposed to a synthetic medium supplemented with HDL-insulin-FGF and transferrin had a life span similar to that of cultures exposed to serum and FGF (60 population doublings), the life span of cultures repeatedly passaged and seeded in total absence of serum was much shorter (26 population doublings). This could reflect an incomplete recovery from damage to the cells caused by trypsin when cells are

exposed to HDL versus their complete recovery when they are exposed to serum for 6 or 15 hours.

When the abilities of plastic versus ECM-coated dishes to support the proliferation of low density cultures of bovine adrenal cortex cells maintained in serum-free medium were compared, plastic did not support the proliferation of cells exposed to F-12 medium supplemented with HDL-insulin-transferrin and FGF unless cells were initially seeded in the presence of serum. When fibronectin-coated dishes were used, low density adrenal cortex cells seeded in the presence of serum proliferated actively when exposed 15 hours later to medium supplemented with HDL-insulin-transferrin and FGF. They would not grow, however, unless FGF was present. This contrasts with cells grown on ECM, which were able to sustain proliferation in media supplemented only with HDL-insulin-transferrin. The average growth rate of cultures on fibronectin was nevertheless always 1.7 times that of cultures seeded on ECM. When the life span of cultures seeded in the presence of serum and then exposed to F-12 medium supplemented with HDL-insulin-transferrin and FGF were compared as a function of the substratum upon which cells were maintained (either fibronectin- or ECM-coated dishes), the life span of cultures maintained on fibronectin-coated dishes was nearly half (34 population doublings) that of cultures maintained on ECM-coated dishes (60 population doublings). When cultures were seeded in the complete absence of serum and then exposed to the same medium, the life span of cultures maintained on fibronectin was again half (13 generations) that of ECM (26 generations). The observation that cells plated in either the presence or absence of serum and maintained identically (in F12 medium supplemented with HDL-insulin-transferrin-FGF) on either fibronectin- or ECM-coated dishes survive for nearly the same number of days in culture suggests that still

undefined factor(s) must be present for sustained proliferation. Furthermore, the results shown in Fig. 6C indicate that this factor(s) may be nutritional, and not hormonal, since for the first 10 generations, the growth-rate of cells plated in the absence of serum and maintained in F-12 media supplemented with HDL-insulin-transferrin-FGF on either fibronectin or ECM-coated dishes is nearly identical.

These observations are similar to those already made on the comparative life spans in culture of lens epithelial cells or A-431 carcinoma cells maintained on fibronectin- versus ECM-coated dishes and grown in a synthetic medium supplemented with either HDL-insulin-transferrin and FGF, in the case of lens cells, or transferrin and HDL in the case of the A-431 carcinoma cells (17, 19).

The growth requirements of adrenal cortex cells and those of other cells that are capable of producing steroids, such as granulosa cells, are quite similar. In both cases cells are extremely sensitive to the mitogenic stimulus provided by insulin. Adrenal cortex cells are the most sensitive, since insulin at concentrations as low as 5 ng/ml was already optimally active, while the optimal concentration in the case of granulosa cells was 300 ng/ml. As already observed with granulosa cells (33), the mitogenic effect of HDL on adrenal cortex cells was quite limited in comparison to that observed with cells that do not have a steroidogenic capacity, such as vascular endothelial and smooth muscle cells (12, 37, 38), corneal endothelial cells, and lens epithelial cells (19). Although HDL is required in order for adrenal cortex cells to respond to insulin, the concentrations which were found to be optimal were extremely low (25 µg protein/ml), and at concentrations above 50 µg protein/ml HDL became cytotoxic. When the ability of subfractions of HDL to support cell proliferation were compared, HDL_3 was found to be a stronger

mitogen than HDL_2. This may reflect the fact that in adrenal cortex cells HDL_3 is a more potent activator of HMG CoA reductase than HDL_2 (unpublished results). It could also reflect the fact that low density adrenal cortex cells when maintained in defined medium and actively growing are very sensitive to the cytotoxic effect of LDL. The ability of HDL_2 to interact with LDL receptors by virtue of its apoprotein E could explain its lesser effect on cell proliferation.

The difference in the biological activity of HDL for lens epithelial cells, vascular endothelial and smooth muscle cells, or corneal endothelial cells, versus its effect on granulosa cells and adrenal cortex cells, may reflect the fact that both cell types are capable of actively synthesizing cholesterol. Their growth requirement for the cholesterol provided by either HDL_2 or LDL is therefore minimal. Exposure of adrenal cortex cells to either lipoprotein could lead to cholesterol overload and cell death when cells are actively proliferating. In contrast, vascular endothelial and smooth muscle cells, as well as corneal endothelial cells, have a limited ability to make their own cholesterol and may therefore rely more heavily on the activation of HMG CoA reductase by HDL (which will result in the increased synthesis of cholesterol, among other products) in order to proliferate.

Adrenal cortex cells grown and repeatedly passaged in serum-free medium supplemented with HDL-insulin-transferrin and FGF retain their ability to produce steroids. Due to the rapid loss of 11 hydroxylase in such cultured cells (35), the release of 11 Deoxycortisol rather than that of cortisol was measured by radioimmunoassay. Analysis of the basal rate of release of 11 Deoxycortisol, although it is the main steroid produced by cultured adrenal cortex cells, does not reflect the full potential of the cells to synthesize and release steroids, since this cell type can produce other steroids as well (35).

As reported for adrenal cortex cells grown in the presence of serum and exposed to FGF, basal steroidogenesis, as reflected by the ability of the cells to release 11 Deoxycortisol into their medium, decreases as a function of passage number (24). Although the addition of ACTH stimulated steroidogenesis, this stimulation was observed only in early-passage cultures and reflects the rapid desensitization of the cultured cells to ACTH (23). Cultured cells retained their ability to respond to db cAMP by increased steroidogenesis, however, and this could be observed in both early- (P_3) and late- (P_{14}) passage cells grown in absence of serum in medium supplemented with HDL-insulin-transferrin and FGF.

Lipoproteins such as LDL, although highly toxic for actively growing populations of bovine adrenal cortex cells exposed to serum-free medium, were observed to be less cytotoxic once cultures became confluent and cells entered a quiescent stage (unpublished observations). In agreement with previous reports of Kovanen et al (26) and Brown et al (4), addition of LDL to confluent and resting cultures of adrenal cortex cells grown in medium with lipoprotein-deficient serum resulted in a 5- to 10-fold increase in steroid secretion with early-passage cells exposed to either ACTH or db cAMP, and with late-passage cell cultures exposed to db cAMP. In contrast, exposure of cells to HDL did not result in any increase in the basal level of steroidogenesis, in steroidogenesis stimulated by ACTH or db cAMP in early-passage cultures, or in that stimulated by db cAMP only in late-passage cultures. It is therefore likely that LDL, but not HDL, can deliver cholesterol to the cells and provide a substrate for synthesis of steroid hormones.

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TABLE I

Effect of Growth Conditions and ACTH on the Ability of Early-Passage P_2 Bovine Adrenal Cortex Cells Maintained in Defined Medium to Produce 11 Deoxycortisol

	- ACTH		+ ACTH	
Growth Conditions*	Final Cell Density ($\times 10^{-5}$)	11 Deoxycortisol (ng per 10^6 cells)	Final Cell Density ($\times 10^{-5}$)	11 Deoxycortisol (ng per 10^6 cells)
HDL	1.39	147 ± 4.2	1.34	264 ± 18.1
HDL-FGF	1.53	152 ± 3.8	1.49	229 ± 8.3
HDL-insulin	2.77	169 ± 4.1	2.76	436 ± 22.2
HDL-FGF-insulin	2.92	151 ± 3.5	2.87	493 ± 22.0
1% serum	4.94	171 ± 5.7	4.54	326 ± 12.0

Confluent bovine adrenal cortex cells previously maintained on ECM-coated dishes and grown in F-12 medium supplemented with 10% serum (5% horse, 5% fetal calf) on 100 mm ECM-coated dishes were trypsinized as described in "Materials and Methods." After centrifugation in the presence of F-12 medium supplemented with 500 µg/ml of HDL, the cells were resuspended in F-12 medium alone. 4×10^4 cells were then seeded on 35 mm ECM-coated dishes. The various factors (HDL, FGF, insulin) or 1% serum were then added and the cultures were either exposed or not to ACTH (10^{-8} M). Transferrin (1 µg/ml) was routinely added to cultures not receiving serum. FGF (100 ng/ml) and ACTH (10^{-8} M) were added on days 0 and 2. 1% serum (0.5% horse serum and 0.5% fetal calf serum) and all other factors (HDL, 25 µg protein/ml or insulin, 10 ng/ml) were added on day 0 only. The media of 4-day-old cultures were collected and the levels of 11 Deoxycortisol were determined by radioimmunoassay, as described in "Materials and Methods."

TABLE II

Comparison of the Ability of HDL versus LDL to Support the Steroidogenic Response of Bovine Adrenal Cortex Cells Grown in Defined Medium When Exposed to ACTH, db cAMP, or ACTH and db cAMP

Conditions*	P_3 (11 Deoxycortisol ng/10^6 cells/24 hours)		P_7 (11 Deoxycortisol ng/10^6 cells/24 hours)		P_{14} (11 Deoxycortisol ng/10^6 cells/24 hours)	
	HDL	LDL	HDL	LDL	HDL	LDL
No exposure	40 ± 2.3	127 ± 3.6	49 ± 1.1	71 ± 2.3	42 ± 9.1	72 ± 1.3
ACTH	88 ± 7.2	205 ± 18.3	47 ± 3.5	73 ± 0.6	48 ± 4.5	82 ± 6.3
db cAMP	284 ± 20.6	781 ± 12.3	186 ± 6.0	270 ± 4.5	104 ± 8.7	218 ± 0.56
ACTH + db cAMP	419 ± 31.6	826 ± 57.0	197 ± 10.6	290 ± 27.5	108 ± 0.6	224 ± 10.3

Bovine adrenal cortex cells previously frozen at passages 3, 7, or 14 (Fig. 6) were thawed and grown to confluence in 10% serum (5% horse, 5% fetal calf) on 100 mm ECM-coated dishes. Confluent cultures were then trypsinized and seeded in 10% serum at 1 X 10^5 cells per 35 mm ECM-coated dish. Fifteen hours later, cultures were washed twice with F-12 medium. Two ml of F-12 medium supplemented with HDL (25 µg protein/ml), insulin (50 ng/ml), FGF (100 ng/ml), and transferrin (1 µg/ml) was then added to each dish. HDL, insulin, and transferrin were added once at day 0. FGF was added on days 0, 2, 4, and 6. On day 8 media were changed. F-12 medium supplemented with transferrin (1 µg/ml) and with either HDL or LDL (250 µg protein/ml) was then added to the dishes. Cultures were further incubated for 24 hours in the absence or presence of either ACTH (10^{-6} M) or db cAMP (1 mM), or of both agents together. The media were then collected and cultures trypsinized to determine their final cell density. The level of 11 Deoxycortisol released by cells into their media was determined by radioimmunoassays, as described in "Materials and Methods."

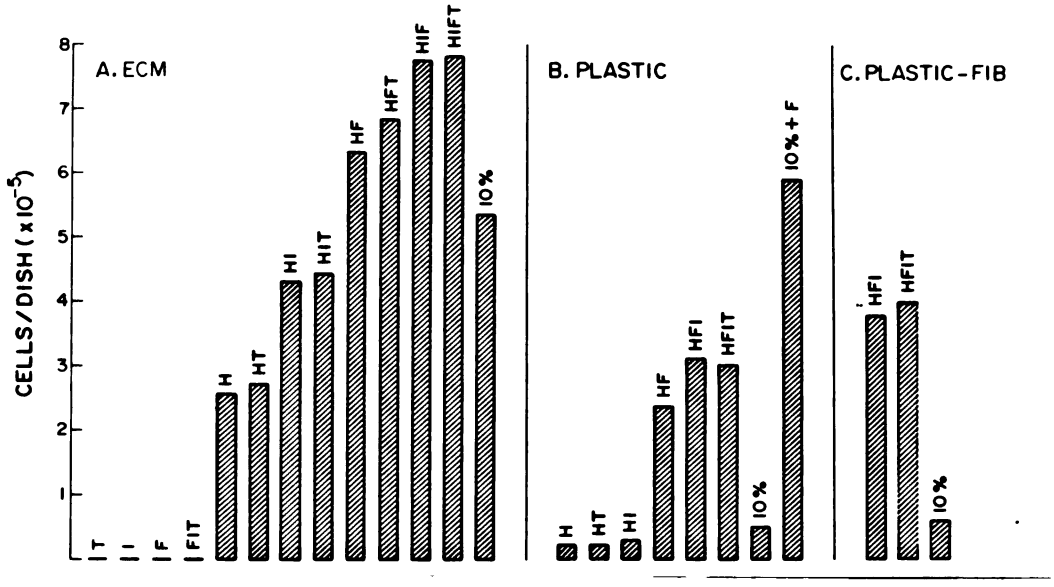

Fig. 1. Comparison of the proliferation of bovine adrenal cortical cells maintained on plastic, fibronectin-coated, or ECM-coated tissue culture dishes and exposed to F-12 medium supplemented with serum or various factors.

(A) Four X 10^4 cells were seeded on 35 mm ECM-coated dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum. Fifteen hours later the dishes were rinsed twice with F-12 medium. Fresh F-12 medium supplemented with transferrin (T, 1 μg/ml), insulin (I, 100 ng/ml), FGF (F, 100 ng/ml), HDL (H, 25 μg protein/ml) added alone or in combination or with 10% serum (10%, 5% horse and 5% fetal calf) was then added to the dishes. Serum, HDL, insulin, and transferrin were added once on day 0, whereas FGF was added every other day. Duplicate cultures were counted on day 8 on a Coulter counter. Cell number for each duplicate never varied by more than 10%.

(B) Four X 10^4 cells were seeded on 35 mm plastic tissue culture dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum. Fifteen hours later the dishes were rinsed twice with F-12 medium. Fresh F-12 medium supplemented with transferrin (T), insulin (I), FGF (F), HDL (H) added alone or in combination or with 10% serum with or without FGF (F) was then added to the plates. Concentrations and schedule of addition were the same as in (A). Duplicate cultures were counted on day 8. Cell number for each duplicate never varied by more than 10%.

(C) Four X 10^4 cells were seeded on 35 mm fibronectin-coated dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum. Fifteen hours later, the dishes were rinsed twice with F-12 medium. Fresh F-12 medium supplemented with HDL, FGF, and insulin (HFI), HDL-FGF-insulin and transferrin (HFIT), or 10% serum (10%) was then added to the plate. Concentrations and schedule of addition were the same as in (A). Duplicate cultures were counted on day 8. Cell number for each duplicate never varied by more than 10%.

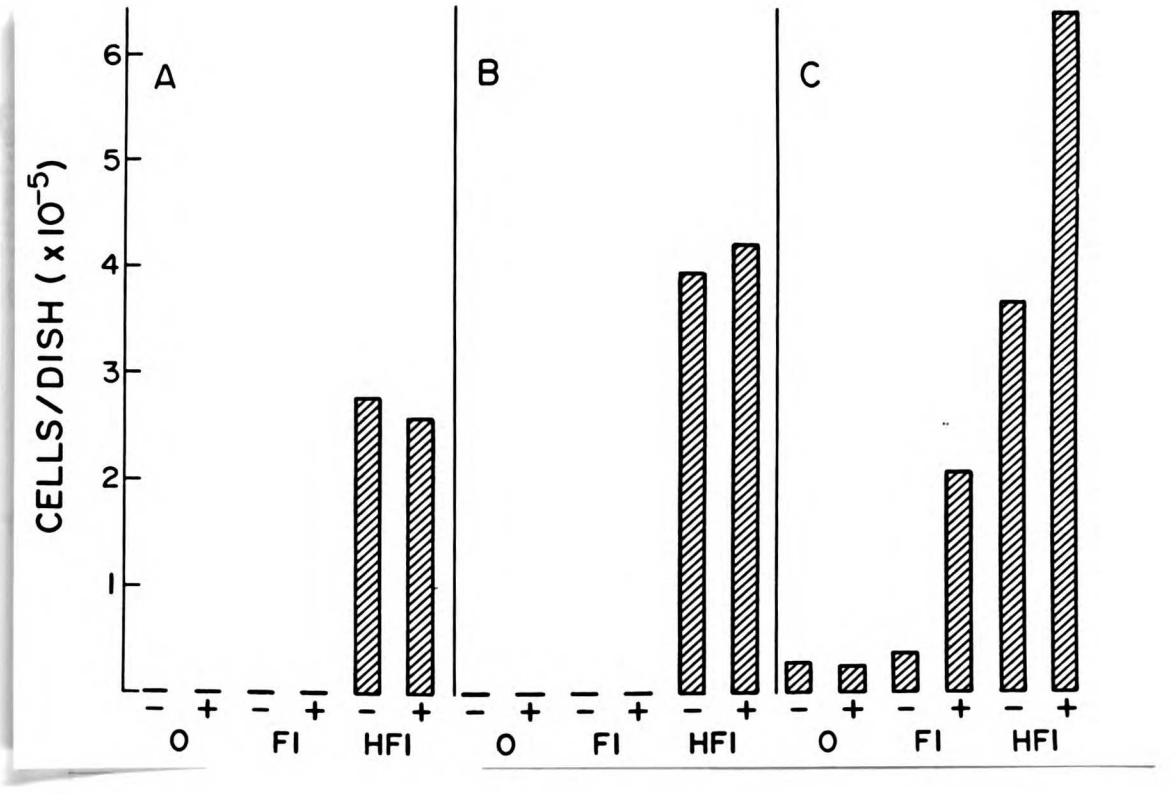

Fig. 2. Effect of prior culture conditions on the transferrin requirement of bovine adrenal cortex cells exposed to F-12 medium supplemented with FGF and insulin or HDL, FGF, and insulin.

Second passage bovine adrenal cortex cells were grown to confluence under the following conditions. (A) Cells were grown on plastic dishes in the presence of F-12 medium supplemented with 10% serum (5% horse serum + 5% fetal calf serum) and FGF (100 ng/ml, added every other day). (B) Cells were grown on ECM-coated dishes in the presence of F-12 medium supplemented with 10% serum alone. (C) Cells were grown on ECM-coated dishes and exposed to F-12 medium supplemented with HDL (25 µg protein/ml), insulin (50 ng/ml), FGF (100 ng/ml), and transferrin (1 µg/ml). HDL, insulin, and transferrin were added once at day 0; FGF was added every other day (until cells reached confluence). Confluent cultures grown under these various conditions were then trypsinized and centrifuged in F-12 medium supplemented with 500 µg protein/ml HDL. The cells were resuspended in F-12 medium alone and seeded at 4×10^4 cells per 35 mm ECM-coated dish in F-12 medium alone (O) or F-12 medium supplemented with FGF and insulin (FI) or HDL, FGF, and insulin (HFI). Transferrin (1 µg/ml) was either added (+) or not (-) for each condition. The HDL concentration was 25 µg protein/ml; those of FGF and insulin were 100 and 50 ng/ml, respectively. HDL, insulin, and transferrin were added only at day 0, whereas FGF was added every other day. Duplicate dishes were counted for each condition on day 8. Cell number for each duplicate never varied by more than 10%.

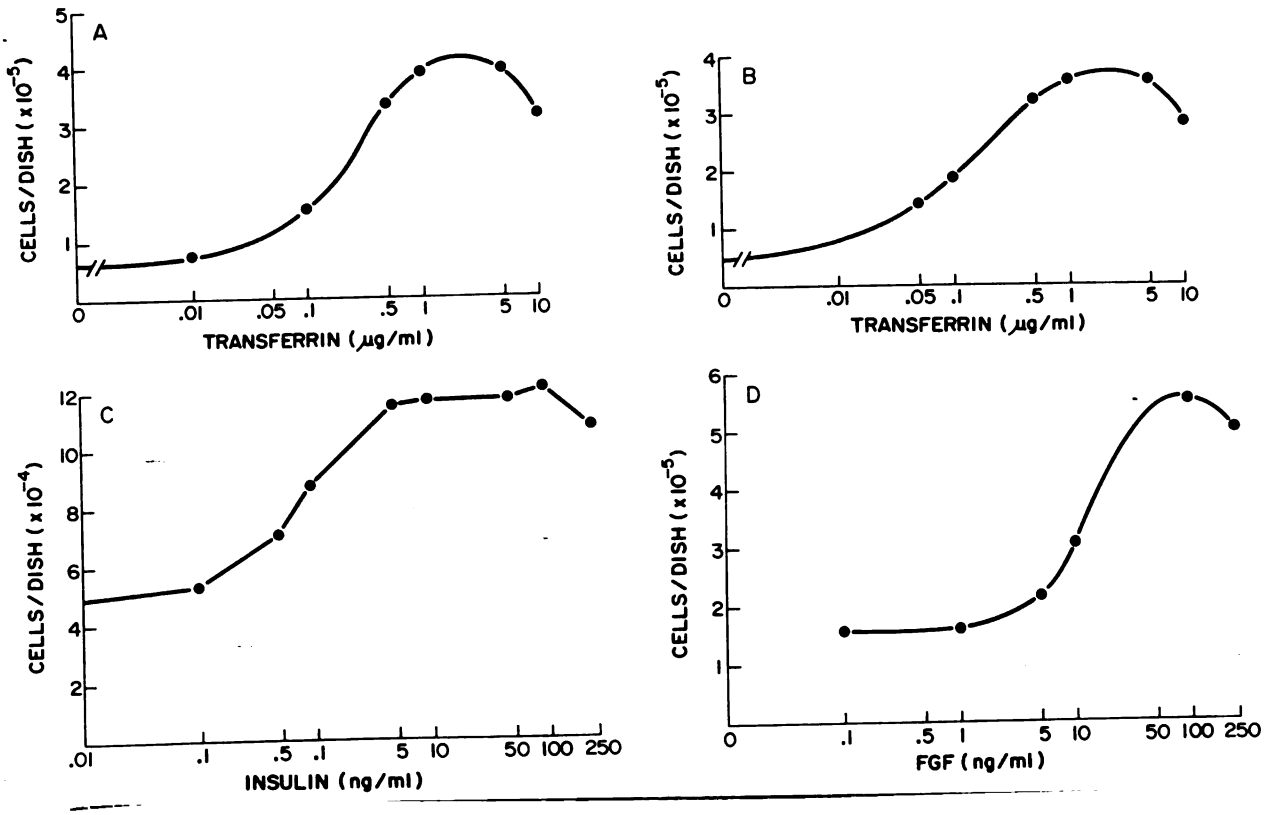

Fig. 3. Effect of increasing concentrations of transferrin, insulin, and FGF on the proliferation of bovine adrenal cortical cells maintained on ECM-coated dishes.

(A) Four X 10^4 cells were seeded on 35 mm ECM-coated dishes in the presence of F-12 medium supplemented with HDL (25 μg protein/ml), insulin (50 ng/ml), and FGF (100 ng/ml). Transferrin was added at concentrations ranging from .01 to 10 μg/ml. (B) Four X 10^4 cells were seeded on 35 mm ECM-coated dishes in F-12 medium supplemented with insulin (50 ng/ml) and FGF (100 ng/ml). Transferrin was added at concentrations ranging from .01 to 10 μg/ml. (C) Four X 10^4 cells were seeded on 35 mm ECM-coated dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum. Fifteen hours later the dishes were rinsed twice with F-12 medium. Fresh F-12 medium supplemented with HDL (25 μg protein/ml), transferrin (1 μg/ml), and insulin at concentrations ranging from 0.1 to 250 ng/ml were then added to the dishes. (D) Four X 10^4 cells were seeded on 35 mm ECM-coated dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum. Fifteen hours later the dishes were rinsed twice with F-12 medium. Fresh F-12 medium supplemented with HDL (25 μg protein/ml), insulin (10 ng/ml), transferrin (1 μg/ml), and FGF at concentrations ranging from 0.1 to 250 ng/ml was then added to the dishes. Cultures in (A), (B), and (C) were counted on day 6, while those in (D) were counted on day 8. Transferrin, HDL, and insulin were added only once at day 0, while FGF was added every other day. All points represent duplicate determinations which never varied by more than 10%.

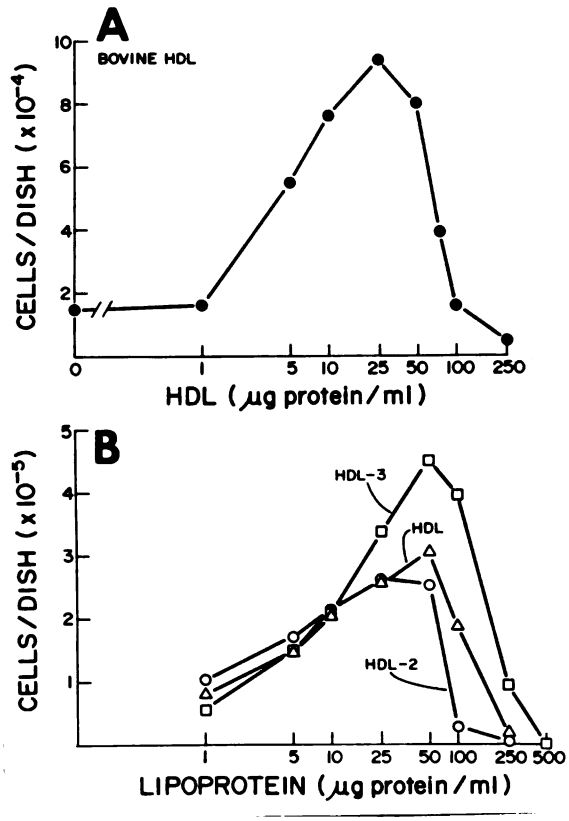

Fig. 4. Effect of increasing concentrations of HDL and its subfractions on the proliferation of bovine adrenal cortical cells maintained on ECM-coated tissue culture dishes.

(A) Two X 10^4 cells were seeded on 35 mm ECM-coated dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum. Fifteen hours later the dishes were rinsed twice with F-12 medium. Fresh F-12 medium supplemented or not with bovine HDL at concentrations ranging from 1 to 250 μg protein/ml was added to the dishes. (B) Four X 10^4 cells were seeded on 35 mm ECM-coated dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum. Fifteen hours later the dishes were rinsed twice with F-12 medium. Fresh F-12 medium supplemented with FGF (100 ng/ml), insulin (10 ng/ml), transferrin (1 μg/ml), and concentrations of human HDL () and HDL-2 () ranging from 1 to 250 μg protein/ml and of HDL-3 () from 1 to 500 μg protein/ml was then added. HDL, HDL_2, HDL_3, insulin, and transferrin were added once at day 0, whereas FGF was added every other day. The final cell number of dishes receiving no lipoprotein but only FGF, insulin, and transferrin was 1.4 X 10^4. Duplicate determinations were made for each point in (A) and (B), and cells were counted on days 6 and 8, respectively. The cell number for each duplicate never varied by more than 10%.

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Fig. 5. Comparison of the growth-rates of low density bovine adrenal cortex cell cultures maintained on ECM-coated dishes and exposed either to medium supplemented with serum or to medium supplemented with HDL, insulin, and FGF added either singly or in combination with transferrin.

(A) Bovine adrenal cortex cells previously grown on ECM-coated dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum were trypsinized and centrifuged in F-12 medium supplemented with HDL (50 μg protein/ml). The cells were resuspended in F-12 medium alone and seeded at 2×10^4 cells on 35 mm ECM-coated dishes. The F-12 medium was then supplemented with the various factors or serum. The growth-rates of cultures exposed to HDL and transferrin (HT,); HDL, FGF, and transferrin (HFT,); HDL, insulin, and transferrin (HIT,); HDL, insulin, FGF, and transferrin (HIFT, o); or 10% serum (10% ser, o) were compared. The concentration of HDL was 25 μg protein/ml, whereas those of transferrin, FGF, and insulin were 1 μg/ml, 100 ng/ml, and 50 ng/ml respectively. 10% serum was a mixture of 5% horse serum and 5% fetal calf serum. Serum, HDL, insulin, and transferrin were added only once at day 0, whereas FGF was added every other day. Duplicate dishes for each point were counted on a Coulter counter on the days indicated, and the cell numbers never varied by more than 10% for each duplicate determination.

(B) Bovine adrenal cortex cells were seeded and maintained as in (A) with the following differences: 1) cells were plated at an initial density of 4×10^4 cells per 35 mm ECM-coated dish, and 2) 1% serum (0.5% horse serum and 0.5% fetal calf serum) was used instead of 10% serum.

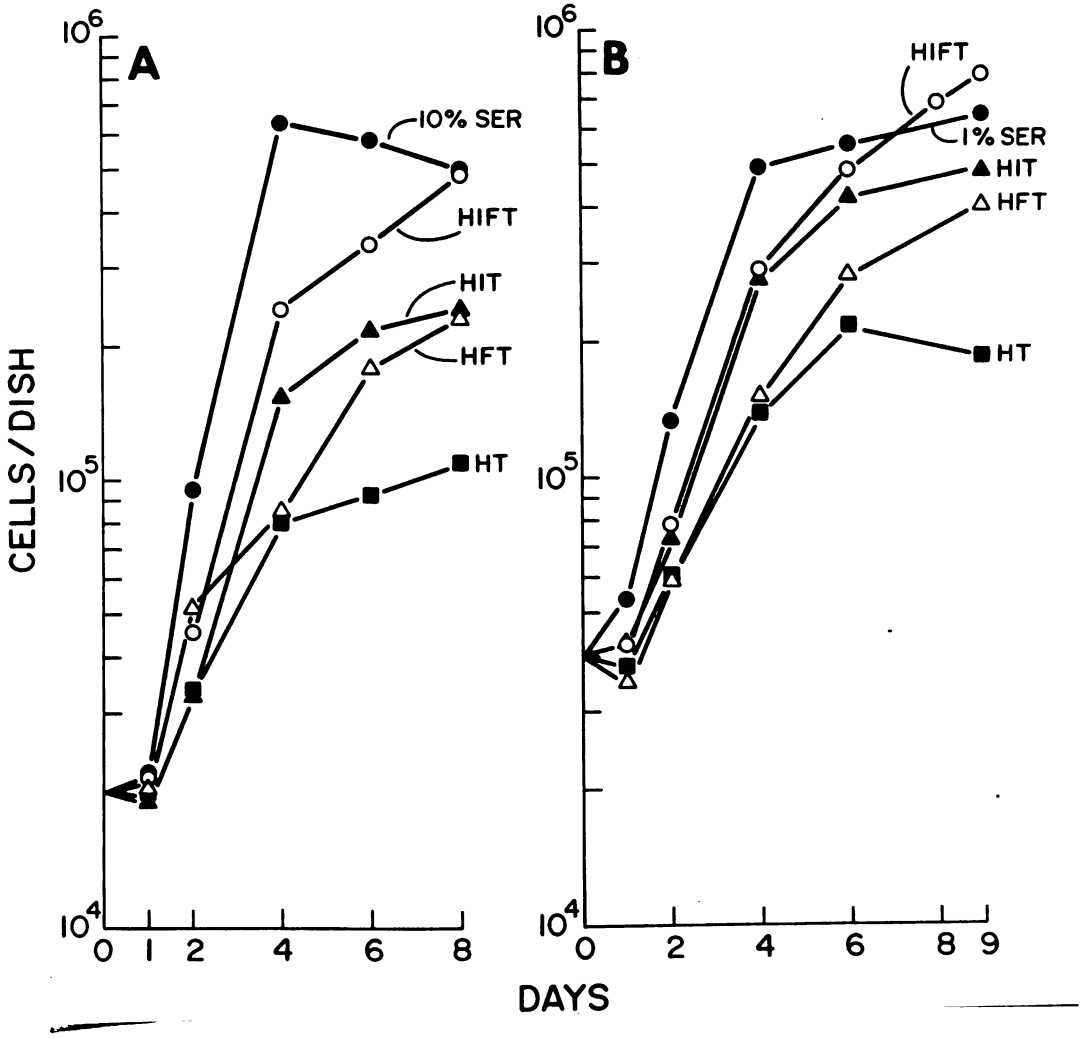
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Fig. 6. Culture lifetime of bovine adrenal cortex cells maintained on fibronectin-or ECM-coated dishes and grown in the presence of F-12 medium supplemented with HDL-insulin-transferin and FGF.

(A) Four X 10^4 cells were seeded at each passage on 35 mm ECM-coated dishes in F-12 medium supplemented with 5% horse serum and 5% fetal calf serum. Either six or fifteen hours later the dishes were rinsed twice with F-12 medium. Fresh F-12 medium supplemented with HDL (25 µg protein/ml), insulin (50 ng/ml), and transferrin (1 µg/ml) with (HITF) or without FGF (HIT) was then added to the dishes. HDL, insulin, and transferrin were added only at day 0, whereas FGF was added every other day. Triplicate plates were counted on day 7 and cell number never varied by more than 10%.

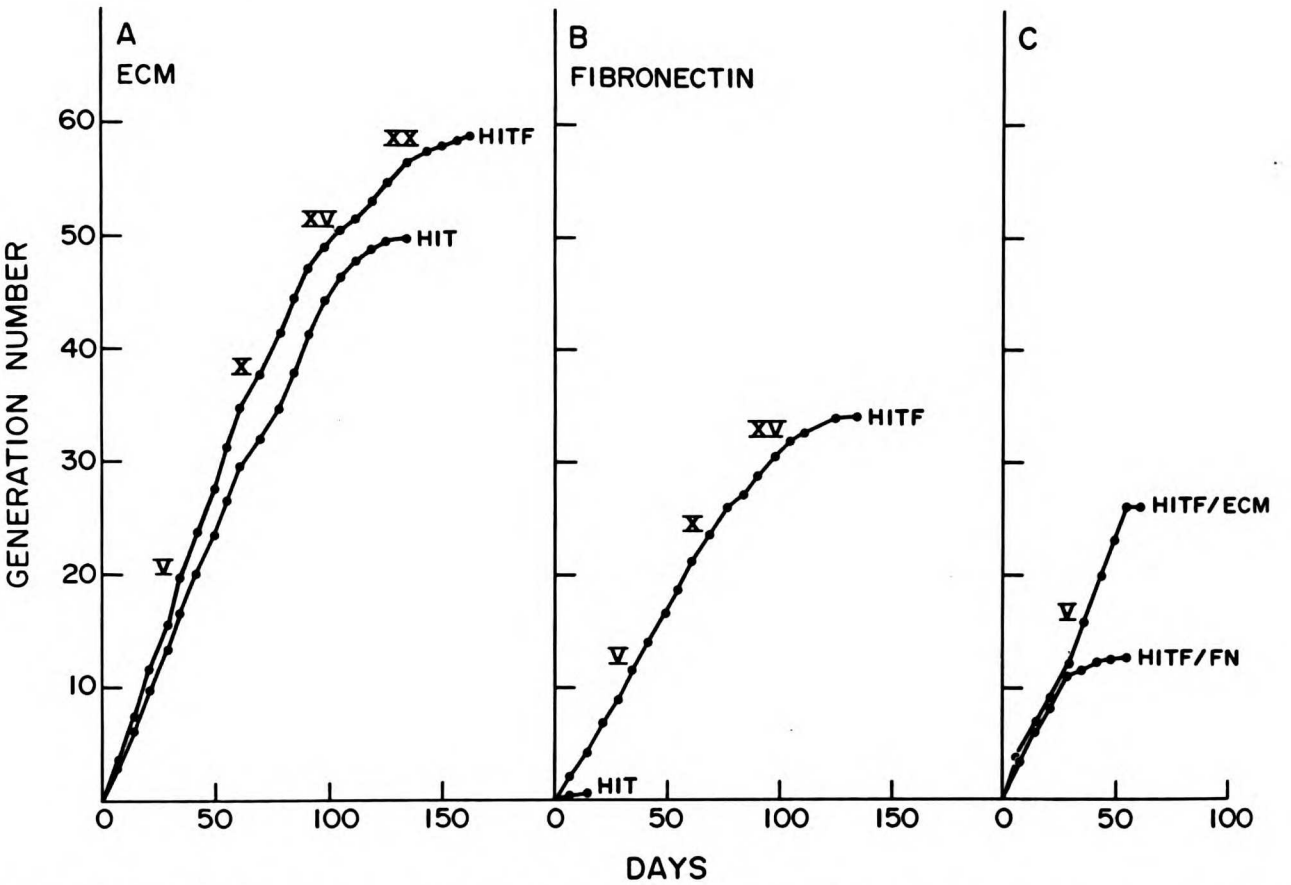
(B) This protocol is identical to that of (A), except that fibronectin-coated dishes were used instead of ECM-coated dishes. The fibronectin-coated dishes were made by adding 100 µg/ml fibronectin to 35 mm Falcon tissue culture dishes. They were then allowed to sit overnight at $4^{\circ}C$ and washed four times with phosphate-buffered saline before use.

(C) Adrenal cortex cells were seeded at each passage in the complete absence of serum. Trypsinized cells were centrifuged in F12 medium supplemented with HDL (500 µg protein/ml) and transferrin (1 µg/ml). The cells were then resuspended in F12 medium supplemented with HDL (25 µg protein/ml) and transferrin (1 µg/ml) and seeded on ECM-coated (ECM) or fibronectin-coated (FN) dishes at 4 X 10^4 cells/35 mm dish. Insulin (50 ng/ml) and FGF (100 ng/ml) were then added to either the ECM-coated (HITF/ECM) or fibronectin-coated (HITF/FN) dishes. HDL, insulin, and transferrin were added only once at day 0, whereas FGF was added every other day. Triplicate plates were counted on day 7. Cell numbers never varied by more than 10% in each triplicate.

(D-F) Morphological appearance of bovine adrenal cortex cells at passage 3 (D), 10 (E), and 17 (F) when maintained on ECM-coated dishes and seeded in the presence of serum. Cells were maintained with FGF, as described in (A).

(G-H) Morphological appearance of bovine adrneal cortex cells at passage 3 when maintained on fibronectin-coated dishes with FGF, as described in (B) for (G) or (C) for (H).

(I) Morphological appearance of bovine adrenal cortex cells maitnained on ECM-coated dishes and seeded in the absence of serum, as described in (C). The cells were from passage 7. Pictures were taken when cultures became confluent (day 6 or 7) with a Nikon phase-contrast photomicroscope (X 100).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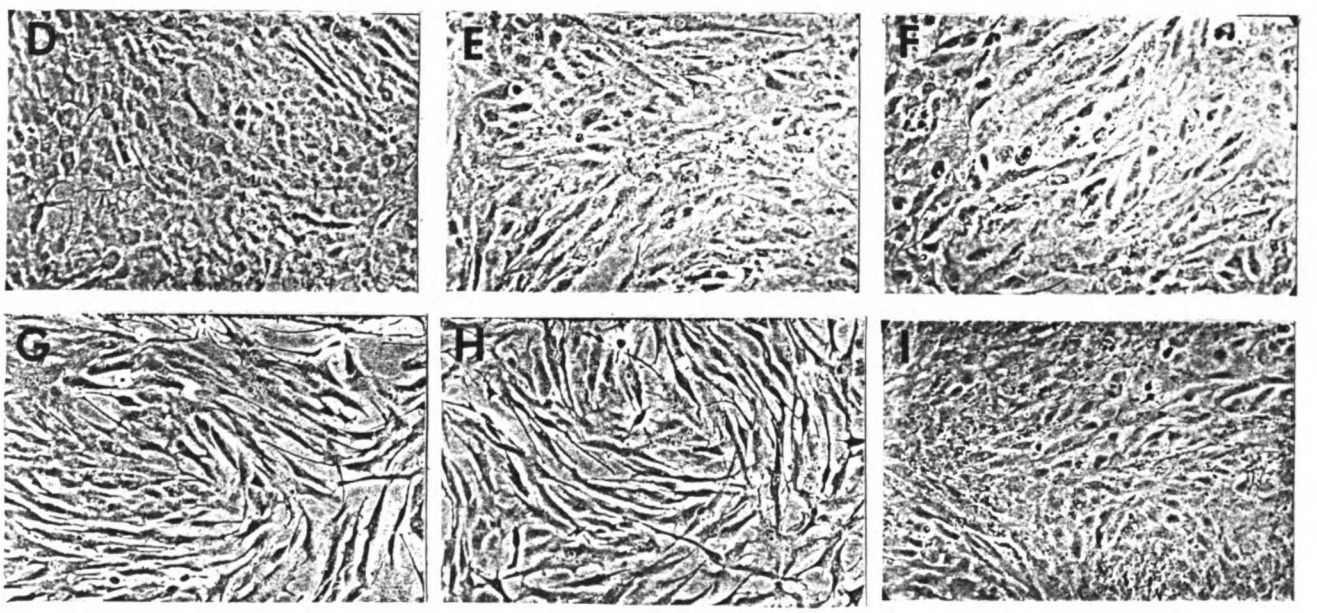
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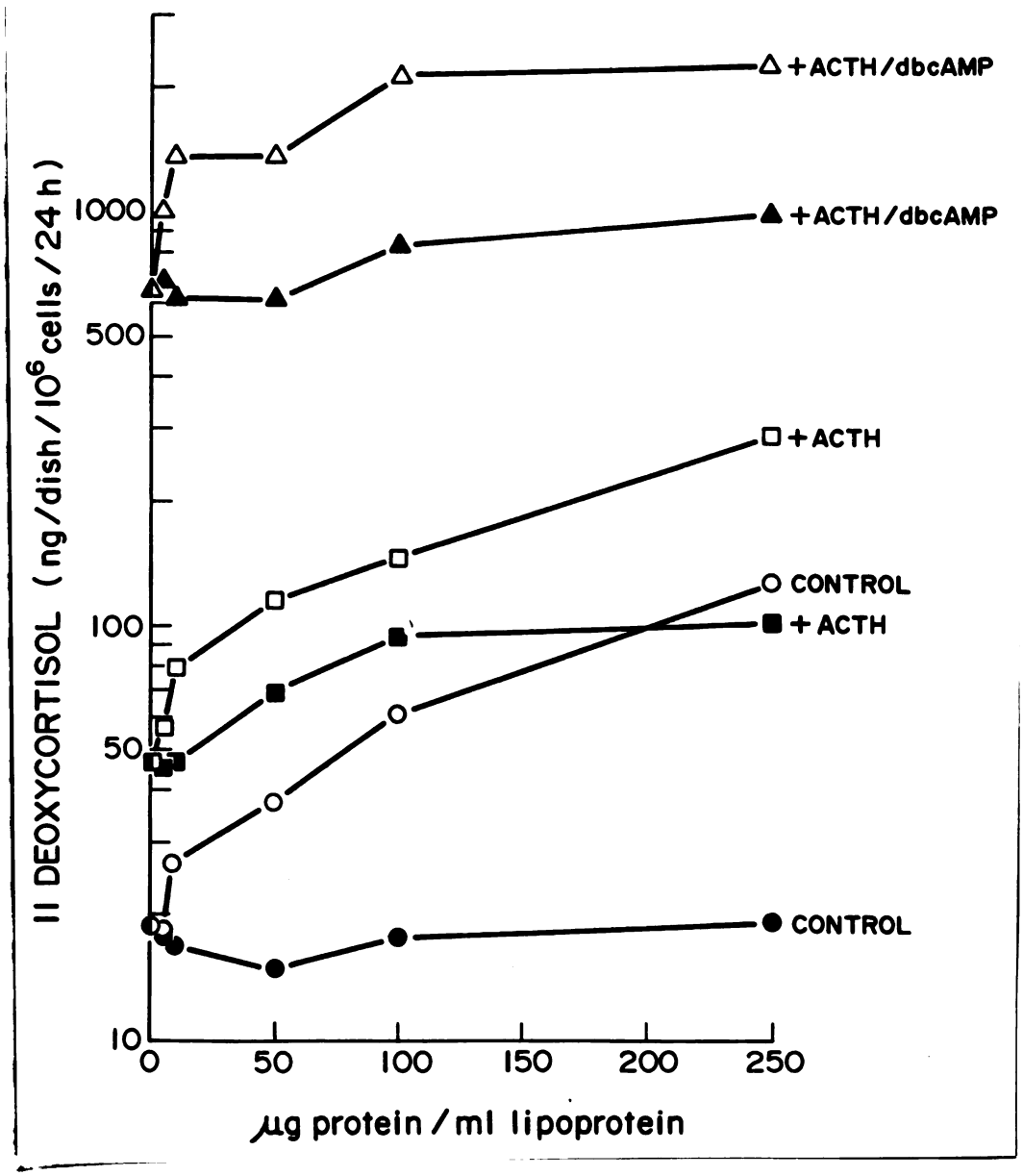
Fig. 7. Effect of increasing concentrations of HDL or LDL, ACTH or db cAMP or both on production of 11 Deoxycortisol by bovine adrenal cortex cells.

Third passage bovine adrenal cortex cells, previously grown to confluence on ECM-coated dishes and exposed to F-12 medium supplemented with HDL (25 µg protein/ml), insulin (50 ng/ml), FGF (100 ng/ml), and transferrin (1 µg/ml), were trypsinized. Cells were then centrifuged in the presence of F-12 medium supplemented with 50 µg protein/ml HDL and resuspended in F-12 medium supplemented with 1 µg/ml transferrin. Four X 10^4 cells were then seeded per 35 mm ECM-coated dish. HDL (25 µg protein/ml), insulin (50 ng/ml), and transferrin (1 µg/ml) were added only once on day 0. FGF was added every other day until day 8, when the cells were nearly confluent. The medium was then renewed, and the cells were allowed to grow for 2 more days. On day 10 the media were changed to F-12 medium supplemented with transferrin (1 µg/ml) and HDL (o) or LDL (o) at concentrations ranging from 5 to 250 µg protein/ml, with or without ACTH (10^{-8} M, ,) or db cAMP (1 mM) and ACTH (,). Twenty-four hours later the media were collected and the cells counted in duplicate. The duplicate determinations never varied by more than 5%. The level of 11 Deoxycortisol released by the cells into their media was determined by radioimmunoassays, as described in "Materials and Methods." Duplicate aliquots from duplicate determinations for radioimmunoassay of 11 Deoxycortisol never varied by more than 10%.


△ +ACTH/dbcAMP
▲ +ACTH/dbcAMP
□ +ACTH
○ CONTROL
■ +ACTH
● CONTROL
11 DEOXYCORTISOL (ng/dish/10⁶ cells/24 h)
1000
500
100
50
10
0
50
100
150
200
250
µg protein / ml lipoprotein

Chapter 5

Insulin inductin of low density lipoprotein metabolism in bovine adrenal cortex cells as a function of their steroidogenic activity

I. Introduction

Cultured bovine adrenal cortex (BAC) cells maintained in serum-free medium require the presence of insulin in order to produce optimal amounts of steroid when cells are exposed to ACTH, cholera toxin, or dibutyryl cyclic AMP (1). Since plasma cholesterol, delivered in great part by low density lipoprotein (LDL) (3, 5), is the natural substrate for steroid formation in BAC cells, the stimulatory effect of insulin on LDL receptor-mediated endocytosis has been analyzed. The results of the present studies suggest that, when BAC cells maintained under serum-free conditions are stimulated by cholera toxin to produce steroids, insulin greatly increases the number of available LDL receptors. This in turn increases the ability of the cells to internalize and degrade LDL.

II. MATERIALS AND METHODS

Materials

Fibroblast growth factor (FGF) was purified from bovine brains as previously described (7). Brain FGF yields a single band on polyacrylamide gel electrophoresis at pH 4.5 and on an isoelectric focusing column (total volume 110 ml, pH range 3.4 to 11.0). The activity focused within a single peak having an isoelectric point of 9.6. When analyzed by SDS slab gel polyacrylamide electrophoresis, a single band (13,000 MW) was observed when gels were stained with silver nitrate (7). Fibronectin was purified from bovine plasma as described by Engvall, Ruoslahti, and Miller (8). When analyzed by slab gel polyacrylamide gel electrophoresis under reduced conditions, the purified bovine plasma fibronectin ran as a doublet with a molecular weight in the range of 220,000.

Crystalline bovine serum albumin was obtained from Schwarz-Mann (Orangeburg, N.J.). Insulin, transferrin, and dibutyryl cyclic AMP (db cAMP) were obtained from Sigma Chemical Co. (St. Louis, Mo.). ACTH 1-24 (Organon, West Orange, NJ) was a gift from the Dept. of Pediatric Endocrinology (Univ. of Calif., San Francisco). Dulbecco's modified Eagle's medium (DME) and Ham's F-12 medium were obtained from Grand Island Biological Co (Grand Island, N.Y.). Calf serum and fetal calf serum were obtained from Irvine Serum Co (Irvine, Ca.). Horse serum was obtained from Colorado Serum Co. (Denver, Colo.).Tissue culture dishes were from Falcon Plastics, Gentamicin from Schering Co. (Kenilworth, N.J.), and Fungizone from Squibb (Princeton, N.J.).

Preparation of low density lipoprotein (LDL) and high density lipoprotein (HDL)

Human LDL (1.019 d 1.063 g/cm^3) and HDL (1.07 d 1.21 g/cm^3) were obtained from human plasma by differential ultracentrifugal flotation (9). In order to remove contaminating plasma proteins and lipoprotein fractions of higher density, the LDL and HDL were washed by flotation through a solution corresponding to their respective upper densities. Protein concentrations were determined as described by Lowry et al (11) and modified by Maxwell et al (12). Each lipoprotein fraction migrated as an homogeneous band on agarose electrophoretic gel.

The purified HDL and LDL preparations were analyzed by double immunodiffusion to determine the degree of cross-contamination of the HDL preparation by LDL and vice-versa (13, 14). When HDL or LDL preparations were analyzed by double immunodiffusion, 0.2 μg of HDL protein gave a single precipitin line against rabbit anti-human $_1$ apoprotein (anti-HDL) (Behring Diagnostics, American Hoechst Co., Somerville, N.J.). In the case of LDL, no

precipitin line could be observed even at a 500-fold higher protein concentration (100 μg protein). Likewise, LDL (0.2 μg protein) gave a single precipitin line when tested against rabbit anti-human -apoprotein (anti-LDL) (Cappel Laboratories, Cochranville, Pa.), whereas HDL at 500-fold higher protein concentration did not give a precipitin line. These results demonstrate that HDL preparations contained less than 0.2% LDL, if any at all, and vice versa (13, 14). To eliminate the possibility of a contamination by plasma proteins, the purity of the LDL and HDL preparations was analyzed by slab gel electrophoresis (10 to 18% and 5 to 18%, respectively, exponential polyacrylamide gel gradient containing 0.1% sodium dodecyl sulfate) with or without prior delipidation with tetramethyl urea (32, 33). When the electrophoretic patterns of HDL and LDL preparations were compared to that of plasma or LPDS, no obvious contamination by plasma proteins was observed.

Cells and Culture Conditions

Cultures of BAC cells from passage 1 to 5 were trypsinized and centrifuged in F12 medium containing HDL (500 μg protein/ml) and transferrin (2 μg/ml). The pellet was resuspended in F12 medium supplemented with HDL (30 μg protein/ml) and transferrin (1 μg/ml). Cells were seeded at 4×10^4 (or greater) per 35 mm ECM-coated tissue culture dish and grown to confluence (6-8 days) in F12 medium supplemented with HDL (30 μg protein/ml), transferrin (μg/ml), insulin (50 ng/ml), and FGF (100 ng/ml). FGF was added every other day until confluence. The cell densities of confluent cultures were between $5\text{-}7 \times 10^5$ cells/dish.

Cultures of BAC cells were also seeded directly into 10% serum- (horse serum:fetal calf serum at a ratio of 1:1) supplemented F12 on 35 mm ECM-coated dishes. Four hours later, the media were changed to F12 supplemented with HDL (30 μg protein/ml), transferrin (1 μg/ml), insulin (50

ng/ml), and FGF (100 ng/ml). FGF was added every other day until the cells reached confluence. The various additions of lipoproteins, insulin, cholera toxin, and chloroquine were then made as described in the figure legends, usually in the presence of F12 medium supplemented only with HDL (1 μg protein/ml) and transferrin (1 μg/ml). HDL (1 μg protein/ml) and transferrin (1 μg/ml) were routinely added to confluent BAC cultures during the course of experiments to protect the cells from lysing, which was found to occur in their absence.

Determination of Lipoprotein Binding, Internalization, and Degradation

Confluent BAC cultures were grown as described above and exposed to F12 medium supplemented with transferrin (1 μg/ml) and HDL (1 μg protein/ml). Cholera toxin (CT, 10 ng/ml) or insulin at the concentrations indicated in the figure legends were then added either alone or in combination and cells were incubated for 20 to 24 hours. Various concentrations of ^{125}I-LDL, with or without a 20-fold excess of unlabeled LDL, were then added to triplicate plates and cultures. The cultures were further incubated at either 4°C or 37°C for various time periods. At regular intervals the media were collected, and each monolayer was washed 10 times with cold (4°C) PBS containing 0.2% BSA. Cellular bound, internalized, or degraded ^{125}I-LDL was determined. To release the ^{125}I-LDL that was bound to the cell surface, the washed plates were exposed to 1 ml of a solution containing 50 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and 10 mg/ml of dextran sulfate for 1 h at 4°C (15). The dextran sulfate-released radioactivity was then determined in a Beckman Gamma Counter (310 series). After incubation with dextran sulfate, plates were further washed 6 times with cold (4°C) PBS containing 0.2% BSA. Cells were then dissolved in 0.1 M NaOH, and counted to determine the amount of ^{125}I-LDL that had been

internalized in the cell and hence was not accessible for release by dextran sulfate. In order to determine the amount of ^{125}I-LDL degraded, an equal volume of cold 25% (wt/vol) tricholoracetic acid was added to the collected medium. After centrifugation, the supernatant was extracted with hydrogen peroxide and chloroform to correct for free radioiodide (16), and the radioactivity of an aliquot of the aqueous phase was then determined. The nonspecific value for each LDL concentration was determined by incubating the monolayer with ^{125}I-LDL in the presence of a 20-fold excess of unlabeled LDL. In order to express the results in terms of cell number, duplicate cultures were trypsinized and counted in a Coulter counter for each experiment.

Steroid measurement

11 deoxycortisol (11 DOC) was measured by radioimmunoassay after ether extraction from the medium. The antibody was purchased from Dr. G. Abraham, (Division of Reproductive Biology, Harbor General Hospital, Torrance, CA). Radioimmunnoassays were performed as described (17) on duplicate aliquots from duplicate dishes. Differences between duplicate aliquots and dishes were less than 10%. Assay linear dose response was between 5 and 10,000 pg per tube, and samples were diluted to fall on the linear part of the standard curve. The media samples after ether extraction were assayed without prior chromatography. Each figure represents the data of at least one of three separate experiments.

III. RESULTS

Characterization of the receptor-mediated low density lipoprotein pathway in bovine adrenal cortical cells maintained in a serum-free medium

The ability of BAC cells to bind and metabolize LDL has been studied with BAC cell cultures maintained on ECM-coated dishes and grown in the

presence of F-12 medium supplemented with HDL, insulin, transferrin, and FGF. As shown in Fig. 1, these cells express an LDL receptor-mediated pathway whose characteristics (time-course of binding, internalization and degradation of ^{125}I-LDL) are similar to that which has been described for granulosa cell cultures grown and maintained in defined medium supplemented with transferrin, HDL, insulin, and FGF (18). The interaction of ^{125}I-LDL (100 μg protein/ml) with the cells was analyzed as a function of time. At 37°C, binding was observed to be maximal after 30 minutes at 37°C. It gradually decreased to approximately 30% of this value over the next 20 hours of incubation. This probably reflected the down-regulation of the LDL receptor following prolonged exposure to the ligand. The amount of internalized ^{125}I-LDL reached a maximum at 2 hours and remained constant over the next 6 hours (Fig. 1). Degradation of ^{125}I-LDL, as expected, lagged behind the internalization of ^{125}I-LDL. This may indicate the time required for the LDL to get into the lysosomes, where degradation of the internalized ^{125}I-LDL receptor complex occurs. Degradation was linear between 1 and 20 hours and had a rate of 0.18 μg protein/10^6 cells/1 h (Fig. 1).

To characterize further the LDL receptor-mediated pathway in BAC cells grown under serum-fee conditions, the binding, internalization, and degradation of ^{125}I-LDL as a function of concentration were determined after a 4 h exposure at 37°C. As shown in Fig. 2A-C, binding, internalization, and degradation of ^{125}I-LDL saturated at a concentration of about 150 μg protein/ml. When the results shown in Fig. 2A were submitted to Scatchard analysis (using a molecular weight of 6.3 X 10^5 for the protein portion in the LDL complex (19)), a single class of high affinity receptor sites with a K_D of 2 X 10^{-8} M was observed. The total number of cell surface LDL receptors under the described conditions was 3.5 X 10^3.

The effect of insulin on low density lipoprotein binding and metabolism and 11 deoxycortisol released by bovine adrenal cortex cells

Previous studies performed with BAC cells in vitro have demonstrated that insulin plays a role in both their proliferation and steroidogenic capacity (1). Since increased steroidogenesis in cells exposed to various trophic factors and insulin could reflect an activation of LDL receptor-mediated pathways, we have examined the effect of insulin on ^{125}I-LDL binding, internalization, and degradation. As shown in Fig. 3, a 20 h exposure to cholera toxin (CT, 10 ng/ml), with increasing times of exposure (from 15 min to 20 h) to insulin (100 ng/ml) resulted in increased binding, internalization, and degradation of ^{125}I-LDL (100 μg protein/ml, 4 h, 37°C). BAC cells exposed to insulin in the presence of CT (but not in its absence) for 20 h (Fig. 3) had 3.3-fold more bound, 2.1-fold more internalized, and 2.1-fold more degraded ^{125}I-LDL than in the absence of insulin. In the absence of CT,preexposure to insulin had little effect (Fig. 3) on ^{125}I-LDL binding, internalization, or degradation.

When BAC cells were preincubated with increasing concentrations of insulin (ranging from 1 to 10,000 ng/ml) in the presence and absence of CT (10 ng/ml), an insulin dose-dependent increase in the binding, internalization, and degradation of ^{125}I-LDL (100 μg protein/ml, 4 h, 37°C) was observed in cultures incubated with CT (Fig. 4A-C). In the absence of CT, insulin, regardless of concentration, had no effect (Fig. 4A-C). A parallel increase in 11 deoxycortisol (11 DOC) secretion with increasing insulin concentrations in the presence of CT, but not in its absence, was also observed (Fig. 4D).

Correlation between ^{125}I-LDL binding, internalization, and degradation and 11 deoxycortisol released by bovine adrneal cortical cells

The time-course analysis of the binding, internalization, and degradation of ^{125}I-LDL (10 μg protein/ml, 37°C) was analyzed in conjunction with the

release of 11 DOC by BAC cells incubated for 24 h with CT (10 ng/ml) and insulin (100 ng/ml). The binding of ^{125}I-LDL was maximal at 30 min, while the amount of ^{125}I-LDL internalized reached a steady state by two hours; degradation, which lagged behind the ^{125}I-LDL internalized for the first two hours, increased at a linear rate of 0.15 μg protein/10^6 cells/h (Fig. 5). When the release of 11 DOC was analyzed, it was found to increase in a time-dependent manner and followed the rate of ^{125}I-LDL internalized (Fig. 5). In Table I, control values for the experiment in Fig. 5 are presented. From these values it can be seen that preincubation of cells with insulin alone did not markedly affect ^{125}I-LDL internalization or degradation. Preincubation with CT alone increased ^{125}I-LDL internalization and degradation after 4 h by 10- and 5-fold, respectively, and 11 DOC release by 2.7-fold. When insulin was added with CT, ^{125}I-LDL internalization and degradation after 4 h increased by 45- and 22-fold, respectively. This correlated with a 9.6-fold increase in 11 DOC released into the medium.

The contribution of cholesterol to 11 deoxycortisol production after lysosomal degradation of ^{125}I-LDL was studied by titering the effect of chloroquine, an inhibitor of lysosomal functions, on ^{125}I-LDL degradation and 11 deoxycortisol production. Confluent cultures of BAC cells were preexposed to cholera toxin (10 ng/ml) and insulin (100 ng/ml) for 24 hours and to increasing concentrations of chloroquine for 2 hours. ^{125}I-LDL degradation (10 μg protein/ml, 4 h, 37°C) was then analyzed in parallel with 11 DOC release (Fig. 6). As shown in Fig. 6A, 100 μM chloroquine inhibits more than 95% of the ^{125}I-LDL degraded. This was paralleled by an 80% inhibition of 11 deoxycortisol secretion (Fig. 6B). The total inhibition of 11 deoxycortisol secretion did not occur, presumably because of endogenous cholesterol synthesis.

The effect of insulin on the binding of ^{125}I-LDL to BAC cells exposed to cholera toxin

The binding of ^{125}I-LDL (30 min, 37°C) to BAC cells preexposed to cholera toxin (10 ng/ml) with and without insulin (100 ng/ml) for 24 hours was analyzed as a function of ^{125}I-LDL concentration (Fig. 7A & B). Scatchard analysis of the binding of ^{125}I-LDL to cells preexposed to cholera toxin (Fig. 7A) or cholera toxin and insulin (Fig. 7B) is presented. In both cases, high affinity sites (K_D 2.2 X 10^{-8} M) were present, as were lower affinity sites (K_D approximately 1.3 X 10^{-7}). The number of high affinity sites in cells preexposed to cholera toxin was calculated using a molecular weight of 6.3 X 10^5 for the protein portion in the LDL complex (19), and found to be approximately 9 X 10^4 LDL cell surface receptors per cell (Fig. 7A). When insulin (100 ng/ml) was added to BAC cells in the presence of CT, for the 24 hours preexposure the number of LDL cell surface receptors increased by 12.7-fold (3.6 X 10^5 LDL receptors per cell) (Fig. 7B). No change in affinity of either the high or low affinity site occurred after preexposure or not with insulin. While ^{125}I-LDL binding (30 min, 37°C) has been studied with BAC cells not preexposed to either cholera toxin or insulin, the scarcity of LDL cell surface receptors under these conditions resulted in considerable variation in separate experiments when Scatchard analysis of the data was made (i.e. K_D of 1-5 X 10^{-8} M, and 2-5 X 10^4 receptors per cell).

IV. DISCUSSION

When BAC cells were cultured under serum-free conditions and exposed to cholera toxin or cholera toxin and insulin, high affinity (K_D = 2 X 10^{-8} M) LDL cell surface receptors were observed. LDL binding to these receptors was saturable at about 100 μg protein/ml and was maximal after 30 minutes of exposure to cells maintained at 37°C. The bound LDL receptor complex

reached a steady-state rate of internalization 2 hours after the exogenous addition of LDL. Degradation of internalized LDL receptor complexes proceeded at a linear rate of about 0.15 μg protein/10^6 cells/hour in cells stimulated to release steroids by either cholera toxin alone or cholera toxin and insulin. A similar value for LDL degradation has been previously reported for bovine granulosa cells cultured in the presence of lipoprotein-deficient serum (18).

The absolute number of LDL receptors on BAC cells cultured for 8 days under serum-free conditions was exceptionally low. For example, as little as 3 X 10^3 receptors per cell were observed when cells were incubated with increasing concentrations of LDL for 4 hours at 37°C. When BAC cells were exposed for 30 minutes at 37°C to increasing LDL concentrations, preexposure to cholera toxin alone, as determined by Scatchard analysis, increased within 24 hours the number of high affinity LDL receptors by an average of 3-fold, so that the final density was about 9 X 10^4 receptors per cell. Insulin together with cholera toxin increased the LDL receptor density by an additional 4-fold (3.6 X 10^5 high affinity receptors per cell). The dissociation constant of the LDL receptor remained the same (mean value of 2.2 X 10^{-8} M) regardless of whether or not steroidogenesis was stimulated by cholera toxin. Scatchard analysis of LDL binding data was more reliable in the case of cultures exposed to either cholera toxin or cholera toxin and insulin. This reflects the vast increase in receptors per cell (i.e. 0.9 or 4 X 10^5 LDL receptors per cell in cultures exposed to cholera toxin or cholera toxin and insulin, versus 2-5 X 10^4 receptors per cell for control cultures or cultures exposed to insulin alone). The appearance of low affinity sites was demonstrable by Scatchard analysis in cells preincubated with cholera toxin and cholera toxin and insulin. It is doubtful, however, whether such binding sites contribute significantly to the

subsequent internalization process, since both the internalization and degradation of ^{125}I-LDL are saturated at an LDL concentration of 100 μg protein/ml. At this LDL concentration, only the high affinity LDL receptor sites would be operational. This suggests that the low affinity sites play little part in the cholera toxin or cholera toxin-and-insulin-stimulated internalization and degradation of ^{125}I-LDL by BAC cells.

As shown by the time-course analysis of binding, internalization, and degradation of ^{125}I-LDL and the parallel production of 11 DOC, production of 11 DOC appears to be tightly coupled to the delivery of cholesterol by LDL. Furthermore, when degradation was inhibited by chloroquine-inhibition of lysosomal function, 11 DOC secretion followed a similar chloroquine concentration-dependent decrease.

The effects of cholera toxin and Bt_2 cAMP have been previously described as mimicking the effect of ACTH on BAC cells in vitro (22). The mechanisms by which cAMP acts to increase the steroidogenic potential of these and other steroidogenic cells is still unresolved. In granulosa cells, cAMP has been shown to increase the transport and degradation of LDL, thereby increasing the intracellular cholesterol content of the cells, at least in part through an increased affinity of the high affinity LDL receptor sites (18). The current studies on BAC cells show that, although no change in affinity of the high affinity receptor site occurs in response to cholera toxin (which raises intracellular cAMP), a large increase in the number of high affinity receptor sites occurs. Insulin further increases the number of these receptors, which results in the increased delivery of cholesterol to the cells and in as much as a 10-fold increase in 11 DOC secretion in a 4 hour period.

The lack of an effect of insulin on cells not exposed to cholera toxin suggests that cAMP is the initiator of steroidogenesis and that insulin's role is

to support this event. Once steroidogenesis is induced, LDL provides the cholesterol utilized for steroid formation via a receptor-mediated pathway. The studies presented here indicate that insulin may play an essential role in this process by stimulating the biosynthesis of LDL receptors, or by recycling them. The precise mechanism by which insulin does this will require further study.

The tremendous induction of high affinity LDL receptors by insulin when BAC cells were exposed to cholera toxin under serum-free conditions suggests that insulin may be a critical factor for optimal steroid release. It also provides a mechanism of action for insulin stimulation of steroid release. Until now, in vitro studies of LDL receptors have utilized media supplemented with either serum or plasma (3, 20). These media contain insulin, and studies conducted with cells cultured under such conditions would mask the effect of insulin on LDL binding and metabolism, since these cells already exposed to plasma insulin would have an optimal density of LDL cell surface receptors.

The lack of an effect of insulin on cells not exposed to cholera toxin suggests that cAMP itself will stimulate steroidogenesis by activating the various enzymatic systems involved in the production of 11 DOC from cholesterol. The role of insulin would be to increase LDL cholesterol entry within the cell by increasing the density of LDL cell surface receptor sites, therefore increasing the pool of cellular cholesterol available for steroidogenesis.

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TABLE I

Regulation of LDL receptor activity and steroid produciton in bovine adrenal cortex cells with or without prior incubation with insulin, cholera toxin, or both

24 hour preincubation in the presence of:	^{125}I-LDL BOUND		^{125}I-LDL INTERNALIZED		^{125}I -LDL DEGRADED		11-Deoxycortisol PRODUCED
	0 hr	4 hr	0 hr	4 hr	0 hr	4 hr	
	ng protein/10^6 cells						ng/10^6 cells/4hrs
Nothing	—	—	1.74	5.1	2.16	22.6	37 ± 4
Insulin	—	—	3.03	8.7	2.52	40.0	45 ± 5
Cholera Toxin	10.0	4.0	7.32	50.0	2.84	120.0	100 ± 7
Cholera Toxin + Insulin	38.4	48.5	17.0	227.7	8.4	504.0	355 ± 17

Control values for the experiment presented in Fig. 5 are tabularized. Confluent cultures of BAC cells were preincubated in F12 medium supplemented with HDL (1 µg protein/ml), transferrin (1 µg/ml), and insulin (100 ng/ml) or cholera toxin (10 ng/ml) or both insulin (100 ng/ml) and cholera toxin (10 ng/ml) for 24 hours. The media were then renewed (same components) and ^{125}I-LDL was added for 0 or 240 minutes. ^{125}I-LDL (10 µg protein/ml) that was bound to the cell surface, internalized, and degraded, as well as 11 deoxycortisol secreted into the media, were determined for each time point as described under "Experimental Procedures."

SEE NEXT PAGE

Fig. 5. Time-course of ^{125}I-LDL binding, internalization, degradation, and 11 deoxycortisol production at 37°C by bovine adrenal cortex cells following preexposure to cholera toxin and insulin

BAC cells were grown to confluence in the absence of serum in F12 medium supplemented with HDL (30 µg protein/ml), insulin (50 ng/ml), transferrin (1 µg/ml), and FGF (100 ng/ml). HDL, insulin, and transferrin were added only once at the time of cell seeding, while FGF was added every other day. Upon confluence (6-8 days) the media were changed to F12 supplemented with HDL (1 µg protein/ml), transferrin (1 µg/ml), cholera toxin (10 ng/ml), and insulin (100 ng/ml). Twenty-four hours later the media were renewed (same components), and ^{125}I-LDL (10 µg protein/ml) was added for 0, 5, 10, 15, 30, 60, 90, 120, 180, and 240 minutes with and without a 20-fold excess of unlabeled LDL. Binding (o), internalization ( ), and degradation ( ) of ^{125}I-LDL, as well as 11 deoxycortisol ( ) secreted into the media, were determined for each time point. Control values for this experiment are presented in Table I.

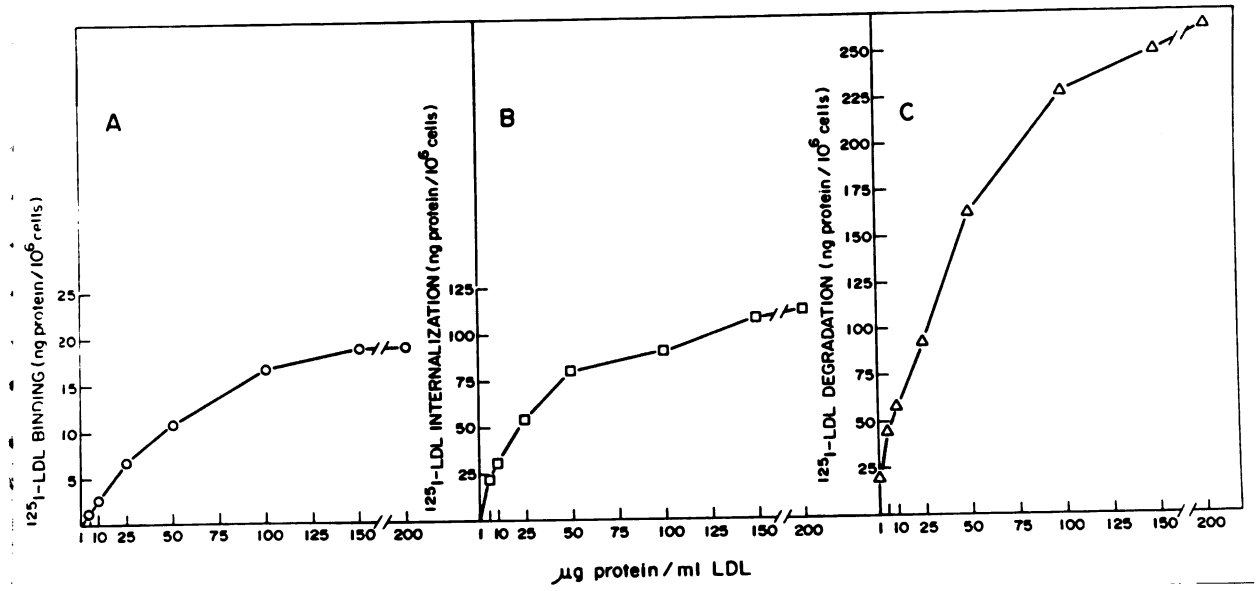


Fig. 2. ^{125}I-LDL binding, internalization, and degradation at 37°C by bovine adrenal cortex cells as a function of ^{125}I-LDL concentration

BAC cells were grown to confluence in the absence of serum in F12 medium supplemented with HDL (30 μg protein/ml), insulin (50 ng/ml), transferrin (1 μg/ml), and FGF (100 ng/ml). HDL, insulin, and transferrin were added only once at the time of cell seeding, while FGF was added every other day. Upon confluence (6-8 days) the media were changed to F12 supplemented with HDL (1 μg protein/ml) and transferrin (1 μg/ml) for 20 hours. The media were renewed again (same components) with the addition of ^{125}I-LDL at concentrations ranging from 1 to 150 μg protein/ml with or without a 20-fold excess of unlabeled LDL. Cultures were then incubated for 4 hours. At the end of the incubation, the cell surface binding (A), internalization (B) and degradation (C) were determined as described under "Experimental Procedures."

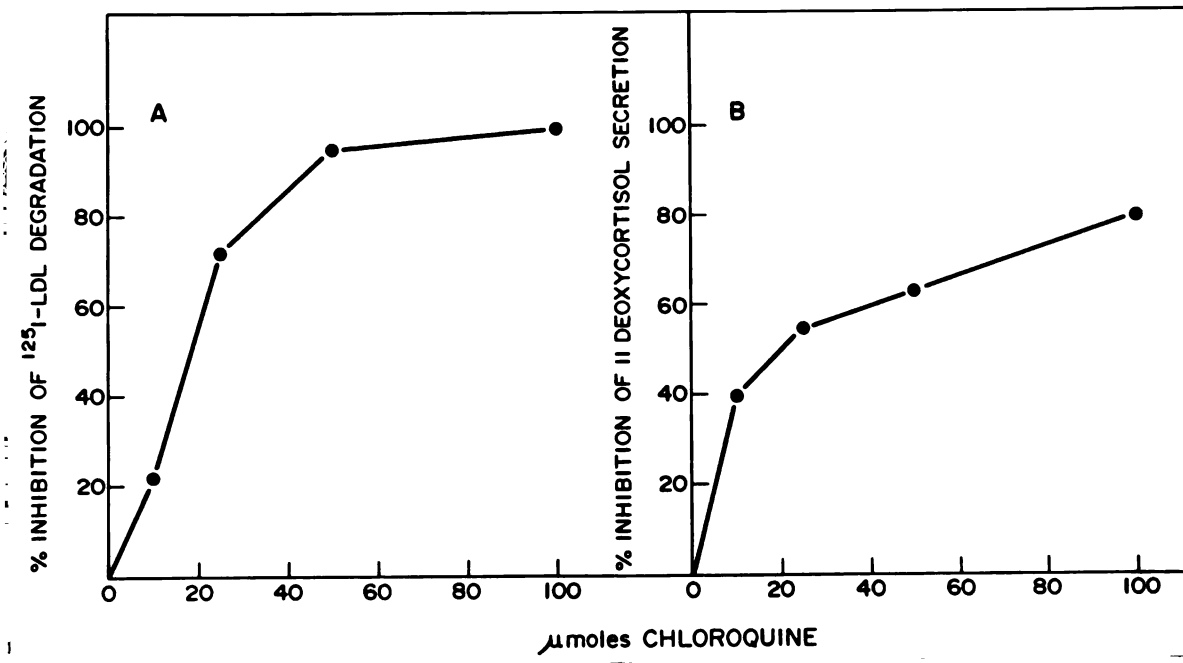


Fig. 6. Inhibition of ^{125}I-LDL degradation and 11 deoxycortisol production by chloroquine in bovine adrenal cortex cells

(A) BAC cells were grown to confluence in the absence of serum in F12 medium supplemented with HDL (30 µg protein/ml), insulin (50 ng/ml), transferrin (1 µg/ml), and FGF (100 ng/ml). HDL, insulin, and transferrin were added only once at the time of cell seeding, while FGF was added every other day. Upon confluence (6-8 days) the media were changed to F12 supplemented with HDL (1 µg protein/ml), transferrin (1 µg/ml), cholera toxin (10 ng/ml), and insulin (100 ng/ml). Twenty-two hours later the cultures were exposed to increasing concentrations of chloroquine. At 24 hours the media were renewed (same components) and ^{125}I-LDL (10 µg protein/ml) was added with and without a 20-fold excess of unlabeled LDL for 4 hours. At the end of the ^{125}I-LDL exposure period the medium was collected, and the amount of ^{125}I-LDL degraded was analyzed as described under "Experimental Procedures." Total degradation of cultures not exposed to chloroquine was 0.17 µg protein/10^6 cells/1 hour. That value was used as a reference to calculate the percentage of inhibition of ^{125}I-LDL degradation in the presence of chloroquine, and these values are presented in the figure.

(B) 11 deoxycortisol production was studied in parallel cultures, as desribed in (A). Total 11 deoxycortisol production by cultures not exposed to chloroquine was 0.15 µg/10^6 cells/1 hour. That value was used as a reference to calculate the percentage of inhibition of 11 deoxycortisol production in the presence of chloroquine, and these values are presented in the figure.

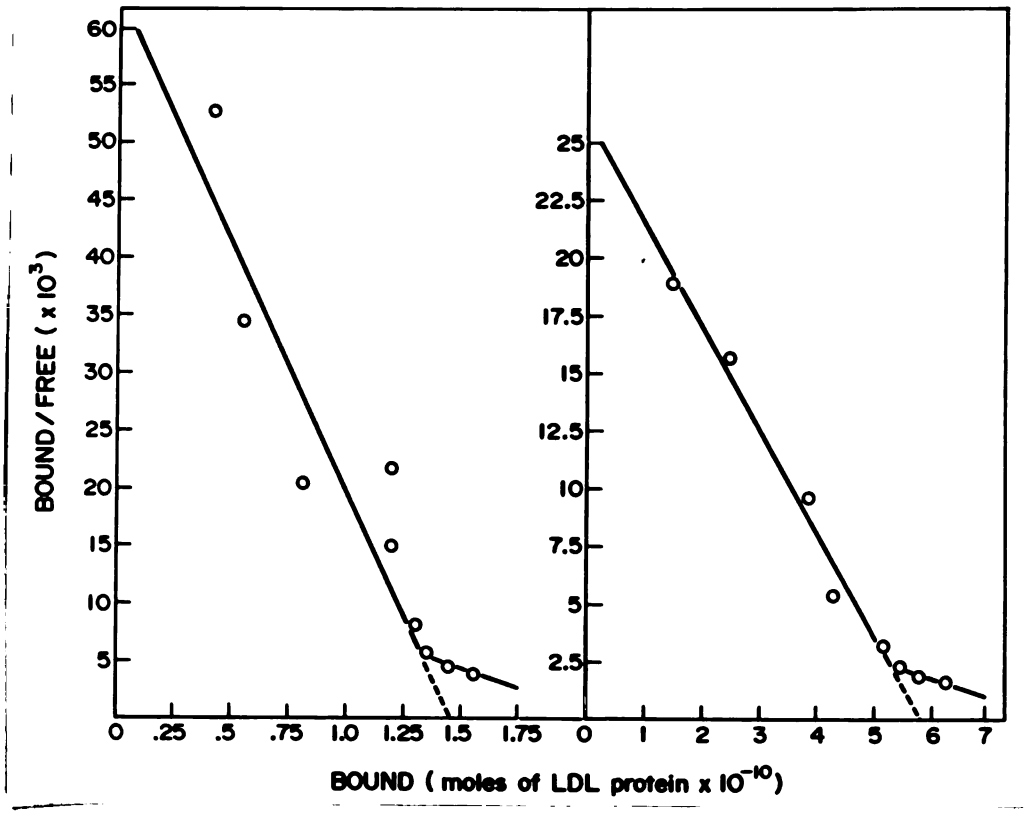
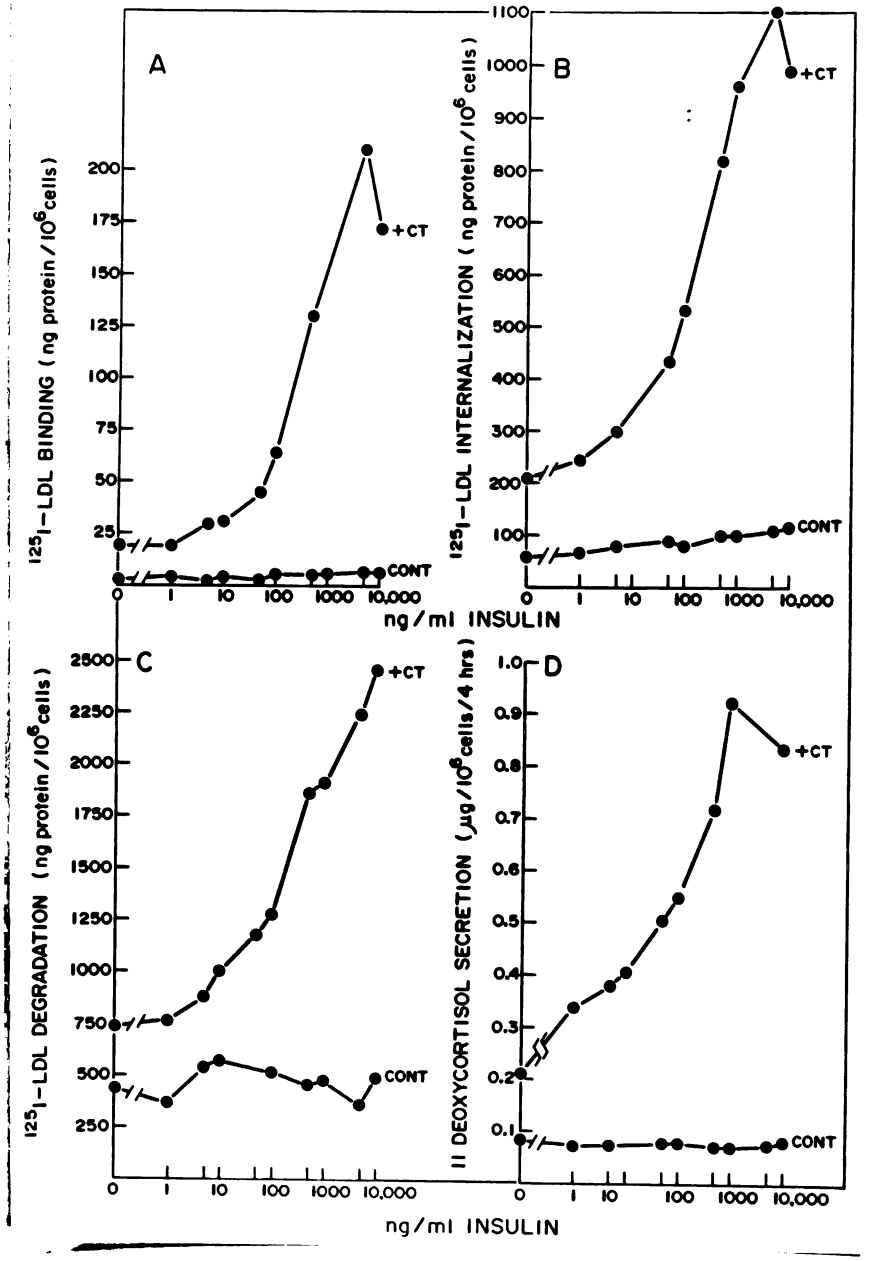


Fig. 7. Scatchard analysis of ^{125}I-LDL binding to bovine adrenal cortex cells at 37°C following preexposure to cholera toxin or cholera toxin and insulin

BAC cells were grown to confluence in the absence of serum in F12 medium supplemented with HDL (30 μg protein/ml), insulin (50 ng/ml), transferrin (1 μg/ml), and FGF (100 ng/ml). HDL, insulin, and transferrin were added only once at the time of cell seeding, while FGF was added every other day. Upon confluence (6-8 days) the media were changed to F12 supplemented with HDL (1 μg protein/ml), transferrin (1 μg/ml), and cholera toxin (10 ng/ml). Half of the cultures were then given insulin (100 ng/ml). Twenty-four hours later the media were renewed (same components), and increasing concentrations of ^{125}I-LDL (5-250 μg protein/ml) were then added with and without a 20-fold excess of unlabeled LDL for 30 minutes at 37°C. Fig. 7A represents the Scatchard analysis of the binding of ^{125}I-LDL to cultures preexposed to cholera toxin, while Fig. 7B represents the Scatchard analysis of the binding of ^{125}I-LDL to cultures preexposed to cholera toxin and insulin. The cell surface ^{125}I-LDL binding was then determined as described under "Experimental Procedures."


A
B
C
D
+CT
CONT
^{125}I-LDL BINDING (ng protein / 10^6 cells)
^{125}I-LDL INTERNALIZATION (ng protein / 10^6 cells)
^{125}I-LDL DEGRADATION (ng protein / 10^6 cells)
11 DEOXYCORTISOL SECRETION (µg / 10^6 cells / 4 hrs)
0 1 10 100 1000 10,000
ng / ml INSULIN

Chapter VI. Significance and Conclusion

In the studies presented in this thesis, experimental results have demonstrated that the type of substrate (plastic, ECM- or FN-coated dishes) upon which adrenal cortical (AC) cells are maintained has a profound influence on their proliferation. The bovine corneal ECM-coated dish, in particular, resulted in the ability to culture AC cells in plasma-supplemented media, and further, to resolve individual components of plasma necessary for proliferation or steroidogenesis. Despite the fact that the exact nature or underlying mechanisms of ECM in enabling these cellular functions were not studied, the results presented emphasize the paramount role of ECM for normal cell proliferation and differentiation.

Evidence has been provided to support the working hypothesis proposed in Chapter 2, that, if AC cells can be cultured under serum-free conditions, specific factors regulating proliferation or steroidogenesis might be determined and studied. The ability of an ECM to support the growth of AC cells in the absence of serum was the initial step in this process. When AC cells were maintained on ECM they no longer required serum but could be cultured in plasma, the physiological *in vivo* humor. The observation by Tauber and Gospodarowicz that high density lipoprotein (HDL) is the most fundamental plasma requirement for the survival and growth of cells *in vitro* was next in the sequence of events needed to culture cells under plasma-free conditions. This observation resulted in the development of a basal media of F12 with HDL (25 μg protein/ml), which were alone, all that AC cells required

for survival, minimal growth and steroidogenic capacity when maintained on ECM (Chapter 5).

In Chapter 3 human fetal adrenal cells were shown to proliferate in response to the growth factors EGF and FGF. Both proliferative rate and final cell density of each zone (fetal and definitive) were greatly increased by these mitogens. Additionally, high affinity ($K_D=10^{-9}$) EGF receptors were demonstrated on the cell surface for both fetal and definitive zone cells. The ability of ECM to stimulate proliferation was also observed for both zones. Although individual plasma components were not studied due to the difficulty in obtaining tissue, the response of these cells to ECM-coated dishes suggests that individual components might be elucidated and studied as has been done for the bovine adrenal cortex (presented in Chapter 4). The recent observation that EGF is detectable in newborn cord blood suggests that this factor is potentially important in the regulation (growth) of this gland.

The results of the studies presented in Chapter 4 demonstrated several new findings. First, with regard to substrates, plastic was inferior while fibronectin-coated dishes with fibroblast growth factor was the equivalent of the growth-promoting ability of the bovine corneal ECM-coated dish. Second, HDL was an absolute requirement for AC cell survival and growth, while the omission of insulin or transferrin resulted in less than maximal proliferative rate and final cell density. Third, AC cells have an exquisite sensitivity to insulin (maximal effective dose on proliferation is 10 ng/ml) which facilitated optimal growth and steroidogenic capacity. Fourth, certain regulatory factors to have effects (e.g. transferrin and LDL), can only be demonstrated when AC cells are cultured for prolonged periods in the absence of serum supplements,

or at least in the absence of such components from the serum. Lastly, AC cells could be repeatedly passaged for extended periods of time (i.e., 60 generations, 3 months) in the absence of serum or plasma supplements and still retain their steroidogenic capacity. This indicated that certain critical growth criteria had been met with the serum-free media and components that were used to culture these cells.

In Chapter 5, the effect of insulin on LDL binding, internalization, degradation, and 11-deoxycortisol secretion could be studied as a result of the serum-free conditions established for AC cells (Chapter 4). This study fulfilled the working hypothesis of Chapter 2 since both the elucidation of a specific factor as well as a particular mechanism by which it acts in the regulation of steroidogenesis was resolved. A previously unknown role for insulin in the augmentation of steroidogenesis was studied by analysis of ^{125}I-LDL binding, internalization, and degradation. The major results of this study indicated that insulin preexposure in AC cells stimulated to produce steroids with cholera toxin increased the binding (via new receptor synthesis), internalization, and degradation of LDL. Furthermore, the activity of the LDL receptor pathway was concentration and temporally related to 11-deoxycortisol synthesis and secretion, which was concentration and temporally related to insulin exposure.

The individual studies presented in this thesis, therefore, form a cohesive study both chronologically and theoretically. The utilization of an ECM resulted in the ability to culture AC cells in plasma. The use of plasma versus serum resulted in the abolition of plasma components required for cell growth and differentiation. Finally, one component, insulin, required for optimal

proliferation and steroidogenic function in AC cells, was studied in detail for its mechanism of action in steroidogenesis. While the results presented in this thesis have identified new factors regulating the functions of adrenal cortical cells, the serum-free system described will also allow for fewer unidentified variables in future studies of these functions.