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Hormonal regulation of growth and differentiation in pancreatic acinar cells in culture

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Hormonal regulation of growth and differentiation in pancreatic acinar cells in culture.

by

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DISSERTATION

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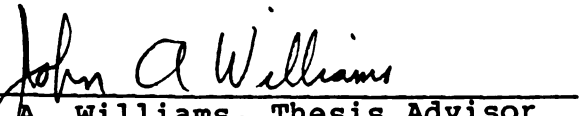
THESIS ABSTRACT: Two pancreatic acinar cell lines were investigated as *in vitro* models for hormonal regulation of exocrine pancreatic cell proliferation and differentiation. Rat pancreatic AR42J cells were compared with normal rat acini with respect to secretory protein expression, secretion, and hormonal regulation. AR42J cell secretory proteins were characterized by biosynthetic labeling, polyacrylamide gel electrophoresis, and autoradiography. Four major secretory proteins were identified: amylase, procarboxypeptidase A, chymotrypsinogen, and rat acinar pancreas secretory protein M_r=68,000 (unknown activity). The secretion of these 4 proteins was stimulated 2 - 3.5 fold by CCK. Dexamethasone treatment for 48 hours caused a 150 - 250 % increase in the biosynthesis of all four proteins. The proportion of total trichloroacetic acid precipitable, biosynthetically labeled protein released into the medium by CCK was equivalent for both dexamethasone-treated and non-treated cells (210 % of control). Dexamethasone inhibited proliferation in AR42J cultures by 95 %: the control generation time, 34 hours, was increased to 850 hours by 10nM dexamethasone. Inhibition of DNA synthesis was detectable after 6 hours, half-maximal after 12 hours, and maximal (9 % of control) after 18 hours of dexamethasone treatment. The half-maximal effective dose for inhibition of DNA synthesis was 0.5 nM; maximal inhibition was achieved with 10nM dexamethasone. The rank order of potency for inhibition of DNA synthesis by steroid hormones was dexamethasone>corticosterone> aldosterone>progesterone. The time-course, dose-response, and steroid specificity for two parameters of differentiation - increased amylase synthesis and increased CCK binding - paralleled inhibition of DNA synthesis. To determine whether inhibition of proliferation

alone would cause differentiation, two alternative methods of growth inhibition were used. Hydroxyurea or serum starvation inhibited growth to the same extent as dexamethasone, but did not induce differentiation, as assessed by amylase content and CCK binding. To determine whether cell proliferation was compatible with cellular differentiation, EGF and insulin were combined with sub-maximal dexamethasone (1 nM). In AR42J cultures released from growth inhibition by EGF or insulin, 1 nM dexamethasone increased cellular amylase content 5.9 - 6.5 fold, equivalent to the amylase increase in growth inhibited cultures. Mouse 266-6 cells responded to 10nM dexamethasone treatment with a 3 - 12 fold increase in amylase content; however, 266-6 cells contained only one tenth as much amylase per cell as AR42J cells and appeared less well differentiated morphologically. In contrast to AR42J cells, proliferation of mouse 266-6 cells was not inhibited by glucocorticoid treatment. It was concluded that, in AR42J and in 266-6 cells, glucorticoid effects on differentiation and cell proliferation were independent and separable. These properties and hormonal responses of rat AR42J and mouse 266-6 cells mirror properties of fetal acinar pancreas at specific stages of development. It was concluded that the AR42J and 266-6 cell lines would provide useful models for the identification and molecular characterization of hormonally induced regulators of cell proliferation, differentiation, and secretory signal transduction.



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DEDICATION

This Thesis is dedicated to my husband, Kent Guthrie, because of his patience and his devoted support for my research.

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Chapter 1

EXOCRINE PANCREATIC CELLS IN VITRO

Introduction.

The exocrine pancreas is a fruitful model for the study of biologic principles basic to all eukaryotic cells. Pancreatic exocrine cells originate in the fetus as undifferentiated epithelial cells of endodermal origin that proliferate and develop into pancreas-specific cell types (1). There are two main categories of exocrine pancreatic cells: ductal cells which secrete electrolytic fluid, and acinar cells which synthesize and secrete digestive enzymes.

Pancreatic acini have proven to be a particularly good model for the elucidation of intracellular pathways leading to the synthesis and secretion of tissue-specific protein secretory products (2). Pancreatic acinar cells are polarized. They receive hormonal stimuli via receptors at their basolateral surface and secrete digestive proteins into the lumen at their apical pole (3).

Hormonal regulation.

The major physiological stimuli for digestive enzyme release are acetylcholine from nerve terminals (4,5) and cholecystokinin (CCK or pancreozymin) from the small intestine (8-10). There is evidence that pancreatic enzyme secretion may be potentiated or modulated by neurotensin, insulin, gastrin, Substance P, bombesin-related peptides, vasoactive intestinal polypeptide (VIP), and secretin (6-8, 11-19). Inhibitory actions on enzyme secretion have been reported for pancreatic polypeptide (20) and somatostatin (21).

The receptors for many of the above named secretogogues are linked with either the adenylate cyclase or phosphoinositide intracellular message systems. Receptors for acetylcholine, CCK, bombesin, gastrin, and Substance P are coupled to phosphorylase C, the activation of which leads to the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) and the subsequent production of inositol trisphosphate (IP_3) and diacylglycerol (DAG) (63). IP_3 causes release of Ca^{2+} from intracellular stores and DAG activates protein kinase C. Increases in intracellular Ca^{2+} and activated protein kinase C lead to increased enzyme secretion by mechanisms yet to be elucidated (63). Receptors for VIP and secretin are linked to adenylate cyclase, the activation of which leads to an increase in cyclic AMP followed by increased enzyme secretion (63). Somatostatin inhibits cAMP production, but the mechanism of somatostatin action on pancreatic acini is not well established (63). Insulin binding to its receptor leads to increased receptor tyrosine kinase activity, however the mechanism by which insulin potentiates secretion has yet to be elucidated (63).

In addition to its acute effects on enzyme release, the secretogogue CCK can produce long-term trophic effects on the acinar pancreas, as evidenced by increased protein and DNA synthesis and content (22-26). Trophic effects have also been reported for insulin (27), epidermal growth factor (EGF) (28), secretin (25,26), and acetylcholine analogs (29). Inhibitory effects on adult rat acinar cell proliferation were reported for somatostatin (30) and glucocorticoids (31).

Interpretation of <u>in vivo</u> work is complicated by the possibility that the hormones studied affect the release of other regulatory factors. <u>In vitro</u> models allow for the assessment of direct hormone effects on the tissue of interest. A long-term <u>in vitro</u> model for the acinar pancreas is needed for

the characterization and molecular identification of the biological mechanisms underlying hormonal regulation of growth and differentiation, protein synthesis, and secretion.

The ideal in vitro model would require an immortal, homogeneous cell line multiplying in culture which displays acinar-specific characteristics such as maintenance of acinar-like morphology and digestive enzyme synthesis and secretion. The hormonal responsiveness of this cell line should mirror that of acini in vivo. The ideal cell model should also be amenable to transfection with foreign DNA. Immortal growth characteristics would also allow for the possibility of genetic manipulation and the development of genetic variants.

Explant and primary cell culture.

Fetal pancreatic rudiments differentiate in culture under appropriate conditions (32-35). Such explant culture systems provide evidence that interactions among stromal, endocrine, ductal, and acinar cells influence development of the acinar pancreas.

In order to determine precisely which hormonal and tissue interactions induce and maintain acinar differentiation, researchers devised methods for the culture of isolated acini and dissociated acinar cells. Pancreatic acini from adult mice were maintained in short-term suspension culture under various hormonal conditions (36). The addition of EGF, carbachol, and insulin to the culture medium was found to promote protein synthesis and secretory responsiveness to CCK after 24 hours(36). In another study dissociated pancreatic acinar cells from adult rat were maintained at high density in suspension culture for up to 4 weeks (37). In the presence of acetylcholine analog, these cells were reported to retain their

differentiated morphology and continued to synthesize protein, but did not replicate (37).

In other studies, differentiated pancreatic cells have been cultured on various surfaces. Isolated adult guinea pig acinar cells were reported to replicate on plastic culture dishes for 10 days, during which their differentiated characteristics declined (38). Adult rat acinar cells, over 5 days in culture, retained responsiveness to acetylcholine analog (amylase secretion) and to insulin (amylase synthesis) (39). The use of a basal lamina matrix promoted the reorganization of rat acinar cells into acini-like structures with several differentiated morphological and biochemical features, including digestive enzyme content and polarity around lumina (40). However, these cultures were maintained for only 5 days (40). Mouse acinar cells were induced to replicate on collagen gels to form monolayer cultures which were maintained for several weeks (41,42). These cultures responded to EGF, insulin-like growth factor I, and CCK with increased protein and DNA synthesis, and recovery of a semi-differentiated morphology. However, content of digestive enzymes declined and was not restored by hormonal treatment (41,42). Thus, primary culture systems have proven valuable for the study of short-term hormonal effects on differentiated acinar cells and longer-term effects on relatively de-differentiated cells. For the elucidation of long-term hormonal effects on cells which display acinar characteristics, a tumor cell line would prove useful.

Transplantable tumors and immortal cell lines.

Several immortal cell lines have been established from human pancreatic tumor specimens: MIA PaCa (49), PANC-1 (50), BxPC-3 (51),

AsPC-1(52-54), Hs766T(55), and CAPAN-1 and -2 (56,57). These cell lines typically displayed ductal characteristics, did not synthesize or secrete digestive proteins, and did not respond to normal acinar regulatory hormones. These findings reflect pathological reports that 90 % of human pancreatic tumors are of a ductal histiotype (58). It is not known whether human pancreatic carcinomas arise from de-differentiating acinar cells, from ductal cells, or from putative stem cells (centroacinar cells). The one report of hormonal responsiveness in culture described the regulation of proliferation in the MIA PaCa-2 cell line by EGF and by somatostatin (59). Since EGF and somatostatin regulate growth in many different cell types, this response is not indicative of acinar character. Because of their predominantly ductal characteristics, the human exocrine pancreatic cell lines did not prove useful for the study of normal cell biology of the acinar pancreas.

Transplantable tumors of the exocrine pancreas have been established from human, hamster and rat by treatment with chemical carcinogens (43-48). Human tumors were raised from fetal pancreas explants which were cultured with the carcinogen methylnitrosourea and subsequently transplanted into nude mice (43). Related chemical carcinogens were utilized through feeding or injection to induce transplantable tumors in animals. The human and hamster-derived tumors displayed no acinar markers, but possessed ductal characteristics such as positive histological staining for mucin and expression of duct cell surface antigen (43,44). In contrast, the rat tumors were heterogeneous in cell type and contained a relatively high proportion of differentiated acinar cells as assessed by morphology and enzyme content (45-48, 64,65). When cells of the Reddy and Rao tumor (45) were dispersed and analyzed, they were found to

resemble day-19 embryonic pancreas in their pattern of secretory protein content (64,65) When most of these rat tumor cells were placed in culture, however, the acinar-type cells de-differentiated to resemble duct cells which did not re-differentiate with hormonal treatment (47). Each culture displayed different and heterogeneous characteristics, since the cells did not all arise from the same subclone. Thus, these transplantable tumors did not yield a cultured cell system useful for the study of long-term acinar cell hormonal regulation.

Two immortal cell lines have been established from an azaserine-induced rat pancreatic tumor (48,60). In preliminary studies, I found one of the cell lines - designated ARIP- to be unsuitable for study of the acinar pancreas. ARIP cells displayed an undifferentiated apearance at both the light microscopic and ultrastructural level (flattened, fibroblastic appearance, no prominent secretory apparatus, data not shown). Moreover, ARIP cells did not synthesize amylase (determined by amylase activity) nor did they secrete any detectable proteins (determined by biosynthetic labeling, polyacrylamide gel electrophoresis, and autoradiography, not shown). The second immortal cell line derived from the azaserine-induced rat pancreatic tumor - AR42J- has been shown to possess several acinar specific characteristics. AR42J cells display a relatively differentiated acinar morphology, as evidenced by well-developed secretory organelles such as Golgi apparatus, rough endoplasmic reticulum, and zymogen granules.(61) Moreover, AR42J cells contain mRNA for several digestive enzymes (67,70), synthesize amylase protein (61) and possess receptors for and secretory responses to CCK and bombesin (68,69). Also, a cell line derived from a transgenic mouse tumor - 266-6 - was also invetigated as an acinar pancreatic model as part of this

thesis (62).

Glucocorticoid hormones have profound effects on the acinar pancreas both in vivo (30,31,70-73) and in explant organ culture (34,74). Glucocorticoid effects on acinar pancreatic cell proliferation and enzyme synthesis vary with the age, health, and dietary status of the fetus or adult animal (1), which implies that glucocorticoid effects can be modulated by other regulatory factors. In order to further elucidate direct effects of glucocorticoids on acinar cells, herein I will assess the suitablility of the AR42J and 266-6 cell lines as models for acinar pancreas, and describe hormonal regulation of differentiation and growth in these two cell lines.

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Chapter 2

Hormonal regulation of protein biosynthesis and secretion in AR42J cells.

The exocrine pancreas has been extensively studied with regard to organ development and differentiation, the biosynthesis and secretion of protein products, and the hormonal regulation of secretion (5,10,18,22). Studies in whole animals and in isolated pancreatic acini suggest cellular and molecular questions that can be best approached through the use of a cell line established in culture. The AR42J cell line was developed from a rat tumor of the exocrine pancreas (7). It has been reported that AR42J cells contain mRNA for several digestive enzymes (19). To date, however, protein synthesis and secretion have been described only for amylase (9,11,12,14). Here we report that AR42J cells synthesize at least three additional digestive enzyme proteins, the biosynthesis and secretion of which are hormonally regulated in similar fashion as for normal rat acini.

MATERIALS AND METHODS

Materials: The following were purchased: bovine plasma albumin (BSA, fraction V) from ICN, Naperville, IL; soybean trypsin inhibitor (SBTI, type 1-S), bacitracin, Hoechst 33258, calf thymus DNA type 1, HEPES, and dexamethasone from Sigma Chemical Co., St. Louis, MO; L-[^{35}S]methionine (1083 Ci/mmol) and Enlightning from New England Nuclear, Boston,

MA; IgG Sorb (protein A) from Enzyme Center, Inc., Boston, MA; Dulbecco's modified Eagle's medium (DME-H21) with and without methionine, penstrep, fungizone, fetal calf serum, trypsin/EDTA, and minimum Eagle's medium essential amino acid supplement from Gibco, Grand Island, NY; dithiothreitol, TEMED, molecular weight markers, ammonium persulfate, acrylamide and BIS from Bio-Rad Laboratories, Richmond, CA; CCK-8 from Bachem Inc., Torrence, CA. Bovine trypsin,and porcine procarboxypeptidases A and B were purchased from Sigma. Amylase was purified from rat pancreas. **Antisera**: polycolonal antisera were raised in rabbits (16),against amylase, trypsin, procarboxypeptidases A and B, and total secreted protein from rat acini. For production of rat total pancreatic-secretory protein antiserum, medium was collected and lyophilized from isolated normal rat acini that had been incubated for 2 hours in the presence of 1nM CCK. Antisera against rat anionic trypsinogen, chymotrypsinogen, and elastase were gifts from Dr. C. Largman, Martinez V.A. Hospital, Martinez, CA. All antisera immunoreactivities to rat proteins were identified by immunoprecipitation from biosynthetically labeled rat acinar cell lysates followed by SDS-PAGE and autoradiography.

Cell culture: AR42J cells were obtained from the American Type Culture Collection, Rockville, MD, in the 19th passage and divided at a ratio of 1:4 through the 80th passage. The cells were maintained in a 5% CO_2 atmosphere in DME-H21

containing penstrep, fungizone, and 10% fetal bovine serum. Cells were detached by treatment with trypsin/EDTA and plated at 300,000 cells per 16 mm-well in microcluster plates. The cells were cultured for 24 hours in the same medium, after which medium was changed and dexamethasone added where indicated.

Biosynthetic labeling and immunoprecipitation: AR42J cells were plated 72 hours before the start of pulse-labeling experiments. The cells were then washed once with methionine-free medium, pre-incubated for 30 minutes in 1 ml of methionine-free DME-H21 containing 0.5 mg/ml BSA plus SBTI (0.1 mg/ml), then incubated for the indicated times in the same medium containg 150 uCi of [^{35}S]methionine (pulse medium). All incubations were carried out at 37^{o} in a 5% CO_2 atmosphere. At the conclusion of the labeling period, cells were washed and cell lysates were prepared by incubation at 23^{o} in 500 ul PBS containing 1% Triton X-100, bacitracin (1mg/ml), benzamidine (10mM), SBTI (5ug/ml), and PMSF (0.2 mM). After centrifugation at 10,000 g for 2 minutes at 4^{o} C, the supernatants were stored at -20^{o} C or processed immediately. For collection of secreted proteins, radiolabeled cells were incubated in complete DME-H21 containing 50 ug/ml BSA, 10 ug/ml SBTI, plus benzamidine, bacitracin, and PMSF in the above concentrations. The medium and cell lysate samples were prepared for SDS-PAGE as described below for immunoprecipitated samples. When medium

samples were to be used for quantification of total radiolabeled protein, samples were precipitated with 10% trichloroacetic acid (final concentration) and the pellets were washed three times with ice-cold 10% trichloroacetic acid. The washed precipitate was dissolved in 0.5 ml of 0.1 M NaOH, then added to 5 ml Liquiscint and its radioactivity determined. The sorting ratio for newly synthesized secretory protein was calculated as ^{35}S (cpm/ug DNA) divided by unstimulated secretory ^{35}S (cpm/ug DNA) (1). For assessment of non-specific trapping of radioactivity, unlabeled medium samples from parallel wells were spiked with 1 uCi of [^{35}S]methionine and treated identically to biosynthetically labeled samples. Non-specific trapping of radioactivity in the precipitate was less than 1% of total counts for each point.

Normal rat acini were prepared as previously described (16), and their proteins were biosynthetically labeled essentially as described above for AR42J cells.

Immunoprecipitation of [^{35}S]methionine labeled proteins from AR42J and normal rat acinar cell lysates was carried out as previously described (11,16). Briefly, antisera (20-30 ul) were incubated with protein A (S.aureus) for 3 hours at 4^{o}, then combined with cell lysates (50-75 ul containing 0.75 ug DNA) and incubated for 18 hours at 4^{o}, followed by centrifugation and washing of the pellet. The adsorbed immune complexes were dissociated by boiling for 3 minutes in

2% SDS, 0.1 M dithiothreitol, 10 mM sodium phospate, and 0.01% bromophenol blue.

SDS PAGE: Electrophoresis of the reduced proteins was carried out as previously described (11) in 10% polyacrylamide slab gels (12.5 X 12.5 X 1.5 mm). Proteins in medium samples (40 ul out of 500 ul/16 mm well) and cell lysates (10 ul out of 500 ul/well) were denatured and reduced by boiling for 3 minutes in the same solution used for preparation of immunoprecipitates. In initial studies, molecular weights were determined through the use of a cocktail of molecular weight markers (BioRad) containing purified proteins of 92.5, 66.2, 45, 31, 21.5, and 14.4 kilodaltons, followed by staining with Coomassie Blue. The use of pre-stained markers yielded identical results in subsequent experiments. For quantification of proteins, autoradiograms were scanned with a densitometer (Hoefer GS300) attached to a chart recorder. The peaks from the tracings were cut out and weighed on a balance. Generally, a given gel was exposed to film for a short time (6-12 hours) for comparison of amylase bands, and for a longer time (20-72 hours) for densitometry of lighter bands. This was necessary to ensure that autoradiogram density was proportional to radioactivity.

DNA assay: AR42J cell lysates were assayed for DNA content on the basis of quantitative binding of DNA to Hoechst dye 33258 as previously described (3,4, and see Chapter 3).

RESULTS

In order to assess the degree of similarity between AR42J cells and normal rat acini, their patterns of protein synthesis and secretion were compared (Fig.2.1). Cells and acini were labeled with [^{35}S]methionine for 60 minutes; labeled cells were then washed, collected, lysed and electrophoresed on polyacrylamide gels. During the one-hour labeling period, rat acini synthesized proteins corresponding to 8 major and 2 minor bands (Fig.2.1) After radioactive medium was removed, and followed by a 30 minute chase period, 10 nM CCK was added with fresh medium. All 10 of the acinar protein species then appeared in the medium over the next 2.5 hours. This result is in agreement with reports that synthesis of secretory products comprises 90% of total protein biosynthesis in normal acinar pancreas (18). Thus, development of the autoradiographic film after only a few hours of exposure revealed label exclusively in secretory proteins. When AR42J cells were similarly studied, many protein bands were observed after the 60 minute labeling period. However, from 30 - 90 minutes of secretion, only 4 of these appeared as major secetory products. These proteins had apparent M_r values of 68,000, 53,000, 45,000, and 29,000. The M_r=53,000 band is similar to the amylase band which was previously reported to be synthesized and secreted by AR42J cells (9,11,12). In the present work, three additional major

secreted protein bands were revealed. These four proteins migrated on a 10% polyacrylamide gel to a similar level as secreted proteins from normal rat isolated acini (Fig.2.1, compare four proteins marked on the right, AR42J/M and rat/M).

To determine whether these 4 secretory proteins were similar to proteins secreted by normal rat acini, immunoprecipitation of cellular lysates was carried out with antisera raised against normal rat secretory proteins (Fig.2.2). Lane B shows the protein pattern from AR42J cells immunoprecipitated by a normal rat acini total-secretory protein antiserum. Four proteins were immunoprecipitated which corresponded to the four proteins secreted by AR42J cells. To further establish the identity of these proteins, antisera raised against specific pancreatic secretory proteins were used. The protein band at M_r=53,000 was precipitated by anti-amylase (Fig.2.2, lane C) as previously reported (11). The AR42J protein at M_r=45,000 was identified as procarboxypeptidase A (lane G). The AR42J protein band at M_r=29,000 was mainly composed of chymotrypsinogen (lane E); however, a small component of this band was immunoprecipitated by anti-elastase (lane F). This may reflect antigenic similarity between chymotrypsinogen and elastase, or may be due to trace amounts of chymotrypsin which were present in the original elastase antigen preparation. There was no specific immunoprecipitation from AR42J cells by anti-rat anionic trypsinogen (lane D), anti-

bovine trypsin type 1 (not shown), or by anti-procarboxypeptidase B (lane H). The light band at M_r=53,000 in lanes A, D, E, and H represents non-specific precipitation of amylase which occasionally occurred due to the abundance of radiolabeled amylase present in cell lysates.

Since CCK stimulates the secretion of several digestive enzymes from normal rat acini, and CCK receptors are present on AR42J cells, CCK regulation of secretion from AR42J cells was studied. The time course of unstimulated secretion of radiolabeled proteins from AR42J cells was first established. After a 60 minute pulse of [^{35}S]methionine, the greatest labeled protein secretion occurred during the first 30 minutes of chase, followed by a gradual decrease over a four hour period (not shown). Moreover, the secretion of some proteins became undetectable after 2 hours whether or not CCK was added. Therefore, in order to study regulated secretion, the following protocol was chosen: after a 60 minute labeling period, the cells were placed in unlabeled medium for 30 minutes; this medium was collected and replaced with fresh medium with or without 10 nM CCK. After a further 60 minutes, the medium was again collected. Medium samples were analyzed on 10% polyacrylamide gels (Fig.2.3) and protein bands were quantified by densitometry of autoradiographs (Table 2.1). In addition to the four major secreted bands, there were several minor bands that appeared in the medium mainly during the first 30 minutes of secretion. From 30 - 90 minutes of secretion, CCK stimulated the secretion of all

4 major proteins, plus several minor bands. When data was averaged over 4 experiments, it was found that CCK increased the secretion of the 4 major proteins to 200 - 350 % of control (Table 2.1).

It was previously shown that treatment with glucocorticoid hormones increased amylase synthesis in AR42J cells (11). In order to determine whether glucocorticoids affect the biosynthesis of other secretory protein products, or whether the effect is specific to amylase, AR42J cells were maintained in 10 nM dexamethasone (DEX) for 48 hours prior to a 15 minute pulse-label with [^{35}S]methionine. This short pulse protocol allowed detection of the same 4 major protein bands that were detected with a 60 minute labeling period. DEX treatment inhibits AR42J cell proliferation (4,11) thus the labeled cell lysates were normalized to DNA content before immunoprecipitation with the anti-rat acinar secretion antiserum (Fig.2.4). As shown in Fig.2.4, all four secretory proteins were increased by DEX treatment. The following increases in biosynthesis were demonstrated by densitometry: amylase, 214 $\pm$ 42 %; procarboxypeptidase A, 254 $\pm$ 55 %; chymotrypsinogen, 237 $\pm$ 44 %; M_r=68,000 protein, 215 $\pm$ 17 %, of control (means $\pm$ S.E. of 3 experiments).

To determine whether the regulatable secretory protein pool is affected by DEX, cells were pretreated with DEX for 48 hours followed by [^{35}S]methionine labeling. The effect of CCK on secretion of labeled TCA precipitated proteins into medium was assessed at various times after incubation in

fresh medium (Table 2.2). Unstimulated secretion from DEX treated cells was approximately 350 % of unstimulated secretion from control cells. However, CCK stimulated protein secretion was 210 ± 17 % of unstimulated secretion in both control and DEX-treated cells at all time points. This corresponds to a sorting ratio of 2.1, as defined by Arvan and Castle (1). It was concluded that the proportion of newly synthesized secretory protein directed to the regulatable pool was not changed by DEX.

DISCUSSION

AR42J cells were found to be an interesting and appropriate *in vitro* model for the exocrine pancreas. Four major AR42J cell secretory protein bands were identified which are immunoreactive in a manner similar to normal rat acinar secreted proteins (Fig.2.2). Amylase, procarboxypeptidase A, and chymotrypsinogen were identified by immunoprecipitation with corresponding specific antisera against these pancreatic enzymes. The M_r=68,000 protein secreted by AR42J cells is immunoreactively similar to the rat secreted protein of similar size, the activity of which is unknown (2,17,18). This similarity was demonstrated by immunoprecipitation with anti-rat acinar total secretory protein anti-serum. In order to identify possible lower molecular weight proteins, AR42J cell secretion samples were also run on 9.5 - 15 % gradient polyacrylamide gels. No major protein bands were detected below M_r=29,000.

Previous studies showed that AR42J cells released amylase in response to CCK and bombesin (9,13). Here it is shown that AR42J cells secrete several proteins in addition to amylase. The secretion of four major proteins from AR42J cells is stimulated in parallel by CCK. AR42J cells do not express all digestive enzymes to as high a level as normal acinar cells. However, some of the same pathways for activation of secretion appear to be present. AR42J cells possess receptors for many normal pancreatic regulators including CCK, gastrin-related-peptide (bombesin), somatostatin, Substance P, VIP, and insulin (9,13,14,20,21). Moreover, Substance P activates phosphoinositide turnover (6) and CCK and Substance P increase intracellular free calcium in AR42J cells (23, unpublished data).

It was previously reported that glucocorticoid treatment increased amylase mRNA and amylase protein biosynthesis in AR42J cells, and that this increase is dependent on prior protein synthesis - a secondary steroid effect (11,12). Here I report that DEX treatment for 48 hours also increases the biosynthesis of procarboxypeptidase A, chymotrypsinogen, and M_r=68,000 protein. While the magnitude of increase in amylase protein biosynthesis was not as great as that found in previous studies, it is significant that the biosynthesis of the proteins studied here increased in parallel and to a similar extent. This result suggests the existence of a single initial glucocorticoid response which leads to

increased synthesis of several pancreatic acinar-specific products.

In previous studies it was found that a maximal dose of CCK (10nM) stimulated secretion of amylase from AR42J cells in direct proportion to total amylase content (9). It is postulated that protein whose secretion is to be regulated by secretogogue must first be "sorted" into a regulatable intracellular compartment. Avan and Castle quantified secretory response by a simple calculation of sorting ratio: the amount of protein secreted under stimulation divided by the amount secreted without stimulation (1). Using this calculation, for both DEX and non-DEX treated cells, CCK stimulated amylase release with a sorting ratio of 2.6. Here I show that secretion of total secretory proteins labeled with [^{35}S]methionine is stimulated by CCK with a sorting ratio of 2.1. Thus, AR42J cells possess a regulated secretory pathway that is readily testable in a short experimental protocol comparable to that which has been utilized by Moore and Kelly to determine sorting index (15). [The term sorting index, however, refers to a calculation performed on data collected from a protocol (15) that is different and more complex than the protocols used for assessing sorting ratio (1).] Moreover, the equivalence of sorting ratio in control and DEX treated AR42J cells suggests that the action of glucocorticoids in this cell is not to switch differentiation-dependent proteins from a constitutive to a regulated pathway. Rather, these results suggest the

existence of a glucocorticoid regulated trafficking activity (8) which is induced in coordination with differentiation-specific acinar secretory proteins, and which mediates the sorting of those proteins into a regulated secretory pathway.

Protein sorting in AR42J cells is imperfect in comparison with normal acini. CCK stimulates amylase secretion from acutely isolated rat acini with a sorting ratio of 10 or greater while pulse-labeled total protein is secreted with a sorting ratio of 4.2 (ref.1 and our unpublished observation). These results can be attributed to a low level of basal (constitutive) secretion from normal acini which reflects efficient storage of digestive enzymes (1). Immortal cell lines which synthesize secretory proteins generally display a high level of "leakage" of a protein product whose secretion in vivo is highly regulated (15). While the AR42J cell line also secretes protein at a high basal level, secretion is readily stimulated by CCK. CCK stimulation acts on AR42J cells to increase intracellular free calcium (our unpublished observation), probably via the breakdown of phosphatidyinositol 4,5-bisphosphate (PIP_2) (6). Thus, the AR42J cell line may prove useful in the molecular characterization of secretory signaling and sorting mechanisms.

Summary

In this study I have shown that glucocorticoid treatment of AR42J cells increased the synthesis of not only amylase,

but three other differentiation-dependent enzymes. This enzyme response mirrors the response of fetal acinar cells at key stages of development (24). Moreover, under certain conditions, inhibition of acinar cell multiplication accompanies glucocorticoid induced differentiation in acini(24). Thus AR42J cells were chosen for investigation of the dual regulation of cellular differentiation and proliferation by glucorticoids, which is the subject of Chapter 3.

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Table 2.1

CCK regulation of protein secretion in AR42J cells.

	% control
amylase	226 ± 20
procarboxypeptidase A	320 ± 47
chymotrypsinogen	351 ± 67
unidentified M_r=68,000 protein	216 ± 28

Secretory proteins were biosynthetically labeled, collected, and electrophoresed as described in Fig.2.3. The relative amounts of individual proteins in each lane were assessed by densitometric scans of autoradiographs. The effect of CCK on secretion of proteins during the 30 to 90 minutes after labeling was calculated as percent control, or lane B/lane A (Fig.2.3). Results shown are the means ± S.E. of 4 experiments.

TABLE 2.2

Sorting of secretory protein into a CCK-regulatable pool.

^{35}S (cpm/ug DNA)

	no DEX		plus DEX	
	-CCK	+CCK	-CCK	+CCK
minutes after labeling				
60-100	246 ± 27	581 ± 90	809 ± 121	1707 ± 205
120-160	200 ± 16	384 ± 54	685 ± 62	1541 ± 224
240-280	59 ± 7	110 ± 14	216 ± 24	454 ± 210

After 48 hours with or without 10 nM DEX, cells were incubated for 15 minutes in [^{35}S]methionine, washed, and incubated in fresh unlabeled medium for 60, 120 or 240 minutes. Medium was then replaced with fresh medium with or without 10 nM CCK. After a further 40 minutes, medium was collected and labeled proteins were TCA precipitated and counted for radioactivity. Results are expressed as radiolabeled protein secreted (cpm) divided by ug DNA contained by cells in the well. Values are the means ± S.D. of 4 wells from a representative experiment out of 3 experiments.

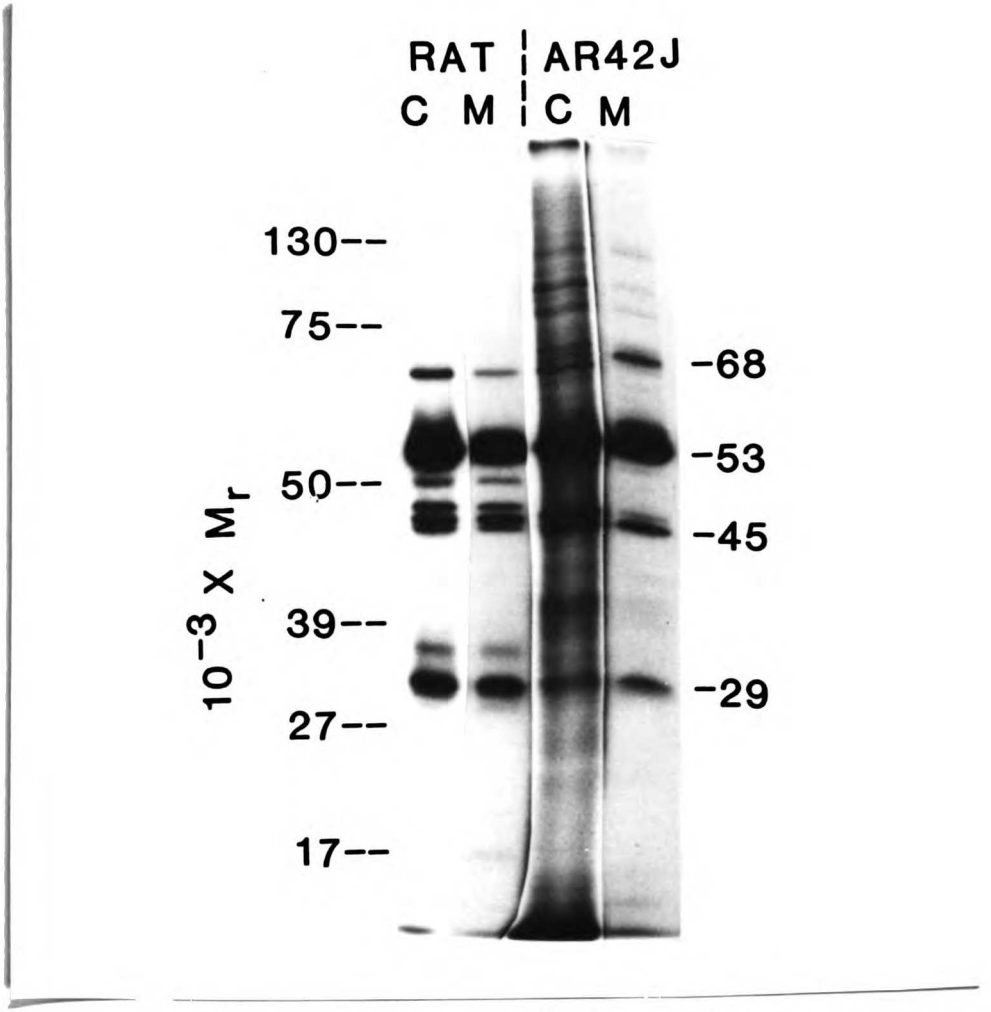

Fig.2.1. Biosynthetically labeled proteins in AR42J cells compared with normal rat acini analyzed by SDS-polyacrylamide gel electrophoresis. Rat,C: rat acini, total cell lysate after 60 minutes labeling time in [^{35}S]methionine. Rat,M: Proteins secreted by rat acini over subsequent 2.5 hours in the presence of 1nM CCK. AR42J,C: AR42J total cell lysate after 60 minutes labeling time. AR42J,M: Proteins secreted by AR42J cells during 60 minutes (after 60 minutes labeling and 30 minutes chase). Numbers on the left indicate the positions of molecular weight markers. Numbers on the right represent the estimated M_r of each of the 4 major secreted protein bands of AR42J cells.

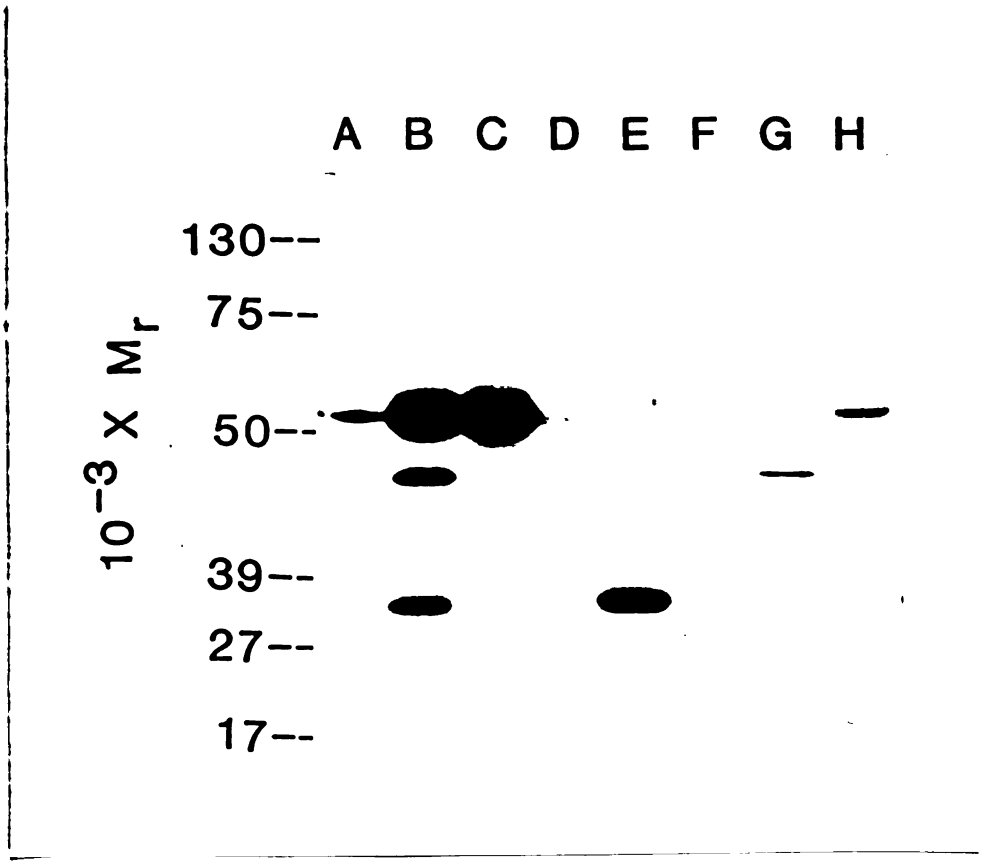

Fig.2.2. Identification of AR42J cell secretory proteins by immunoprecipitation. AR42J cells were incubated for 60 minutes in [^{35}S]methionine, lysed, immunoprecipitated, and the precipitated cell products were subjected to SDS-PAGE. The following antisera were used: Lane A: normal rabbit serum. B: Antiserum from rabbits that had been immunized with the combined secretory products of isolated rat acini. C: Anti-amylase. D: anti-anionic trypsinogen. E: anti-chymotrypsinogen. F: anti-elastase. G: anti-procarboxypeptidase A. H: anti-procarboxypeptidase B.

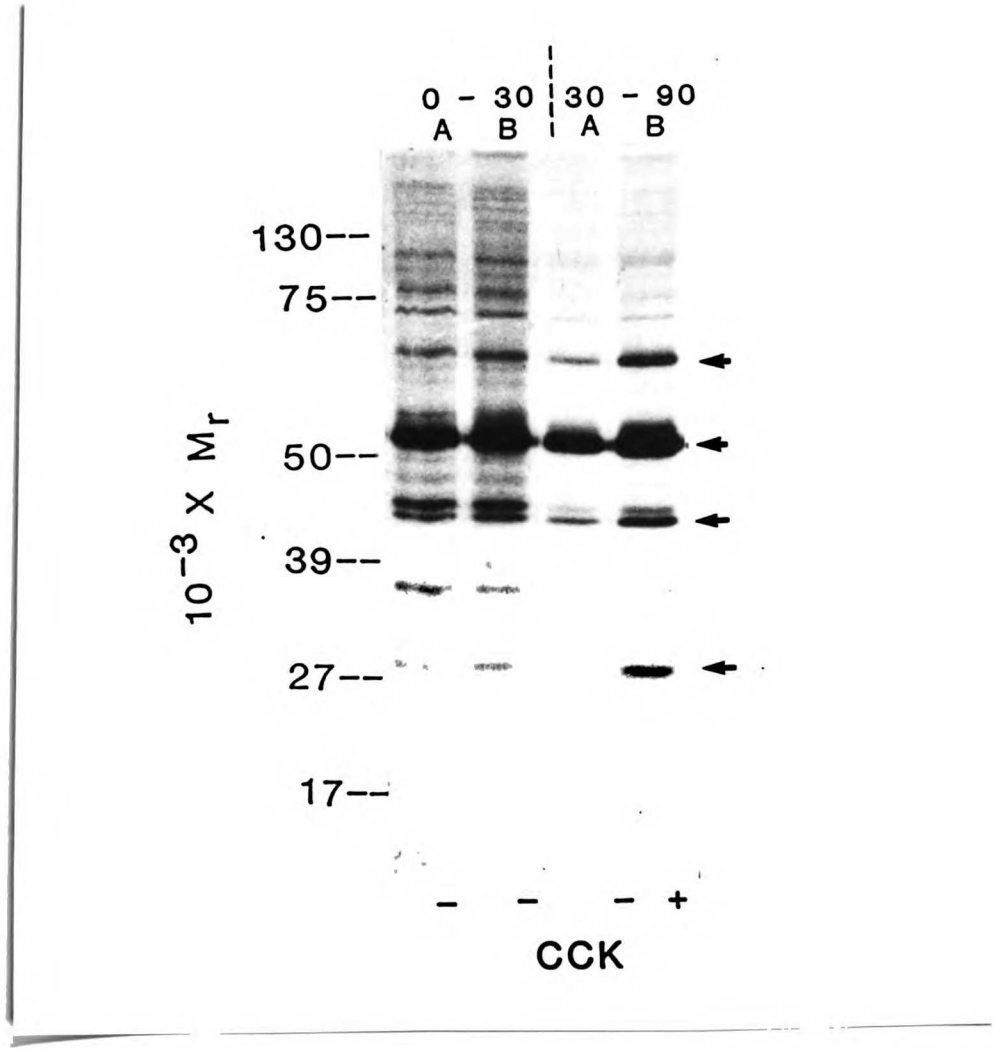

Fig. 2.3. Protein secretion in AR42J cells, regulation by CCK. Proteins were first biosynthetically labeled with [^{35}S]methionine for 60 minutes. After 30 minutes of incubation in unlabeled medium (chase, 0-30, lanes A,B), medium was collected from wells A and B . Fresh chase medium was added with or without 10 nM CCK and collected after a further 60 minute incubation (30-90, lanes A,B). For all lanes, 40 ul out of 500 ul total medium sample was loaded. The proteins in the medium samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Arrows indicate the four major secretory proteins.

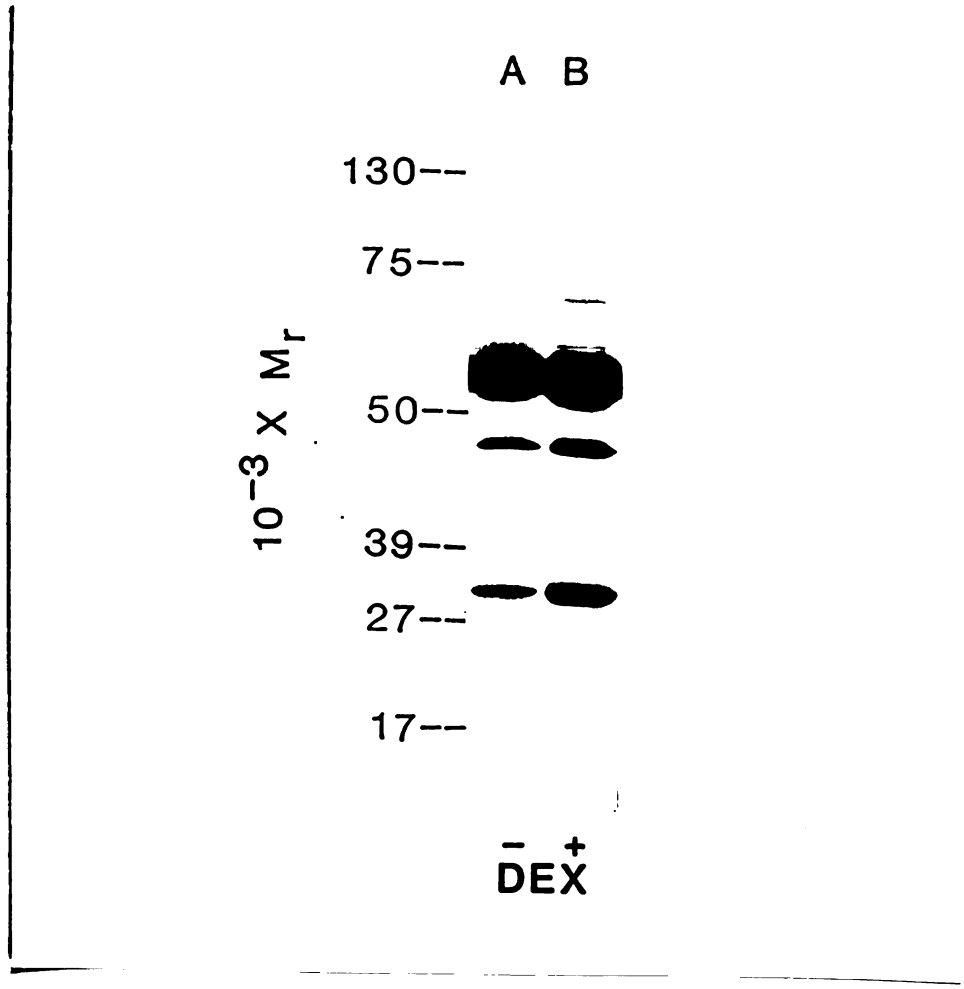

Fig.2.4. Effect of DEX on protein biosynthesis in AR42J cells. After 48 hours with or without 10 nM DEX, cells were incubated for 15 minutes in [^{35}S]methionine, lysed, and subjected to immunoprecipitation with antiserum from rabbits that had been immunized with the combined secretory products of rat acini (see Fig.2.2, lane B). The sample volume used for immunoprecipitation was normalized to 0.75 ug DNA for both lanes (A: 50 ul; B: 75 ul out of 500 ul total cell lysate per well).

CHAPTER 3: INDEPENDENCE OF DEXAMETHASONE-INDUCED DIFFERENTIATION AND GROWTH INHIBITION IN PANCREATIC ACINAR AR42J CELLS.

Growth and differentiation are mutually exclusive events in a number of tissues and cultured cell systems [1-8]. Growth inhibition *in vitro* induced by confluency [2-5] or by serum starvation [6,7] often leads to alterations in patterns of protein biosynthesis and morphological changes characteristic of the differentiated state. In many cases hormonal treatments which inhibit growth also induce differentiation [4,5], and these effects are often reversible [6].

The acinar pancreas is known to be dependent on the presence of glucocorticoids for normal amylase synthesis [9-11]. It has previously been reported that glucocorticoid treatment of a rat pancreatic acinar cell line, AR42J, induces differentiation while inhibiting growth [12]. Treatment with 10nM dexamethasone (DEX) for 48 hours induced an increase in the following differentiated features: amylase gene transcription, rough endoplasmic reticulum, secretory granules, and CCK binding [12-14]. It is unknown, however, whether the induction of differentiated features is linked to growth inhibition.

In the present work, the time and concentration dependence and steroid specificity of DEX induced growth inhibition were characterized and found to parallel induction of amylase. However, inhibition of growth by two alternate methods, hydroxyurea treatment or serum starvation, did not induce differentiation of AR42J cells, as assessed by amylase content and CCK binding. Moreover, reversal of growth inhibition with

EGF or insulin did not prevent glucocorticoid induction of amylase synthesis in AR42J cultures. Thus, in AR42J cells, growth and differentiation are not mutually exclusive and can be independently regulated.

MATERIALS AND METHODS

Cell Culture: AR42J cells were obtained from the American Type Culture Collection (Rockville, MD) in the 19th passage and divided at a ratio of 1:4 every 4 to 7 days through the 80th passage. The cells were maintained in Dulbecco's modified Eagle's medium H-21 containing penstrep, fungizone, added glutamine, and 10% fetal bovine serum (Hyclone and Gibco). Cells were detached by treatment with Trypsin-EDTA (Gibco) and, except where indicated, plated at 300,000 cells per 16 mm-well in microcluster plates. In experiments for Fig. 1, cells were plated at 5 x 10^6 cells per 35 mm-well. Plating efficiency was greater than 98% at both densities, as assessed by recovery of DNA after one day in culture. The cells were cultured for 24 hours in the same medium before the beginning of each experiment (hour 0) at which time cells were collected for assays. Remaining wells received the indicated doses of hormone(s) or changes of medium. Medium was also changed in control wells at hour 0. For serum starvation, the medium contained 10% chemical serum substitute which was purchased from the Cell Culture Facility at the University of California, San Francisco. When a peptide hormone was used, additional peptide was added every 48 hours.

Assays: For measurement of amylase and DNA content, cells were collected with a rubber policeman in 400 ul of PBS and sonicated with a probe type sonicator for 10 seconds. Amylase activity was assayed in aliquots of the sonicated homogenate by the method of Jung [15] which is based on procion yellow dye coupled to starch as substrate. Standards were prepared using amylase Type VI-A from porcine pancreas with an a-amylase activity of 10 U/mg (Sigma). DNA was measured in an assay based on the quantitative binding of DNA to Hoechst Dye 33258 [16]. Briefly, aliquots of 25-200 ul of cell sonicate were mixed with 10 ul of 1% SDS, then incubated with 2 ml of 10^{-6} M Hoechst dye for 10 minutes. Standards were prepared using calf thymus DNA type I (Sigma). Fluorometer readings were taken at excitation wavelength 360nm, emission wavelength 450nm.

DNA synthesis: Newly synthesized DNA was measured by the incorporation of (^{3}H)-thymidine into TCA precipitated material. AR42J cells were incubated in 1 ml of the designated culture medium containing 1 uCi (methyl-^{3}H)-thymidine (20 Ci/mmol, New England Nuclear). The specific activity of serum substituted medium was adjusted to that of medium containing defined fetal bovine serum by adding unlabeled thymidine. Parallel wells were used for DNA and amylase determination. The cells were incubated for one hour at 37o in a 5% CO_2

incubator. The cells were then rinsed once with ice-cold PBS, collected with a rubber policeman into 500 ul PBS containing 0.01% BSA as carrier, sonicated for 10 seconds and precipitated with 10% TCA (final concentration). The washed precipitate was dissolved in 0.5 ml of 0.1 M NaOH, then added to 5 ml Liquiscint and its radioactivity determined. Incorporation of (^3H)-thymidine was expressed as counts per minute per ug DNA.

^{125}I-CCK binding: Porcine CCK-33 (Gastrointestinal Hormone Laboratory, Karolinska Institute, Stockholm, Sweden) was iodinated by conjugation with ^{125}I-labeled Bolton-Hunter reagent as described [17] and subsequently purified by HPLC to a specific activity of 2000 Ci/mmole (3400 cpm/fmole at a counting efficiency of 70%). CCK receptor binding was assessed as previously described [14]. Briefly, to each 16 mm well was added 1 ml of Hepes-Ringer solution enriched with essential amino acids, 0.5% (w/v)BSA, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, and 10 pM ^{125}I-CCK-33. After a 1 hour incubation at 23o, the cells were quickly rinsed twice with PBS at 4o, then collected into 0.5 ml PBS and counted for gamma radiation. Non-specific binding was determined in the presence of an excess of unlabeled hormone (100 nM CCK-8, Bachem Inc., Torrence, CA). Non-specific binding was generally less than 10% of total binding. DNA content was determined as described above in parallel wells without radioactivity. Results were expressed as fmoles CCK specifically bound/mg DNA.

Statistical analyses: Statistical analyses were carried out using Student's t-test or, when appropriate, one-way analysis of variance. Tests were run on a statistical computer program (CRISP) on an IBM PC. Tests were performed on raw data before conversion to %control values.

RESULTS

GROWTH INHIBITION AND INDUCTION OF DIFFERENTIATION BY DEXAMETHASONE

In order to establish the normal rate of growth and the effects of DEX on growth, AR42J cell cultures were followed over 144 hours (Fig.3.1). Between 4 and 12 hours there was a relatively rapid increase in DNA content consistent with partial synchronization by routine plating. At 24 hours there was no significant difference in DNA content between control and DEX-treated cultures. Between 48 and 144 hours, the control cultures proliferated at a rate consistent with a generation time of 34 hours, calculated according to the formula $n=n_o 2^{t/Tg}$, where n_o=ug DNA at 48 hours, n=ug DNA at 144 hours, t=96 hours, Tg=34 hours [18]. In contrast, the greatly inhibited growth of the DEX-treated cultures between 48 - 144 hours was consistent with a generation time of 850 hours. Thus, after an initial period of DNA synthesis, DEX inhibited growth by over 95 %.

Amylase content is high in fully differentiated acinar

cells and reduced in fetal [9] or dedifferentiated cells [12]. Therefore, amylase content was measured as an indicator of differentiation. As previously reported [12], control cultures showed no change in amylase/DNA, whereas DEX treated cultures showed an increase in amylase/DNA ratio which was maximal by 72 hours with an 11- fold increase (Fig.3.1b).

As an additional indicator of differentiation in AR42J cells, CCK binding was assessed. Assay conditions were based on previous studies which established the preparation of radioiodinated CCK-33 and its binding characteristics to AR42J cells [14,17]. As previously reported [14], CCK binding increased 2 - 3 fold with DEX treatment for 48 hours (Table 3.1).

In order to determine the duration of DEX treatment necessary for inhibition of DNA synthesis, (^{3}H)-thymidine incorporation was measured for 1 hour after variable durations of exposure to DEX (Fig.3.2). Hour 24 was chosen as a representative time to sample DNA synthesis in the cultures after the earlier period of partial synchronicity (Fig.3.1a). DNA synthesis was significantly reduced (83 $\pm$ 1% of control, $P<0.03$) after 6 hours of prior DEX treatment (DEX added at hour 18). Half-maximal inhibition occurred after DEX had been added at hour 12; maximal inhibiton occurred after 18 hours of DEX treatment (9.0 + 1.0 % of control).

To establish the dose-dependency of the DEX effects, AR42J cells were cultured in the presence of various concentrations of DEX, and DNA synthesis and amylase content were then measured.

Inhibition of DNA synthesis was half-maximal at 0.5nM and maximal at 10nM DEX (Fig.3.3). The doses which achieved maximal and half-maximal efficacy in amylase content were similar to the doses for inhibition of DNA synthesis (Fig.3.3). The effectiveness of various steroids in inhibiting DNA synthesis were then examined (Fig.3.4). Corticosterone was next in potency to DEX (half-maximal effect at 200nM) followed by aldosterone (600nM); both hormones achieved maximal inhibition at 1uM. Neither progesterone or estradiol significantly inhibited DNA synthesis. These results were consistent with specific action on the glucocorticoid receptor and confirmed a previous report on steroid specific effects on amylase content [12]. Thus, growth inhibition and differentiation induction responses were parallel in dose-response and steroid specificity.

INHIBITION OF GROWTH WITHOUT INDUCTION OF DIFFERENTIATION

In some cell systems, inhibition of growth alone is sufficient to induce differentiation [2-4]. To test whether inhibition of growth would induce differentiation in AR42J cells, growth was inhibited by two different means, treatment with hydroxyurea or serum starvation. Serum starvation inhibits growth by removing serum growth factors. Hydroxyurea treatment inhibits DNA synthesis, and thus inhibits cell growth without affecting RNA or protein synthesis [19].

Both hydroxyurea and serum starvation inhibited (^{3}H)-thymidine incorporation in AR42J cells to a similar extent as DEX treatment (Fig.3.5). In contrast, neither hydroxyurea (Fig.3.6a) or serum starvation (Fig.3.6b) induced an increase in amylase content. Furthermore, DEX increased amylase content in the presence of hydroxyurea (Fig.3.6a) or serum starvation (Fig.3.6b), indicating that these growth inhibitory treatments did not have deleterious effects on the cells' ability to synthesize protein.

As a further measure of differentiation, CCK binding was assessed (Table 3.1). Treatment with hydroxyurea or serum starvation did not increase CCK binding, while DEX caused a 2 -3 fold increase in the presence or absence of hydroxyurea or serum starvation. Thus, inhibition of growth, per se, did not induce differentiation of AR42J cells as assessed by two criteria: amylase content and CCK binding. Under growth arresting conditions, however, the cells were still capable of differentiating when treated with DEX.

REVERSAL OF GROWTH INHIBITION: CONCOMITANT DEX-INDUCED DIFFERENTIATION

Since growth inhibition was not sufficient to induce differentiation, I wished to determine whether growth inhibition was necessary for differentiation. This question was addressed by treatment of AR42J cells with EGF and with insulin, both of which stimulate proliferation (Table 3.2).

In the presence of a sub-maximal (1nM) dose of DEX, EGF treatment reversed the DEX-induced growth inhibition (Table 3.2). Moreover, the increment in DNA increase was equivalent between the two conditions (EGF increase over control = 3.4 $\pm$ 1.4 ug DNA; EGF plus DEX increase over DEX alone = 3.5 $\pm$ 0.4 ug DNA; $P>0.05$). At 72 hours of DEX and EGF co-treatment, DNA synthesis was equivalent to control. In the presence of EGF, DEX increased amylase content to the same extent as in the growth inhibited state (Table 3.2). To assess whether AR42J cells could continue to proliferate while retaining their differentiation, cultures were maintained and divided every 5 days in the presence of 1nM DEX and 1nM EGF; after 3 weeks, their amylase/DNA ratio remained elevated to 684 $\pm$ 3% of control, equivalent to that achieved by 1nM DEX alone (695 $\pm$ 12 % of control).

It has previously been shown that insulin stimulates growth in AR42J cells [20]. In the present study, 1uM insulin treatment for 72 hours increased DNA content to approximately 200 % of control (Table 3.2). In the presence of 1nM DEX, insulin also increased DNA content to 200 % of cultures treated with DEX alone (Table 3.2). Most importantly, in cultures which received this combination of DEX plus insulin, the DNA content and DNA synthesis was similar to that seen in untreated controls. The amylase/DNA ratio in the cultures treated with 1uM insulin plus 1nM DEX was equal to that in cultures treated only with 1nM DEX.

DISCUSSION

This study was initiated in order to elucidate the relationship between the two major effects of DEX on AR42J cells, inhibition of growth and increased differentiation. We found that DEX inhibition of AR42J cell DNA synthesis and DEX stimulation of AR42J cell differentiation share several characteristics.

Both the stimulatory effects of DEX on AR42J cell amylase gene expression and the inhibitory effects of DEX on AR42J cell DNA synthesis require prior treatment of 6 - 12 hours before an effect is seen. This delayed time of action is consistent with a secondary steroid effect which requires prior glucocorticoid induction of an undetermined regulatory protein [13, 22-24]. Further evidence in support of a secondary effect of DEX on amylase mRNA is that the DEX effect requires protein synthesis [13]. Whether DEX inhibition of DNA synthesis likewise requires protein synthesis is unknown. Syms, et al. [25,26] reported evidence for glucocorticoid induced growth repressor proteins in a ductus deferens smooth muscle tumor cell line (DDT_{1MF-2}). In their system, inhibition of protein synthesis for 6 hours with cycloheximide reversed the glucocorticoid inhibition of entry into S-phase. In AR42J cells, however, cycloheximide or puromycin alone profoundly inhibited DNA synthesis (not shown). Thus we could not use this approach to determine whether

inhibition of growth requires protein synthesis. The time delay in inhibition of DNA synthesis could also be accounted for by a primary mechanism. If AR42J cells produce a growth stimulatory factor, and if DEX acts by inhibiting the production of this factor, then the 6 hour lag period could represent the delay in disappearance of the putative protein.

Another common feature of both the ability of DEX to inhibit cell growth and to increase cell differentiation is sensitivity to DEX. AR42J cells respond at lower doses than have been reported for most other cell types (ED_{50}=5-20nM, refs.27-30). For inhibition of DNA synthesis the half-maximal effective dose in AR42J cells was 0.5 nM. This is similar to the half-maximal doses of 1 and 2nM for increased amylase gene expression [13] and for increased CCK binding [14], respectively. The analog specificity displayed by AR42J cells for both inhibition of DNA synthesis and stimulation of differentiation are also similar. These data suggest that these effects are mediated by the Type II glucocorticoid receptor [27]. The similarity in dose-response and steroid specificity of effects on DNA synthesis and effects on differentiation suggests that the early events involved in both induction of differentiation and inhibition of growth are mediated by the same adrenocorticoid receptor subtype.

In several cell types, inhibition of growth by confluency or serum starvation is sufficient to induce the process of differentiation [2-7]. The effect of confluency is not

testable in AR42J cells because they do not become confluent, but rather grow in clumps. When growth of AR42J cells was inhibited by hydroxyurea or serum starvation, growth inhibition alone was not sufficient to induce differentiation. However, the cells were able to differentiate under growth inhibited conditions when DEX was supplied. Therefore, AR42J cells require the specific action of glucocorticoids in order to increase synthesis of amylase and of CCK receptors.

Glucocorticoids are known to have profound effects on proliferation in many different tissues and cell systems. Inhibitory effects have been reported in variants of fibroblasts, hepatoma cells, and embryonic mesenchymal cells [31-36], while growth stimulation has been reported in a variant of 3T3 mouse fibroblast [37]. In addition, glucocorticoids have been reported to enhance or inhibit the action of peptide growth factors [38-40]. Conversely, exogenously applied growth factor has been reported to reverse glucocorticoid mediated growth inhibition [25]. In order to determine whether differentiation of AR42J cells could occur concomitantly with growth in a culture, cells were treated with either EGF or insulin plus sub-maximal DEX. While both EGF and insulin significantly reversed the DEX inhibition of DNA synthesis, the full effect of DEX on differentiation was achieved. It is inferred from the data in Table 3.2 that the same population of cells responded to both DEX and growth factor. The amylase/DNA ratio at 72 hours was equivalently high among all cultures which received DEX,

regardless of their proliferatory state. This strongly suggests that a given cell which proliferated under the influence of EGF or insulin was also induced by DEX to produce increased amylase at some point in the cell cycle. This view is strengthened by the evidence that DEX plus growth factor treated cells continued to proliferate while maintaining a high amylase/DNA ratio during 21 days of treatment. While concomitant DEX treatment reduced the stimulatory effect of peptide growth factor, it is most significant that proliferation occurred at a rate similar to that of untreated cells. Thus, the events involved in cell proliferation and induction of differentiation are not mutually exclusive. However, when the maximal dose of DEX (10nM) was used, neither peptide growth factor reversed growth inhibition (data not shown). This suggests that glucocorticoids act in AR42J cells on a molecular event that is essential for peptide growth factor stimulation of growth, and that the inhibitory influence of DEX prevails.

Summary

This study indicates that, in pancreatic acinar AR42J cells, DEX-induced growth inhibition and differentiation are mutually independent. The question remains whether there is a single regulatory molecule induced by DEX which is responsible for initiating molecular events leading to both inhibition of DNA synthesis and to induction of differentiation-dependent proteins. Further pursuit of this question may lead to the discovery of a key gene, the regulation of which switches AR42J cells from a state of

rapid proliferation to a growth inhibited, differentiated state appropriate to the pancreas.

If the mechanisms operating in AR42J cells are relevant to the normal acinar pancreas, then a different acinar cell line should share certain regulatory responses with the AR42J cell line. The transgenic mouse tumoral cell line - 266-6 - was next investigated as an alternative model for hormonal regulation of acinar pancreas (see Chapter 4).

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TABLE 3.1

CCK binding to AR42J cells in the presence or absence of DEX.

	fmoles CCK bound /mg DNA	
	control	DEX added
complete medium	13.8 ± 1.4	37.3 ± 2.7
complete medium plus hydroxyurea	14.1 ± 4	35.2 ± 4
serum starvation	12.9 ± 1.9	33.8 ± 8

CCK binding was assessed utilizing 10 pM ^{125}I-CCK-33 after 72 hours under the indicated serum conditions. Where indicated, 10nM DEX was added for the last 48 hours. Values given are for specific binding for one representative experiment from a total of 5 (means ± s.d., 4 wells each for total and non-specific binding determinations).

TABLE 3.2

Effects of peptide growth factors in combination with sub-maximal DEX on growth and amylase content.

	ug DNA/well	mU amylase/ug DNA	DNA synthesis (% control)
control	9.8 ± 1.4	9.9 ± 0.8	100 ± 12
insulin	19.8 ± 2.5 *	10.1 ± 2.0	191 ± 18 *
EGF	13.2 ± 0.6 *	9.5 ± 1.2	135 _ 17 *
DEX	5.3 ± 0.4 *	57.0 ± 2.0	36 ± 4 *
insulin plus DEX	12.3 ± 1.9 **	64.0 ± 4.0	108 ± 12 **
EGF plus DEX	8.8 ± 0.4 **	58.5 ± 2.0	89 ± 9 **

Twenty-four hours after plating, cells from 4 wells were collected for assay (2.3 ± 0.5 ug DNA/well). Cells were next cultured for 72 hours with insulin (1uM), or EGF (1nM), or DEX (1nM), or growth factor combined with DEX (same concentrations). [3H]thymidine incorporation was then performed, and separate wells were assayed for DNA and amylase content. DNA synthesis is expressed as % control, cpm/ug DNA. *P<0.03 compared to control; **P<0.03 compared to DEX treatment alone. Values shown are means ± s.e. for 5 experiments.

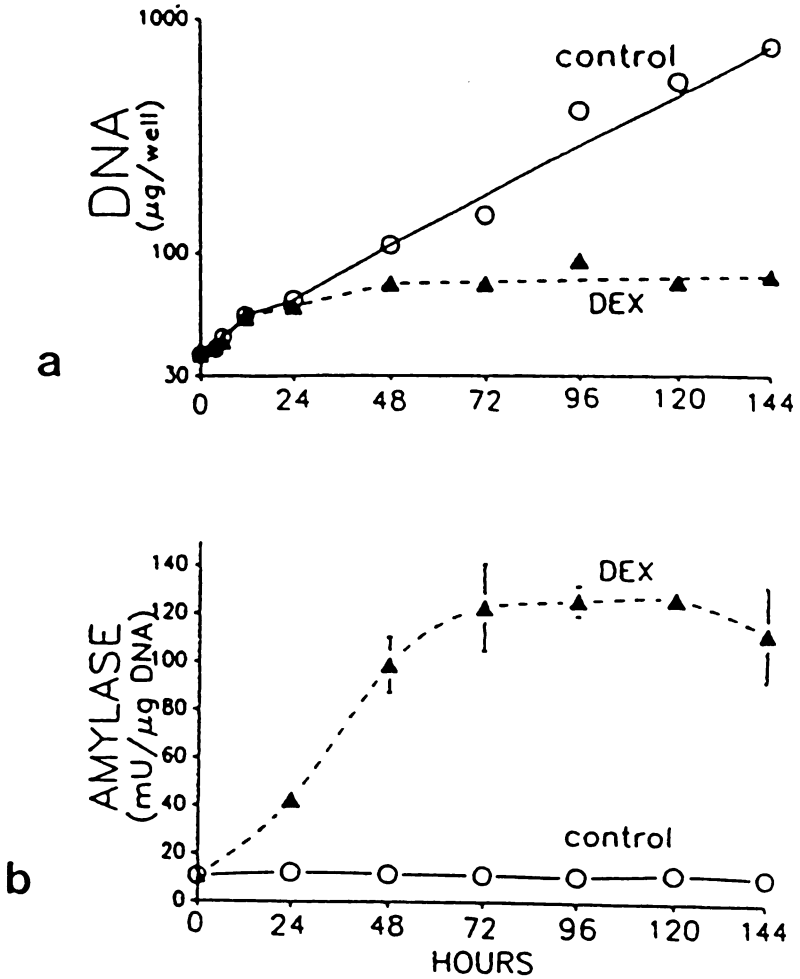

Fig. 3.1: Time course of DEX action on DNA and amylase content. Cells were plated at 5 x 10^6 cells per 35 mm cluster-type tissue culture well 24 hours before initiation of the experiment. Four wells were then collected for analysis (hour 0), the medium was changed and DEX added (10nM final concentration) where indicated. Medium was also changed at 72 hours. Cell viability as assessed by trypan blue exclusion was greater than 98% at each time point. a: Cell growth, expressed as ug DNA/well. b: Cellular amylase content, expressed as mU amylase/ug DNA. Each point represents the mean $\pm$ s.e. of 3 - 7 experiments. Where no error bar appears, the standard error is smaller than the data point mark.

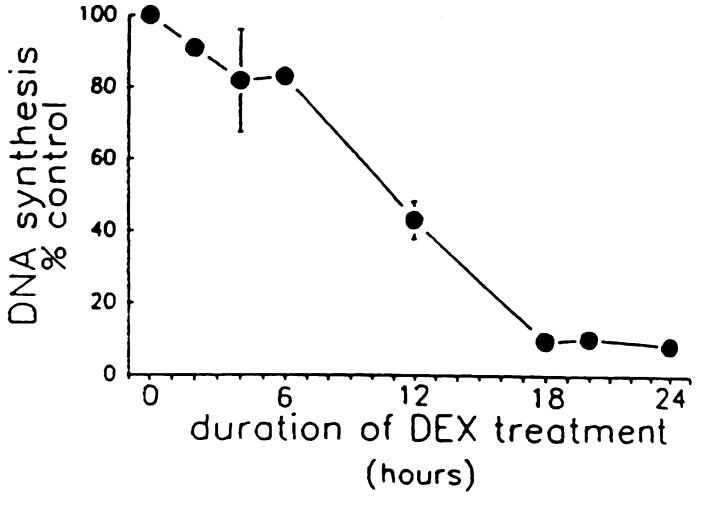

Fig. 3.2: Effect of DEX on DNA synthesis at hour 24. (^{3}H)-thymidine incorporation was assessed over 1 hour, from hour 23.5 to hour 24.5 after prior 10 nM DEX treatment for the indicated durations. (^{3}H)-thymidine incorporation is expressed as %control (cpm/ug DNA). The values for cpm/ug DNA ranged from 7500 (control) to 630 (24 hours DEX). Each point represents the mean $\pm$ s.e. of 3 - 7 experiments with 4 wells per point in each experiment.

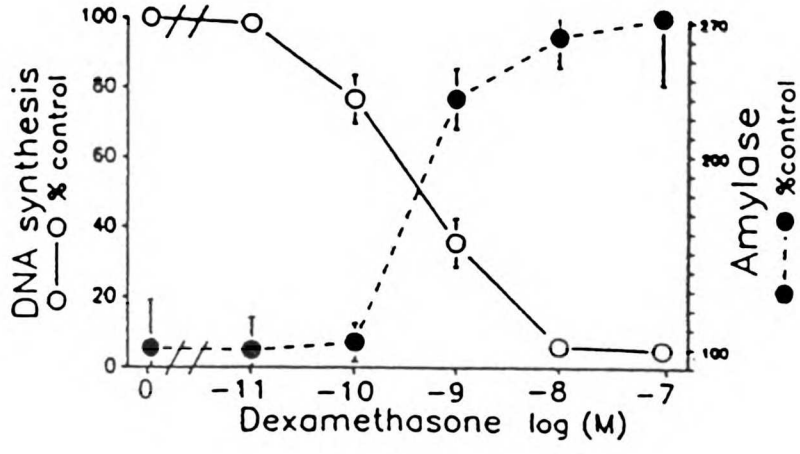

Fig. 3.3: Dependence of DNA synthesis and cellular amylase content on DEX concentration. (^{3}H)-thymidine incorporation over 1 hour and amylase at hour 24 were measured after treatment with DEX at the specified concentrations. Left axis:(^{3}H)-thymidine incorporation, %control(cpm/ug DNA). Values for cpm/ug DNA ranged from 7300 (control) to 700 (DEX at 100nM). Right axis: amylase content, %control (mU amylase/ug DNA). Values for mU amylase/ug DNA ranged from 9.7 (control) to 26.4. Each point represents the mean $\pm$ s.e. of 3 - 7 experiments with 4 wells per point in each experiment.

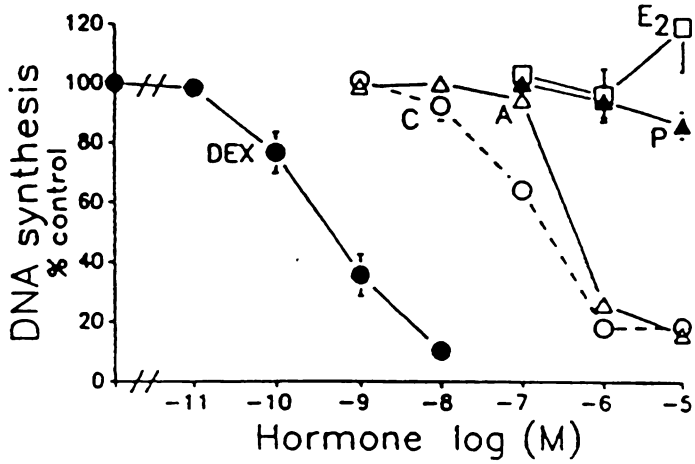

Fig. 3.4: Effect of various steroid hormones on DNA synthesis. Cells were maintained for 20 hours in the specified concentration of DEX, corticosterone (C), aldosterone (A), progesterone (P), or estradiol (E_2) prior to 1 hour of (^{3}H)-thymidine incorporation. Results are expressed as %control (cpm/ug DNA), means $\pm$ s.e. for 4 - 7 experiments.

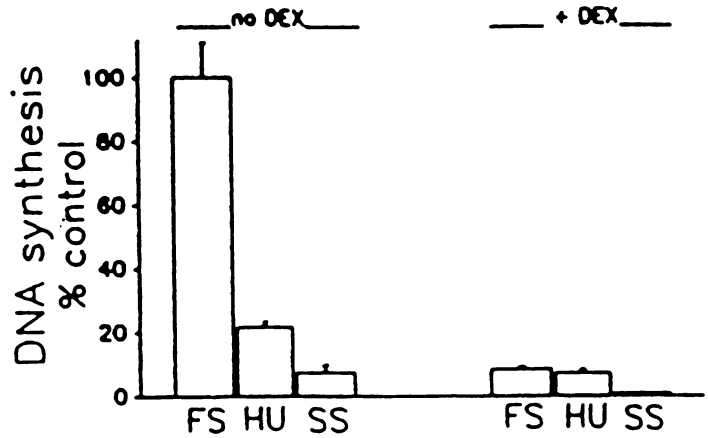

Fig. 3.5: Inhibition of DNA synthesis by hydroxyurea or serum starvation in the presence or absence of DEX. Twenty-four hours after plating, medium was changed to the following conditions, with or without 10nM DEX: complete medium with serum (FS), complete medium containing 0.2 mM hydroxyurea (HU), or medium supplemented with serum substitute (SS) which was adjusted to the concentration of unlabeled thymidine in serum. After a further 24 hours in culture, (^{3}H)-thymidine incorporation was measured over 1 hour and expressed as %control (cpm/ug DNA). Each bar represents the mean $\pm$ s.e. of 3 experiments.

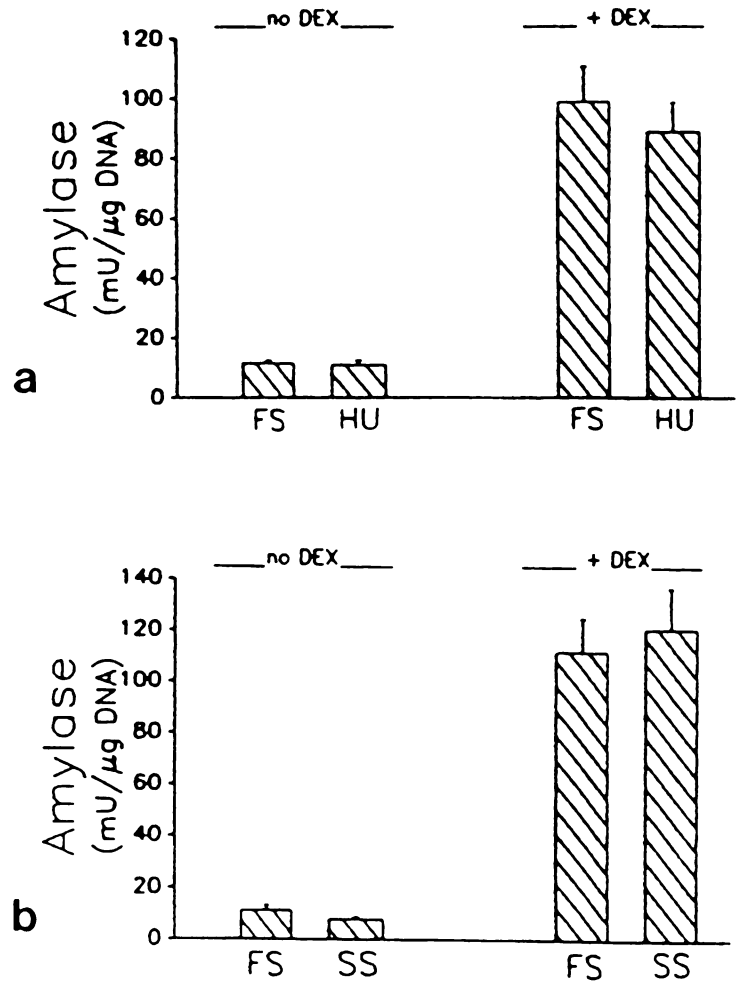

Fig. 3.6: Effects of hydroxyurea (a) or serum starvation (b) on cellular amylase content. Beginning at hour 0, cells were incubated for 48 hours (panel a) or 72 hours (panel b) under conditions stated in Fig.5. Each bar represents the mean $\pm$ s.e. of 3 experiments.

Chapter 4

Regulation of differentiation and growth in 266-6 cells.

The 266-6 monoclonal cell line was generated by the culture of a pancreatic tumor nodule from a transgenic mouse (1). The mouse was produced by the injection of foreign DNA into the pronucleus of a fertilized egg which was then allowed to develop in utero. The resulting animal carried copies of the foreign DNA integrated into one of its chromosomes (4). In this case, the foreign DNA consisted of the transforming gene from the simian virus 40 (SV40) genome fused to the enhancer elements from the rat elastase I gene. Since the elastase regulatory elements are responsive to unidentified factors specific to pancreatic acinar cells, the fused gene was expressed specifically in the acinar pancreas of the recipient mouse. Expression of the T antigen protein of SV40 leads to cell transformation and tumorigenicity by an unidentified mechanism (5). The pancreatic tumors arose in adult mice, long after the onset of specific transcription of the elastase promoter/T antigen fusion gene at day 14 of fetal development (1). It is postulated that this late transformation response is the result of secondary genetic effects initiated by T antigen protein (1). Cells from one pancreatic tumor were successfully cultured to produce the immortal cell line 266-6 (1).

The fact that the fusion gene was expressed via the elastase promoter specifically in pancreatic acini suggested that the 266-6 cell line might retain differentiated features of the exocrine pancreas.

The 266-6 cell line is currently in use for the examination of tissue-specific enhancers produced by these cells (2). In the present study, I determined that 266-6 cells retain several additional pancreas specific features, but are less differentiated than AR42J cells.

Mouse 266-6 cells were obtained from Raymond MacDonald, University of Texas, Dallas, and maintained in DME-H21 with antibiotics and 5% fetal bovine serum, in a 5% CO_2 atmosphere. The cells were grown in 150 cm^2 T-flasks (Corning) and routinely divided at a ratio of 1:6 every 4 days. Their gross appearance was flattened and fibroblast-like, with many pseudopodia. In contrast to AR42J cells, the 266-6 cells did not grow in clumps, but rather spread to cover the surface of the culture dish.

In initial experiments we examined the effects on 266-6 cell morphology of hormones known to affect acinar pancreas and AR42J cells. Dexamethasone, EGF, insulin, or caerulein were added to the medium and 266-6 cells were maintained for 48 hours in the presence of 5% defined fetal bovine serum from Hyclone. The peptide hormones, which were refreshed every day, had no obvious effects on 266-6 cell culture appearance. DEX treatment under these conditions, however, caused the cultures to appear much more confluent, suggesting stimulation of proliferation. There was no change in cell shape with DEX treatment.

Cells were routinely prepared for electron microscopy as described previously (3). Electron micrographs revealed an unremarkable ultrastructure similar to that of an undifferentiated fibroblast (Fig. 4.1). There were none of the differentiated structures found in normal pancreatic acinar cells (dense zymogen granules,

polarity, highly developed parallel arrays of rough endoplasmic reticulum) which appear to a lesser extent in AR42J cells (3). There were, however, numerous ribosomal rosettes, suggestive of active cytoplasmic protein synthesis (Fig. 4.1). Another remarkable feature of 266-6 cell morphology was the appearance of small sections of dilated rough endoplasmic reticulum, the lumens of which appeared swollen with slightly electron-dense contents (Fig. 4.1). There were no obvious changes in ultrastructure caused by 48 hour treatment with DEX, EGF, insulin, or caerulein (not shown).

One differentiated feature retained by 266-6 cells is the ability to synthesize amylase. This was established by the measurement of cellular amylase activity and DNA content as described in Chapter 2. Without hormone treatment, 266-6 cells contain approximately 1.00 mU amylase/ug DNA, which is 10-fold less than that contained by untreated AR42J cells (see Chapter 3). The amylase content was increased 3 - 12 fold by 48 hours of 10 nM DEX treatment (Table 4-1). This amylase increase is consistent with the DEX effect on AR42J cells, but of a lesser magnitude. Forty-eight hour treatments with either caerulein, insulin, EGF, or gastrin had no significant effect on 266-6 cell amylase content (Table 4.1).

Hormonal regulation of 266-6 cell proliferation was next examined. DEX treatment profoundly inhibited AR42J cell proliferation (see Chapter 3), but this effect was not seen in 266-6 cells. Depending on the type and amount of serum used, DEX either stimulated or had no effect on 266-6 cell proliferation (Table 4.1). Thus, in 266-6 cells, induction of differentiation as assessed by amylase synthesis is not tied to growth inhibition. This observation

complements the finding in AR42J cells that growth inhibition is not necessary for DEX induction of differentiation (Chapter 3). It is not known which component of serum confers growth stimulation in combination with DEX in 266-6 cells (Table 4.1).

Insulin treatment had a consistently stimulatory effect on 266-6 cell proliferation (Table 4.1). Whether insulin acts through its own receptor or via the IGF-1 receptor on 266-6 cells in unknown. Neither caerulein, EGF, nor gastrin had significant growth stimulatory effects.

The amylase activity present in medium bathing the 266-6 cells was measured and found to be proportional to, but considerably less than, cellular amylase content (Table 4.1). This suggests that 266-6 cells do not store a high proportion of total amylase synthesized, but rather secrete constitutively at a high rate. AR42J cells also secrete a portion of their amylase content without stimulation. However AR42J cells possess receptors for CCK, which stimulates amylase secretion to 200 - 350 % of basal (see Chapter 2). To determine whether 266-6 cells also respond to CCK, the cells were biosynthetically labeled with [^{35}S]methionine, stimulated with the CCK analog caerulein, and their cellular and secreted proteins subjected to polyacrylamide gel electrophoresis followed by autoradiography and densitometry as described in Chapter 2. Figure 4.2 shows the cellular and secreted protein pattern of 266-6 cells. In the secreted products there appears a prominent protein band of M_r=53,000, which is the apparent molecular weight of amylase in the PAGE system used here (see Chapter 2). For both control and DEX-treated cells, the density of this M_r=53,000 band in the secreted

products from caerulein stimulated cells is approximately 250 % of non-stimulated secretion. This suggests that the CCK analog caerulein does stimulate secretion of amylase from 266-6 cells.

Biosynthetic labeling of 266-6 cells also revealed the presence of several other secreted proteins which were not identified. It was noted that 48-hour DEX treatment induced the synthesis of an M_r=50,000 secreted protein which was not detectable in secretion products from untreated cells (Fig.4.2). The secretion of this and three other proteins also appeared to be stimulated by caerulein. One preliminary binding experiment suggested that 266-6 cells possess a small number of specific and saturable CCK binding sites (not shown).

Summary

Herein I show that 266-6 cells possess certain acinar-specific features in common with AR42J cells, most notably amylase induction by glucocorticoids. However, in contrast to AR42J cells, 266-6 cells did not acquire a more differentiated morphology with DEX treatment. Also in contrast to AR42J cells, 266-6 cells were not growth-inhibited by DEX. The evidence for DEX-induced amylase synthesis in 266-6 cells independent of inhibition of proliferation supports the conclusion previously reached with AR42J cells (Chapter 4), i.e. glucocorticoid effects on differentiation and growth are independent and separable. Thus, 266-6 cells were useful for these studies, but they had fewer differentiated features than AR42J cells. Thus, AR42J cells were a better in vitro model for acinar pancreas than 266-6 cells.

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TABLE 4.1

Effects of hormones on 266-6 cell amylase and DNA content.

	ug DNA per well	mU amylase per well (cellular)	mU amylase per well (in 1ml medium)	mU amylase/ug DNA
A: 5% FBS (Hyclone)				
T_0	3.14 ± 0.4	3.15 ± 0.46		1.00 ± .15
T_{48}control	4.53 ± 0.4	4.98 ± 0.35		1.10 ± .08
DEX	17.90 ± 2.0	49.20 ± 4.0		2.75 ± .23
caerulein	5.20 ± 0.6	4.80 ± 1.6		0.93 ± ,30
EGF	6.28 ± 0.5	2.89 ± 0.6		0.46 ± .09
insulin	17.67 ± 1.9	7.18 ± 2.1		0.41 ± .18
B: 2.5% FBS (Hyclone)				
T_0	0.81 ± 0.12	nd		
T_{72}control	2.90	nd		
DEX	4.07 ± 0.5	50.4 ± 7.2		12.4 ± 1.8
caerulein	3.25 ± 0.8	nd		
EGF	4.8 ± 0.7	nd		
insulin	13.9 ± 1.8	15.9 ± 4.5		1.14 ± 0.6
C: 5% FBS (Gibco)				
T_{48}control	13.0 ± 1.0	12.15 ± 0.8		0.93 ± .06
DEX	17.0 ± 1.0	73.5 ± 11.0		4.3 ± .6
D: 5% FBS (Gibco) + 5% calf serum				
T_0	10.5 ± 1.5	9.8 ± 1.6	42 ± 8	0.93 ± .16
T_{48}control	17.0 ± 3.0	23.0 ± 1.4	261 ± 33	1.35 ± .08
DEX	16.0 ± 2.0	50.4 ± 2.0	502 ± 21	3.15 ± .13
caerulein	17.0 ± 0.8	27.0 ± 0.8	275 ± 17	1.6 ± .05
insulin	28.0 ± 1.6	52.8 ± 7.0	402 ± 112	1.9 ± .25
gastrin	18.0 ± 0.8	25.0 ± 2.0	241 ± 9	1.4 ± .11

TABLE 4.1, continued

Mouse 266-6 cells were routinely plated in either 16mm (Experiments A, B, D) or 33mm (Experiment C) cluster dishes in DME-H21 plus the indicated amount and type of serum. Initial plating density, #cells/mm^2: A: 1700; B: 400; C: 1200; D: 5000. After 24 hours in culture, four wells were collected for assay (T_0) and the indicated hormones added for an additional 48 or 72 hours, at which time the cells were collected for assay (T_{48} or T_{72}). Concentrations used: DEX, caerulein, 10nM; EGF, 1nM; insulin, 1uM; gastrin, 100nM. Each group of data (separate medium conditions) is from a separate experiment. Results are expressed as the means of 4 wells + S.D. nd=not detectable.

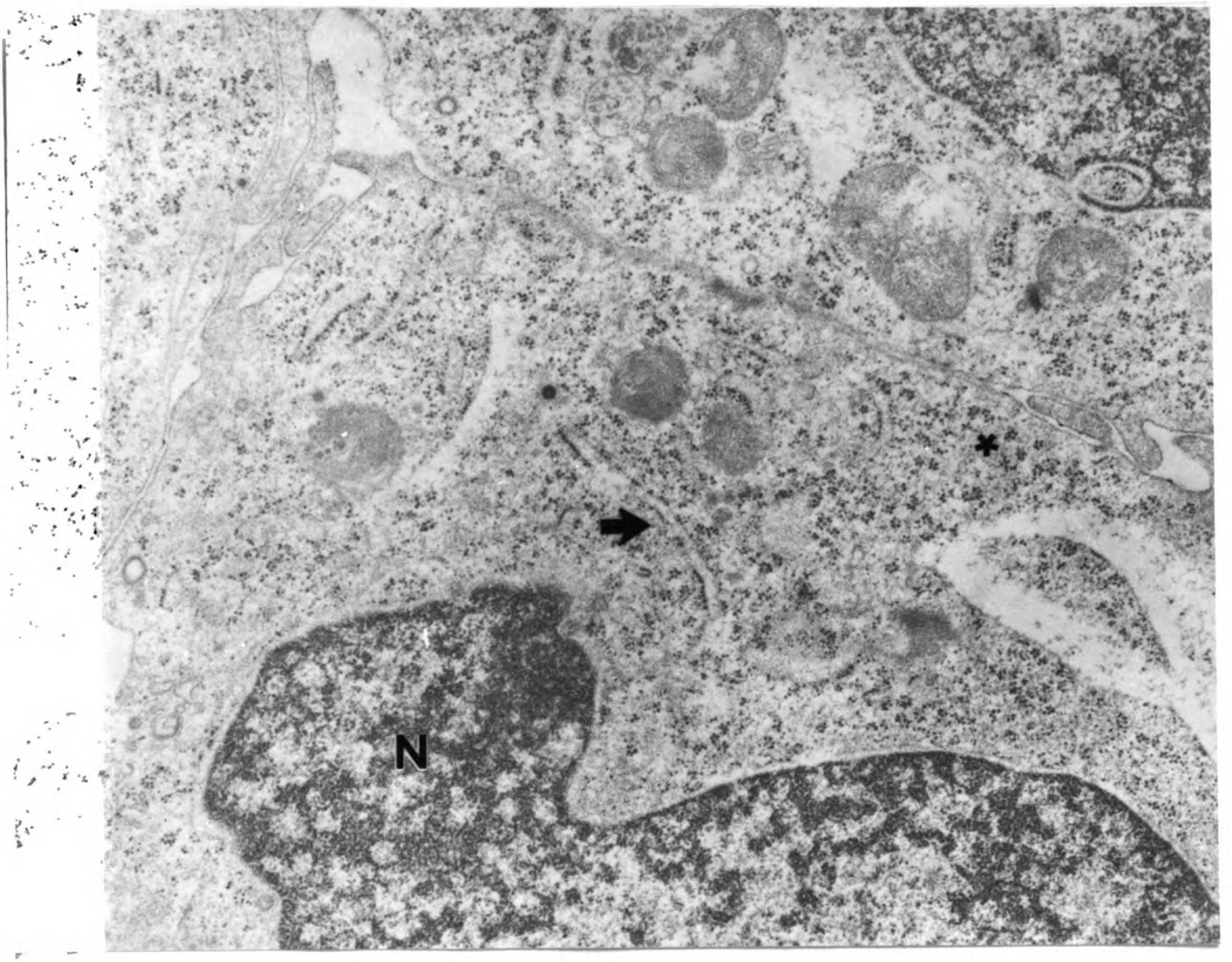

Fig. 4.1: 266-6 cells were plated in 16mm wells in DME-H21 plus 5% fetal bovine serum (Hyclone), maintained for 72 hours, then routinely fixed and prepared for electron microscopy. N=nucleus; *=ribosomal rosette; arrow indicates endoplasmic reticulum.

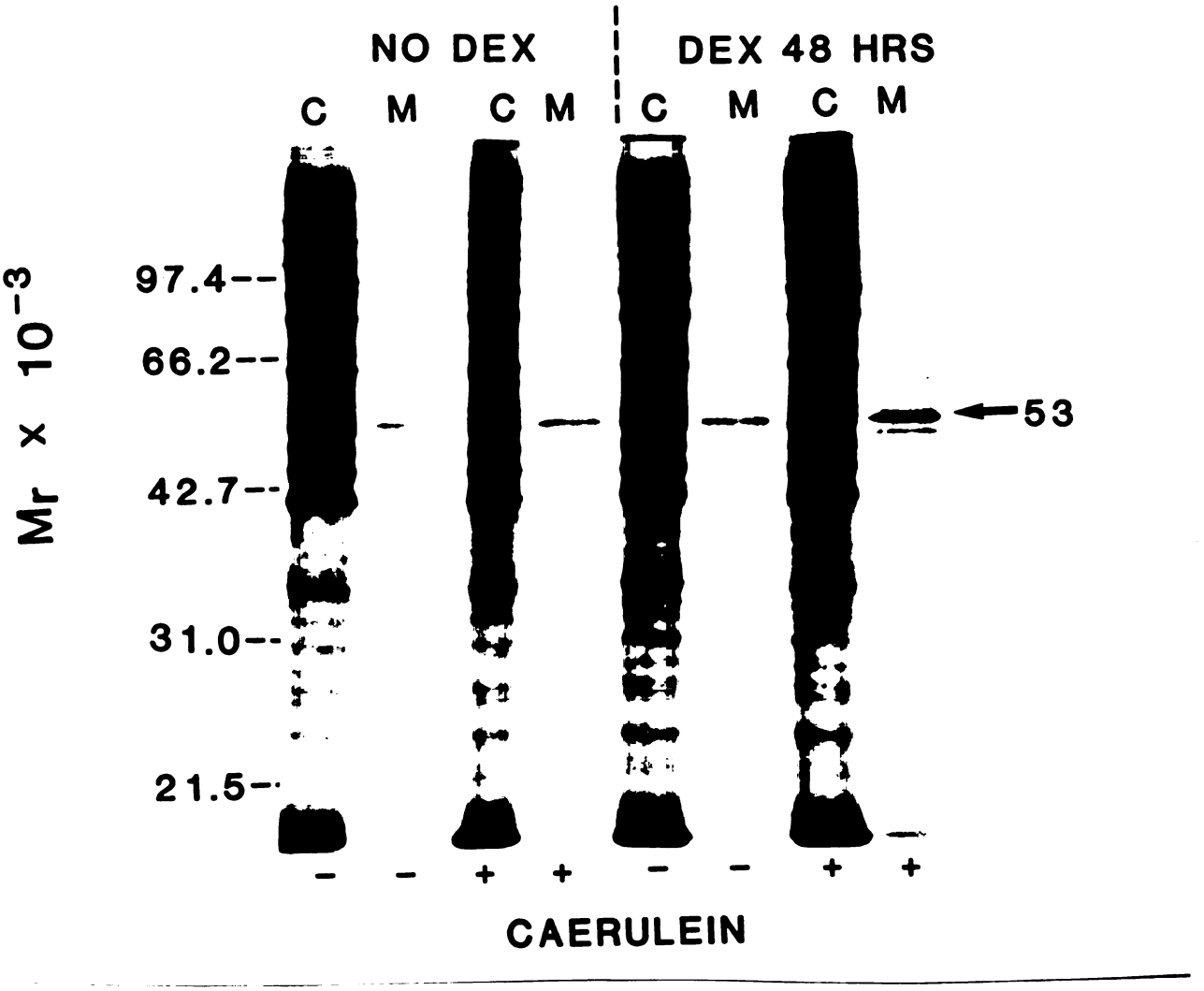

Fig. 4.2: Mouse 266-6 cells were plated in 16 mm cluster wells in DME-H21 containing 5% fetal bovine serum from Hyclone. After 24 hours, 10 nM DEX was added where indicated. After a further 48 hours, cellular proteins were biosynthetically labeled, cells were stimulated with 10 nM caerulein, and their protein products subjected to polyacrylamide gel electrophoresis and autoradiography as described in Chapter 2. The numbers on the left indicate the positions of the molecular weight markers, while the arrow on the right indicates the M_r=53,000 protein band which migrates to the level of amylase in this PAGE system. C=cellular lysate; M=medium.

Chapter 5

Discussion: Comparison of immortal cell lines with normal pancreatic acini.

The AR42J cell line in culture possesses several characteristics of the developing fetal rat pancreas, particularly with respect to glucocorticoid responsiveness. In fetal rat pancreatic rudiments in culture, glucocorticoids stimulate the synthesis of amylase, chymotrypsinogen, and procarboxypeptidases A and B (1). In fetal pancreas, this response is specific for those four digestive enzymes, and is accompanied by morphological differentiation and inhibition of DNA synthesis (1). In AR42J cells, the effects of glucocorticoids on morphological differentiation, enzyme synthesis, and cell proliferation are similar to those in fetal cells. (see Chapters 2 and 3). In contrast, 266-6 cells display a positive amylase response but do not show decreased cell proliferation with glucocorticoid treatment (Chapter 4). These results suggest that glucocorticoid effects on fetal pancreatic differentiation and growth as seen in organ culture may be independently mediated.

Maintenance of normal glucorticoid level is not essential for normal fetal pancreatic development; fetal pancreatic rudiments will differentiate in culture in the complete absence of glucocorticoids, although at a slower rate (1). It appears that a differentiation-inducing event causes a genomic change early in gestation which programs the future acinar cells and their progeny. The proliferation of fetal cells concomitant with synthesis of digestive enzymes (2,3) mirrors the condition of AR42J cells exposed to

sub-maximal glucocorticoid in combination with peptide growth factors (Chapter 3). Cell multiplication is compatible with differentiation.

In the neonatal and adult rat, glucocorticoid levels modulate the level of pancreatic amylase synthesis (4,5), as in AR42J and 266-6 cells.

Other differentiation-dependent characteristics studied here, CCK binding and responsiveness, develop in a non-parallel fashion in the fetal pancreas (6). There is spontaneous enzyme secretion by pre-natal day 15. A full complement of CCK receptors is present by 1 day before birth, and CCK stimulates calcium mobilization at this stage. However, only at 1 day after birth does CCK stimulate amylase secretion. It is postulated that signal transduction mechanisms distal to calcium mobilization mature during the two day period spanning birth (6). AR42J cells possess functional CCK receptors and secrete digestive enzymes in response to CCK (ref. 7 and Chapter 2). The high level of unstimulated protein release from AR42J cells, and their CCK-secretory response, which is diminished in comparison with that of normal acini (Chapter 2) may reflect immaturity of signal transduction mechanisms in this cell line.

Future use of in vitro models for acinar pancreas.

The AR42J and 266-6 cell lines may prove to be valuable tools for the future molecular identification of factors involved in the regulation of cell differentiation and proliferation. For instance, these immortal cell lines could be exploited for mutagenisis and the production of variants which lack glucocorticoid responsiveness. The variants might then be used as recipients of genes donated by wild-type cells. Alternatively, treatment of AR42J or 266-6 cells with DNA hypomethylating agents might cause

Transduction of genomic DNA from differentiated cells might be expected to lead to differentiation and/or growth inhibition of wild-type cells.

The use of immortal cell lines will also be important in the elucidation of molecular events which control the development and maintenance of neoplasia. Cancer can be viewed as the result of derangement of molecular events which normally lead to cell differentiation and regulated proliferation. Thus, the identification of normal cellular regulatory factors may have wide implications for the understanding and treatment of neoplasia.

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