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Differentiation and Transdifferentiation of Adult Pancreatic Cells

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Ph. D.

Dedicated to my parents

Abstract

In vitro studies will contribute significantly to an understanding of cell growth and differentiation in the adult pancreas. This thesis reports on the changes occurring when acinar cells, terminal ducts and islets are grown in culture.

A number of methods for adult pancreatic duct epithelial isolation and culture from different species have been reported. However, there are no reliable methods for the large scale isolation and culture of the terminal segments of the pancreatic duct system. Acinar fragments of the hamster pancreas are isolated by partial digestion with collagenase. Culture is achieved by embedding in a matrix of rat-tail collagen in DME:F12 medium and 10% NuSerum supplemented with epidermal growth factor (EGF) and cholera toxin (CT). The results show that ductal cysts arise within the areas of degenerated acinar tissue. The method developed in the preparation of this thesis gave a high yield of ductal cysts. Autoradiography indicates the duct cysts could have originated from either progenitor cells of ducts or those of acini. In addition, the question of whether these duct epithelial cysts originate from phenotypic transformation of acinar cells needs to be further evaluated.

Acinar fragments isolated from the hamster pancreas were embedded in type 1 collagen and grown in various media. After 34 days in culture, up to 80% of the acinar cells can be shown to be normal, while the remainder were severely degenerated. This occurred in media to which EGF and CT had not been added. When the latter were added, cystic structures developed. However, a few of the cells within the cystic wall still showed amylase positive immuno-reactivity.

Cellular amylase activity decreased over time but a more rapid decline was shown in cells cultured with Media containing EGF and CT. It was also found that the duct-like cells had a limited capacity to redifferentiate into acinar cells. This study suggests that ductal-like epithelial structures may arise from transformation of acinar cells and /or proliferation of ductal cells.

Purified islets from human pancreas were placed into collagen gel matrix and cultured in DME:F12 medium and 10% NuSerum supplemented with EGF and CT. The results showed that the cultured islets underwent a cystic transformation that was associated with (i) a progressive loss of insulin gene expression, (ii) a loss of immunoreactivity for insulin protein, and (iii) the appearance of CK-19, a marker for ductal cells. After the transformation was complete, the cells had the ultrastructural appearance of primitive duct-like cells. Cysts showed a progressive enlargement with cell replication, as reflected in a 1500% increase in the incorporation of tritiated thymidine. These results are consistent with the transdifferentiation of islet cells to ductal cells.

Résumé

Des études *in vitro* feront progresser de façon importante les connaissances sur la croissance et la différenciation des cellules du pancréas chez l'adulte. Cette thèse décrit les changements qui surviennent dans les cellules acineuses, les canaux pancréatiques et les îlots de Langerhans mis en culture.

La documentation décrit plusieurs méthodes pour isoler et mettre en culture les cellules épithéliales du canal pancréatique du pancréas de l'adulte. Cependant, aucune méthode fiable n'a encore été décrite pour procéder sur une grande échelle à l'isolement et à la culture des segments terminaux du canal pancréatique. La digestion partielle par collagénase est utilisée pour isoler des fragments d'acini provenant de pancréas de hamster. Les isolats sont inclus dans une matrice de collagène de queue de rat et placés dans un milieu contenant une proportion égale de DME et F12, et du NuSerum 10% auquel ont été ajoutés le facteur de croissance épidermique (EGF) et la toxine cholérique. Les résultats montrent que les kystes du canal pancréatique se constituent dans les zones de tissu acineux. La méthode de culture élaborée pour préparer cette thèse a permis de provoquer la formation de nombreux kystes du canal pancréatique. Les résultats de l'autoradiographie indiquent que les kystes du canal pourraient s'être formés à partir des cellules souches du canal pancréatique ou à partir des cellules acineuses. D'autres recherches seront nécessaires pour déterminer si la formation de ces kystes de l'épithelium du canal pancréatique est attribuable à un changement phénotypique des cellules acineuses.

Les fragments d'acini isolés du pancréas de hamster ont été inclus dans un collagène de type 1 et mis en culture dans différents média. Après 34 jours en culture, jusqu'à 80 % des cellules acineuses étaient encore normales alors que les autres 20 % présentaient les signes d'une grave dégénérescence. Ce phénomène a été observé dans les milieux de culture auxquels l'EGF et la toxine cholérique n'avaient pas été ajoutés. Des structures kystiques se sont cependant constituées dans les milieux contenant l'EGF et la toxine cholérique. Cependant, quelques-unes des cellules de la paroi kystique continuaient de présenter une immunoréactivité positive de l'amylase. L'activité de l'amylase cellulaire a diminué avec le temps, mais dans les cellules cultivées dans les milieux contenant

l'EGF et la toxine cholérique, elle a diminué plus rapidement. En outre, les cellules de type canalaire arrivaient plus difficilement à se différencier de nouveau en cellules acineuses. Les résultats de cette étude permettent de croire que les structures épithéliales de type canalaire se constitueraient par suite de la transformation des cellules acineuses et/ou de la prolifération des cellules du canal pancréatique.

Des îlots de Langerhans purifiés provenant de pancréas humain ont été inclus dans une matrice de collagène et mis en culture dans un milieu DME:F12 et de NuSerum 10 % auquel ont été ajoutés l'EGF et la toxine cholérique. Les résultats montrent que les îlots mis en culture ont subi une transformation kystique reliée (i) à la disparition progressive de l'expression du gène de l'insuline, (ii) à une perte de l'immunoréactivité à la protéine de l'insuline, et (iii) à l'apparition de CK-19, un marqueur des cellules du canal pancréatique. Lorsque la transformation était terminée, les cellules ressemblaient par leur apparence ultratructurelle à des cellules primitives de type canalaire. À mesure que les cellules se reproduisaient, les kystes augmentaient progressivement de volume comme en témoigne l'augmentation de 1 500 % de l'absorption de thymidine marquée au tritium. Ces résultats appuient l'hypothèse de la transdifférenciation des cellules d'îlots de Langerhans en cellules de canal pancréatique.

List of Abbreviations

BrdU	5-Bromodeoxyuridine
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CCK	Cholecystokinin
CCK-PZ	Cholecystokinin-Pancreozymin
cDNA	Complementary Deoxyribonucleic Acid
CK-19	Cytokeratin 19
CT	Cholera Toxin
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
HBSS	Hank's Balanced Salt Solution
NSE	Neuron Specific Enolase
PBS	Phosphate-Buffered Saline
PDGF	Platelet Derived Growth Factor
PP	Pancreatic Polypeptide
RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic Acid
SBTI	Soybean Trypsin Inhibitor
SDS	Sodium Dodecyl Sulfate
SRP	Signal Recognition Particle

Prologue

This thesis demonstrates my studies on the differentiation of adult pancreatic cells during my Ph.D. training in Pathology. The choice of cell differentiation as the project reflected in part my previous training and in part the laboratory tradition. I had engaged in cancer research for many years before joining this group. I was convinced that the understanding of cell differentiation is the key to elucidate the histogenesis and pathogenesis of cancer. This laboratory is the place where Drs. W.P. Duguid and L. Rosenberg have established an experimental model of nesidioblastosis, induced by partial obstruction of the pancreatic ducts.

The general aim of the project was to study how pancreatic cells differentiate while being cultured in type 1 collagen. The use of collagen as a three-dimensional substrate provides an environment more similar to physiological conditions and has facilitated the growth and differentiation of different cell types in culture.

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It is obvious from the organization of the thesis that the published papers are integrated into the thesis as a whole and are not presented as completely separate entities.

Four contributors did considerable works for this thesis.

Dr. William P. Duguid, my supervisor, has frequently discussed the results of my experiments and helped me to interpreted the experimental data. He has also read the histological specimens with me and guided my interpretation of the specimens.

Dr. Lawrence Rosenberg, my co-supervisor, has given some advice on the research project. He has constantly discussed the results of my experiments and guided me to prepare manuscripts for publication.

Dr. Steven Paraskevas has worked with me as a team to isolate islets for human pancreas.

Dr. Peter Metrakos has helped me to develop the method of isolation and culture of intralobular ducts from the hamster pancreas.

The experiments described in this thesis did not reproduce earlier work and represent original contributions to the field of cell differentiation in the adult pancreas. I consider that the most important original aspects contained in this thesis are:

1. I have developed a new method for isolation and culture of intralobular ducts from the hamster pancreas. This method gives a higher yield of ductal cysts than have been previously reported..
2. I have also developed a new method for isolation and culture of acinar cells from the hamster pancreas. For the first time the acinar cell phenotype can be maintained for over 1 month when cells are cultured in collagen.
3. It is the first time to show that the cultured acini undergo phenotypic changes when EGF and cholera toxin are added to the medium. These duct-like structures are lined by a single layer of cubical or flattened epithelium with a few acinar cells.
4. Despite limited success, I have been the first to try to redifferentiate duct-like cells into acinar cells when cells are cultured in collagen.
5. It is the first time that cultured human islets cells have been shown to transdifferentiate into duct cells in vitro.

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Chapter 1

Introduction and General Objectives

Herophilus of Chalcedon first described the human pancreas in about 300 B.C. (Child III, 1985). Four hundred years later, Ruphos of Ephesus named this important abdominal organ the *pancreas* (from Greek, *pan-kreas*, all flesh). Since then, knowledge of the pancreas has been gradually accumulated due to its physiological and pathological importance appertaining to the human body. Great advances have been achieved in the knowledge and understanding of the pancreas, its biology, function and diseases in the past decades. The scope of this chapter encompasses the basic concepts and knowledge closely related to my research project, including: anatomy and histology of the pancreas, embryological development and cell differentiation in the adult pancreas. Our understanding of this knowledge about the pancreas has provided fundamental information in designing, performing, and interpreting our research results.

1.1 Anatomy and histology of the pancreas

The pancreas is partly an endocrine organ, secreting hormones into the blood stream, and partly an exocrine organ, secreting digestive enzymes that are carried by ducts directly to the duodenum. The exocrine pancreas is composed of the acinar units and the duct system, whereas the endocrine pancreas is formed by the islets of Langerhans. The present section describes the anatomy and histology of acinar, duct, and islet cells in relation to their functions.

1.1.1 Gross anatomy of human and hamster pancreas

The human pancreas is a soft, yellowish-pink gland that appears distinctly lobulated to the unaided eye (Harris, 1979; Bockman, 1993). The connective tissue capsule that separates the pancreatic tissue from adjacent structures is remarkably thin and is covered with peritoneum. Partitions of connective tissue extend in from the capsule and divide the pancreas into macroscopic lobules; each is composed of many microscopic lobules, which are the functional units of the exocrine pancreas. In the adult, the gland is 14 to 18 cm long, 2 to 9 cm wide, and 2 to 3 cm thick. Its weight varies between 70 and 150 g with a mean of 90 ± 16 g in males and 85 ± 15 g in females. The pancreas extends transversely across the posterior wall of the abdomen from the descending part of the duodenum on the right to the spleen on the left. It is long and irregularly prismatic in shape, and has been compared to a human or a dog's tongue. The pancreas has four named parts: head, neck, body, and tail. The head of the pancreas is shaped like the head of a hammer and encircled by the curve of the duodenum. A projection from the lower left

part of the head termed the uncinate process lies to the right of the ascending part of the duodenum. The neck that connects the head to the body of the gland is about 2.5 cm long. The body, which is prismatic in shape with three surfaces: anterior, posterior, and inferior, extends transversely to the left and tapers to the tail. The tail is the narrow part extending left as far as the lower part of the inner aspect of the spleen.

The pancreas of the hamster is a diffuse low dendritic type as in other rodents (Bivin et al., 1987; Greene, 1959). It is a pinkish white structure in the central abdomen and averages approximately 0.4 to 0.5 % of total body weight in the adult. The general shape of the pancreas is similar to the Greek letter λ, with the irregularly shaped central area lying mediodorsally to the cranial duodenum. It is divided into three regions: duodenal, gastric, and splenic lobes. The duodenal lobe is the thin, leaf-shaped, averaging only 12% of the total weight. It is bordered on the right by the duodenum and ascending colon. It extends laterally from the duodenal loop to run caudally enclosed in the mesentery, between the duodenum and cranial ascending colon. The gastric lobe comprises 25% of the total weight. It is attached to the stomach and pylorus by the mesogastric membrane formed from the greater omentum. The splenic lobe follows the greater curvature of the glandular stomach and comprises 40% of the total weight. It is covered by the greater omentum as it runs cranially to the area of the aorta and caudal vena cava. The splenic lobe is caudal to the spleen and has a connective tissue attachment to the cranial descending colon.

1.1.2 The acinar units

The enzyme-secreting units of the exocrine pancreas are the acini (from Latin *acinus*, berry) comprising about 74% and 82 % of the gland volume in

the human and guinea pig, respectively (Tasso et al., 1973; Bolender, 1974). The majority of cells in the acinus consist of acinar cells, with a smaller number of centroacinar cells marking the beginning of the ductular system of the gland. The supporting matrix of the acini is a mixture of several different types of coilagen and other extracellular matrix molecules. The basal lamina, which consists of types IV and V collagen, laminin, and heparan sulfate proteoglycans, is a product of the epithelial cells (acinar cells and cells of the duct system) (Ingber et al., 1985). Collagen types I and II and fibronectin are presumably produced by fibroblasts and are located in the adjacent extracellular space.

1.1.2-1 Microscopic structure of acinar cells

By light microscopy, acinar cells are pyramidally shaped with their narrow apical ends bordering the lumen, which is the finest terminus of the interlobular duct system (Dixon, 1979; Ekholm et al., 1962a). The base of the acinus is broad and rests on a basal lamina and reticular connective tissue. The nucleus is rounded and lies towards the base of the cells; it contains abundant chromatin and one to three prominent nucleoli. Zymogen granules, which are restricted to the apical cytoplasm of the cell and vary in number, are stained as brilliantly acidophilic clusters. The cytoplasm between the nucleus and the base of the cell is basophilic in routine histological preparation because of its content of rRNA. The acinar cells are generally uniform in their appearance throughout the pancreas, although a "halo" phenomenon is seen in acinar cells that are located immediately adjacent to islets in the so-called peri-insular region. Acinar cells in this area have a larger cytoplasm and nucleus and an increased volume of zymogen granules compared to the remainder of the pancreas (Hellman et al., 1962). This

finding may be relevant to the observation that under some conditions the relative amounts of zymogens secreted is different between the basal and stimulated states.

Electron micrographs show that the acinar cell is characterized by a massive amount of rough endoplasmic reticulum (RER) surrounding the basal and lateral borders of the nucleus and an accumulation of zymogen granules in the apical cytoplasm (Dixon, 1979; Kern, 1993; Ekholm et al., 1962a). The rough endoplasmic reticulum consists of parallel membranous cisternae and interconnecting tubules in the cytoplasm with the ribosomes attached to their outer surfaces. Clusters of free ribosomes occur in abundance in the cytoplasmic matrix between the RER. Spherical or elongated mitochondria with typical cristae and a varying number of dense intramitochondrial granules are sandwiched between the layers of the RER. The Golgi complex is located on the apical side of the nucleus. It appears as a horseshoe-like structure composed of three to five stacks of flattened membrane-bound cisternae and numerous vesicles. Each Golgi stack has two distinct faces: a *cis* face and a *trans* face. The *cis* face oriented toward vesicles of the RER that appear dilated and are partly devoid of ribosomes. These vesicles destined for the Golgi complex bud from a specialized region of the RER called the transitional elements. Proteins carried by the transitional elements will enter the *cis* compartment; they then move to the medial compartment that consists of the central cisternae of the stack and finally to the *trans* compartment. At the *trans* face of the complex, different stages in the formation of zymogen granules and lysosomes are observed. The spherical electron-dense zymogen granules are variable in size. Each granule is limited by a smooth membrane and is composed of a homogeneous electron-dense material with no internal structure.

The apical surface of the acinar cell possesses a few short microvilli. The microvilli are slender cylindrical processes about 0.1 μm in diameter and 0.5 μm in length. Bundles of fine filaments run longitudinally in the interior of each microvillus and extend into the apical cytoplasm. The junctional complexes between the adjacent acinar cells seal off the acinar lumen (Farquhar, 1981). There are occluding junctions (continuous tight junctions) close to the luminal space. Below these there are zonular adherens (belt desmosomes), and also spot desmosomes (macula adherens) along the lower parts of the lateral plasma membrane.

1.1.2-2 Secretion products

The acinar cells are specialized to synthesize, store, and secrete digestive zymogens and enzymes. Like all proteins, their synthesis occurs on the aggregations of ribosomes called polysomes, where the genetic code for a particular protein is translated into a specific sequence of amino acids. Following their synthesis, the newly formed proteins are translocated into the ER. The mechanism for the import of proteins into ER lumen is explained by the signal hypothesis (Walter et al., 1984; Scheele & Kern, 1993; Walter, 1994). This hypothesis states that a special sequence code (usually 15-30 amino acid residues) is translated into an amino terminal extension of the protein termed the signal peptide (Devillers-Thiery et al., 1975). The nascent proteins with the signal peptide intact are called "preproproteins". The initial step in translation of mRNA is the attachment of the initiation codon of the mRNA to ribosomes free in the cytosol. The "signal peptide" is then translated. The signal peptide has a high affinity for the signal recognition particle (SRP) that is an 11S ribonucleoprotein complex in the cytoplasm (Walter & Blobel, 1982). The SPR attaches to the signal sequence as soon as the peptide emerges from

the ribosome, which forms a large-molecular-weight SRP-ribosome complex (Nunnari and Walter, 1992). This causes a pause for further protein synthesis. The SRP-ribosome complex binds to the SRP receptor, which is an integral membrane protein on the RER membrane. Formation of the complex between the SRP and SRP receptor allows the hydrophobic segment of the signal peptide to insert into the lipid bilayer of the RER membrane. Then, the remainder of the protein is synthesized and passes into the ER lumen. The signal peptide is cleaved from the precursor protein by a membrane-associated signal protease, while the protein remains segregated within the ER lumen.

The nascent proteins in the ER lumen can undergo modification, including disulfide bridge formation and glycosylation (Palade G, 1975; Scheele & Kern, 1993). Conformational changes that fold proteins into tertiary and quaternary structures also take place in the ER. The secretory proteins are transported successively from the RER to the Golgi complex mediated by transport vesicles. In the Golgi, further protein modification (glycosylation) and concentration occur. The digestive enzymes are sorted and transported to zymogen granules. Zymogen granules discharge their content into the acinar lumen by a complex process referred to as exocytosis. This involves a directional movement of zymogen granules toward the luminal plasma membrane, recognition by the zymogen granule membrane of the appropriate fusion partner and a transient fusion of granule membranes with the plasma membrane. After the zymogen granule contents are released, the membranes are retrieved by the cell and reutilized.

The enzymes secreted by the acinar cells are necessary for the normal digestion of proteins, carbohydrates, fats, and nucleic acids (Rinderknecht, 1993; Desnuelle & Figarella, 1979). Some enzymes such as amylase and lipase

are secreted in active form. To prevent autodigestion of pancreatic tissues, the potent proteolytic enzymes are secreted in inactive zymogen form. In the duodenum, trypsinogen is converted to the active enzyme trypsin by the action of enterokinase through the removal of six amino acids from the amino end of trypsinogen. Enterokinase is an enzyme synthesized by the enterocytes of the proximal small intestine (Hermon-Taylor et al., 1977). Once

Table 1-1 Major enzymes secreted by the pancreas

Enzyme/zymogen	*Substrate*	*Action*	*Product*
Trypsin(ogen) 1, 2 and 3	Protein	Cleavage of Arg- and Lys- peptide bonds	Peptides
Chymotrypsin(ogen) A an B	Protein	Cleavage of Phe-, Tyr-, Trp- peptide bonds	Peptides
(Pro)elastase 2	Elastin	Cleavage of bonds adjacent to aliphatic amino acids	Peptides
(Pro)protease E	Protein	Cleavage of aliphatic and hydroxy amino acid residues	Peptides
(Pro)carboxypeptidase A and B	Protein	Cleavage of carboxy-terminal amino acid residues	Amino acids
(Pro)phospholipase A_2	Phospholipid	Hydrolysis of sn-2-fatty acid esters of phosphoglycerides	Fatty acids+lysophospholipids
Lipase	Triglyceride Diglyceride	Hydrolysis of C_1 and C_3 glycerol ester bonds	Fatty acids Monoglyceride
Carboxylester hydrolase	Various lipid	Hydrolysis of water soluble esters of short-chain fatty acids	Fatty acids
Amylase	Starch Glycogen	Hydrolysis of a-1,4-glucosidic linkages	Branched dextrins
Ribonuclease	RNA	Hydrolysis of Phosphate bonds	3'-Phosphate nucleotide 5'-monoesters
Deoxyribonuclease	DNA	Hydrolysis of Phosphate bonds	3'-Phosphate nucleotide 5'-monoesters

Source: Rinderknecht, 1993; Onstad & Bubrick, 1985.

this conversion occurs, trypsin sets up a positive feedback loop for activating trypsinogen (Rinderknecht, 1993). Trypsin then starts a cascade process, whereby all other pancreatic zymogens are converted into active forms.

Some basic properties of enzymes are listed in Table 1-1. Proteolytic enzymes have a hydrolytic action on the specific peptide bonds (Wormsley, 1981). The endopeptidases, which hydrolyze peptide bonds within the molecules of proteins to be digested, include trypsin, chymotrypsin, elastase and protease E. The exopeptidases, which remove terminal amino acids from proteins or polypeptides, include carboxypeptidase A and B. Lipase acts only when the substrate is present at an oil-water interface (Borgstrom, 1993). The emulsification of dietary fat by bile salts is necessary for optimal lipolytic activity. Bile salts, by themselves, inhibit lipolytic activity but the inhibition can be overcome by co-lipase, which is secreted with lipase by acinar cells in zymogen form and converted into an active form by trypsin. In addition, the acinar cells secrete a substance called trypsin inhibitor to prevent premature activation of trypsinogen in the pancreatic ducts (Rinderknecht, 1993).

1.1.3 The ductal system

1.1.3-1 Microscopic structure of the ducts

The ductal system in the human pancreas can be subdivided into three major parts: the main, interlobular and intralobular ducts (Dixon, 1979; Kern, 1993). The main duct is surrounded by dense connective tissue with some elastic fibers and smooth muscle cells and serves as a 'backbone' for the whole organ. Interlobular ducts emerge from the main ducts and run together with the major branches of vessels and nerves in the interlobular connective

tissue. The interlobular ducts branch and give rise to intralobular ducts that enter the pancreatic lobules.

Interlobular and main ducts: The main duct is lined by tall columnar epithelium with occasional interspersed goblet cells and the interlobular ducts by low columnar epithelium (Dixon, 1979). The main and interlobular ducts are similar at the ultrastructural level (Kern, 1993; Kodama, 1983). The epithelial cells show short microvilli on their luminal surface and abundant mucin granules in their apical cytoplasm. The basal and paranuclear region of the cytoplasm is filled with fairly developed RER and mitochondria, while the Golgi complex and secretory granules are localized in the supranuclear cytoplasm. The cells reveal intact junctional complexes and numerous spot desmosomes along the lateral plasma membrane. One special feature concerns the high frequency of exfoliating cells, which show degenerated organelles, pyknotic nuclei and loss of intercellular connections. Exfoliating cells are replaced by new cells that appear in the basal region of the epithelium.

Intralobular ducts: The intralobular ducts branch extensively and give rise to very small ducts that lead to the acinus and are termed intercalated ducts (ductules) since they are interposed between the secretory acini and the intralobular ducts (Dixon, 1979). The end portions of the intercalated ducts extend between the acini to form the centro acinar cells. The intralobular ducts are lined by low columnar to cuboidal epithelium and the intercalated ducts by flattened cuboidal epithelial cells. The cells of intralobular ducts are characterized by a round or oval nucleus with marginal indentations and a small amount of cytoplasm. The outstanding elements in the cytoplasm are the mitochondria, which are often collected in a group near the center of the cell (Ekholm et al., 1962b; Ekholm & Edlund, 1959). The endoplasmic

reticulum in the cytoplasm is very sparse. Ribosomes are mainly found free and concentrated around the Golgi complex. The Golgi complex is generally found close to the apical aspect of the nucleus. Compared to the corresponding organelle in the acinar cells, the Golgi complex in intralobular duct cells is small and lacks typical secretion granules. A characteristic feature of intralobular duct cells is elaborate interdigitations at the lateral and basal plasma membranes. This structural feature as well as numerous mitochondria, which provide energy for the transport, makes the intralobular ducts the best candidate for bicarbonate secretion. The secretion of fluid and ions by these cells is further supported by the immunocytochemical demonstration of carbonic anhydrase mainly in intralobular ducts of the human pancreas (Spicer et al., 1982).

1.1.3-2 Fluid and bicarbonate secretion by duct cells

The human pancreas secretes approximately 1000 ml of pancreatic juice, containing mostly water, ions, and digestive enzymes, into the duodenum per day (Owyang & Williams, 1995). Sodium and potassium are the major cations, whereas the major anions are bicarbonate and chloride (Harper & Scratcherd, 1979). The secretions also contain small amounts of calcium, magnesium, zinc, phosphate and sulfate. The function of the water and ion secretions is to neutralize gastric acid emptied into the duodenum and raise the PH of fluids to a range that is optimal for the activity of pancreatic digestive enzymes.

The concentrations of cations resemble an ultrafiltrate of plasma and are independent of secretory rates. The anionic composition varies and the concentrations of chloride and bicarbonates depend directly on flow rates (Case et al., 1968; Case et al., 1970). When the duct system is not stimulated,

the concentration of bicarbonate is low and that of chloride high. The concentration of bicarbonate rises asymptotically as the flow rate increases under stimulation of secretin. Secretin evokes the secretion of a bicarbonate-rich fluid that is low in chloride. During maximum stimulation, bicarbonate concentrations usually reach at least 130 mEq/l or more and chloride concentrations fall below 35 mEq/l (Case et al., 1968). Chloride concentrations vary inversely to the bicarbonate concentrations. The concentration of chloride always moves in a reciprocal manner to bicarbonate, so that the sum of the concentrations of bicarbonate and chloride remains constant, and equal to the sum of the concentration of sodium and potassium. This is because of an HCO_3^--Cl^- exchange during passage through the ductal system.

Most of the bicarbonate ion in pancreatic fluid is derived from plasma, but some is contributed by the metabolism of pancreatic cells. The cellular events for bicarbonate ion secretion by the intralobular cells are not well elucidated. Bicarbonate secretion by duct cells is an active process that requires ion transport linking with Na^+, K^+ -ATPase (Ridderstap & Bonting, 1969). The carbonic anhydrase inhibitor acetazolamide significantly depresses pancreatic secretory flow and bicarbonate secretion in human (Dyck et al., 1972).

1.1.4 The islets of Langerhans

The endocrine pancreas of mammals consists of groups of hormone-producing cells diffusely scattered in the parenchyma of the gland (Loubatieres, 1969). These so-called "Islets of Langerhans" were originally described by Paul Langerhans in his doctoral dissertation in 1869 and named in 1893 by Edouard Laguesse, an eminent French histophysiologist (Falkmer, 1985). Each islet of Langerhans has complex structures including different types of cells and functions.

1.1.4-1 General composition of islets

The human pancreas has been estimated to possess over 1 million islets (Volk & Wellmann, 1985). The total volume occupied by the islets is about 1.5 to 2 % of the adult pancreas (Weaver et al., 1985; Bolender, 1974). The islets may contain only a few cells, hundreds of cells, or even thousands of cells, and range widely in size from less than 40 mm to 400 mm in diameter. The islets between 100-225 mm in diameter represent 71% of the total number. The distribution and cellular composition of islets are not the same in each part of the gland. The concentration of islets in the tail is greater than that in the head and body (Wittingen & Frey, 1974). Three-dimensional reconstruction of the islets has shown that there exist two types of islets in humans and rats (Malaisse-Lagae et al., 1979; Baetens et al., 1979). The glucagon-rich, PP-poor islets, comprising 28% A cells but only 2% PP cells, are mainly located in the dorsal part of the pancreas in rats. The PP-rich, glucagon-poor islets, comprising 20% PP cells but less than 2% of A cells, are mainly located in the ventral part of the pancreas. Both the B and D cells remain relatively constant.

The pancreatic islets are round, ovoid, or irregular in shape containing parenchymal cells, supportive tissue, vessels, and neural elements. The parenchymal cells in the islets are different types of endocrine cells. The islets are surrounded by a variable amount of connective tissue fibers which are continuous with those of the interstitial septa of the exocrine pancreas (Deijnen van et al., 1992). In rat and man, the major part of the islet periphery is separated from the exocrine tissue by the peri-insular capsule. In the porcine pancreas, the peri-insular capsule is almost absent, where most of the peripheral islet cells are in direct contact with the exocrine cells. Peri-

insular immunostaining for the rat and human pancreas shows a discontinuous insular capsule that is essentially composed of collagen types I, III, IV, V and laminin (Deijnen van et al., 1992; Deijnen van et al., 1994). A sparse amount of connective tissue is also found within the islets.

As in other endocrine organs, the islet mass is highly vascularized. The islets receive approximately 10% of the pancreatic blood flow in comparison with their relative small volume (Lifson et al., 1980). The microvasculature of the islets is described as glomerular-like. The numbers of afferent vessels to the islets vary depending on the size of the islet (Bonner-Weir & Orci, 1982). Each arteriole gives rise to numerous capillaries that follow tortuous paths through the B cell core and then though the non-B cell mantle. Finally, the efferent capillaries coalesce into collecting venules which drain into larger intralobular venules. The islets also possess peripheral nerve fibers, closely associated with the endocrine cells. The studies from electron microscopy and histochemistry show that the islets are innervated by sympathetic, parasympathetic, and peptidergic nerves (Sundler & Botcher, 1991). Although the functional role of the innervation has not been fully clarified, it is known that insulin secretion is induced by parasympathetic stimulation and inhibited by sympathetic stimulation.

1.1.4-2 Cell types in the islets

The mammalian islets are composed of different endocrine cell types. The major cell types are the insulin-producing B cell, the glucagon-producing A cell, the somatostatin-producing D cells, and the pancreatic polypeptide-producing PP cell (Bonner-Weir, 1991). In addition to their specific hormones, islet cells also synthesize several neuronal-specific markers such

as tyrosine hydroxylase (TH) and neuron specific enolase (NSE) (Alper et al., 1988; Le Douarin, 1988).

The islet cell types can be differentiated by light microscopy, using immunocytochemical techniques. They can also be differentiated to some degree by electron microscopy, based on the appearance of the secretory granules. All islet cells possess the essential constituents associated with biosynthesis and release of peptide molecules (Boquist, 1981). The nucleus lies at the basal side of the islet cell, sparsely surrounded by secretory granules. A rich endoplasmic reticulum is mostly of rough type. The Golgi complex is well-developed and composed of cisterns, vesicles, and vacuoles. The mitochondria are usually medium-sized and possess a moderate number of transverse cristae. Microfilaments and microtubules are present in all islet cells, but the lysosomes remain relatively sparse.

The B cell

The insulin-producing B cells comprise 60-80% of the islet cells (Bonner-Weir, 1991; Boquist, 1981). By electron microscopy, the B cells are characterized by the appearance of the secretory granules. The granules, which are limited by single-layered membranes, are generally diffusely distributed in the cytoplasm. In most mammalian species, the secretory granules of the B cells possess a central core of moderate density surrounded by an electron-lucent space situated between the core and the membrane.

The B cells synthesize, store, and release insulin (Gold, 1989). Glucose is the main physiological regulator of insulin biosynthesis and secretion. The insulin gene is highly conserved among vertebrate species (Steinner et al., 1985). The human insulin gene is located on chromosome 11 and the rat insulin gene on chromosome 1 (Owerbach et al., 1980; Steinner et al., 1985). The insulin molecule is composed of two protein chains linked by two

disulfide bridges. The A-chain contains 21 amino acids and the B-chain 30. Insulin biosynthesis is initiated by synthesizing preproinsulin on the RER (Howell, 1991). Preproinsulin has an amino-terminal peptide extension called a signal peptide. After the membrane-associated cleavage of preproinsulin at the ER, proinsulin is transported into the ER lumen and properly folded. Proinsulin is further translocated to the Golgi complex where it is packaged in vesicles. During the formation and maturation of the secretory granules, proinsulin is cleaved by the proinsulin-processing endopeptidases to insulin and C-peptide in equimolar amounts (Hutton, 1994). Insulin and C peptide are stored together in the granule sac and released by exocytosis.

The A cell

The glucagon-producing A cell makes up approximately 15-20% of the islet cells (Bonner-Weir, 1991; Boquist, 1981). Ultrastructurally, the main distinguishing feature of the A cells is their secretory granules, in which large, high density cores occupy most of the space inside the granule membrane. The A cells synthesize, store, and release glucagon that is a 29 amino acid. The human glucagon gene is located on chromosome 2 (Tricoli et al., 1984) and shows more than 90% homology with hamster (Bell et al., 1983). Glucagon biosynthesis is similar to insulin through two intermediates, preproglucagon and proglucagon (Hazelwood, 1989). Glucagon secretion is regulated by nutrients and hormones (Porsman et al., 1991). Glucose and somatostatin inhibit glucagon release. Inversely, catecholamines and amino acids stimulate glucagon secretion.

The D cell

The D cells comprise 5-15% of the islet cells (Bonner-Weir, 1991; Boquist, 1981). Under electron microscopy, the D cells possess rather large secretory

granules with a rounded core of low or moderate density and a closely applied membrane. The D cells produce, store, and release somatostatin that is a 14 amino acid protein presenting in a cyclical configuration. Somatostatin biosynthesis is similar to insulin through two intermediates, preprosomatostatin and prosomatostatin (Hazelwood, 1989). Somatostatin was originally extracted from the hypothalamus and had the property of inhibiting the release of pituitary growth hormone (GH). In addition, somatostatin also suppresses the release of insulin and glucagon (Efendic & Luft, 1981).

The PP cell

The PP cells account for about 1% of the islet cells in pancreatic body and tail (Bonner-Weir, 1991; Boquist, 1981). The PP-rich cells are mostly found in the posterior part of the pancreatic head. The ultrastructural appearance is subject to species variation. Human PP cells contain elongated, high density granules. The PP cells produce, store, and release pancreatic polypeptide that is a 36 amino acid protein. The biosynthesis of pancreatic polypeptide is similar to insulin through two intermediates, prepropancreatic polypeptide and propancreatic polypeptide (Hazelwood, 1989). Pancreatic polypeptide inhibits exocrine pancreatic secretion of enzyme, ion, and water (Hazelwook, 1993).

1.2 Development of the pancreas

The pancreas develops from several buds appended to the primitive gut as part of the hepato-pancreatico-duodenal complex. The development of this gland can be divided into morphogenesis and cytogenesis (Adda , 1984; McLean, 1979). Morphogenesis is a developmental process involving changes in the morphology or form of embryonic structures. Cytogenesis is the process whereby cells acquire those special structural and functional features which distinguish their cell types.

1.2.1 Morphogenesis

The primitive gut of the embryo, an endodermal tube, forms during body flexion when the yolk sac infolds on the ventral aspect of the embryo and part of the yolk sac is incorporated into the embryo (McLean, 1979; Balinsky, 1972). The primitive gut tube is divided into three parts, the foregut, midgut and hindgut (Adda et al., 1984). The foregut is the anterior portion of the primitive gut and forms the pharynx, esophagus, stomach, duodenum proximal to the biliary tract, liver, biliary apparatus and pancreas. The hindgut is the posterior part of the cavity and gives rise to the distal portion of the transverse colon, descending colon and rectum. The midgut remains a gap where it is in open communication with the yolk sac. The midgut is the origin of the small intestines distal to the opening of the bile duct, ascending colon and the proximal part of the transverse colon.

The pancreas develops from two distinct diverticula, the dorsal and ventral pancreatic buds (Adda et al., 1984; Pictet et al., 1972; Githens, 1993; O'Rahilly & Muller, 1978). The dorsal pancreatic bud, the first to develop,

appears at about 20 somites (approximately 11 days of gestation) in the rat (Pectet et al., 1972; Githens, 1993) and slightly earlier than the 20 somite stage (the fifth week of gestation) in the human (Adda et al., 1984; Lee and Lebenthal, 1993). It forms as an evagination of the foregut growing into the dorsal mesentery in the proximal portion of the duodenum and contains a large dorsal duct opening to the gut lumen. The ventral pancreatic bud arises a little later and forms as an evagination of the proximal liver diverticulum. It is composed of two lobes (right and left) and arises from the lateral aspects of the liver diverticulum at its entry into the gut tube (Odgers, 1930). Each of the two parts of the ventral bud contains a small duct opening to the common hepatic duct. The left ventral bud and its duct usually regress completely during the fifth week of development in the human.

The rotation of the duodenum to the right carries the persisting right ventral pancreatic bud along with the common hepatic duct dorsally where they open into the gut lumen (McLean, 1979; Balinsky, 1972). The dorsal and ventral pancreatic buds come into apposition at this stage, that may remain completely independent throughout life, as in the dogfish (Balinsky, 1972). Finally, the two pancreatic buds fuse with each other to form a single organ at the 7th week in man and the 17th day in rat (Lee and Lebenthal, 1993; Githens, 1993).

In the fetus, the dorsal bud gives rise to the superior portion of the head, the neck, the body, and the tail of the pancreas (O'Rahilly & Muller, 1978; Adda et al., 1984). The ventral bud develops into the inferior portion of the head of the pancreas. The origin of the uncinate process is a subject of much debate (Adda et al., 1984). It is generally considered that it is derived from the ventral bud.

The dorsal and ventral pancreatic buds independently develop as a ramified system of tubules (McLean, 1979; Adda et al., 1984). The duct of the dorsal bud (duct of Santorini) opens directly into the duodenum, whereas the duct of the ventral bud (duct of Wirsung) opens into the common bile duct. Fusion of the two primordia is accompanied by anastomosis of the ducts. In man, the main pancreatic duct is commonly formed distally by the duct of the dorsal bud, and proximally by the duct of the ventral bud. The bile duct and the main pancreatic duct open in the papilla of Vater, which opens into the duodenum. In 5 to 10% of cases, the duct of Wirsung does not anastomose with the duct of Santorini (Adda et al., 1984; Lee & Lebenthal, 1993). In these cases, the duct of Santorini becomes the main duct and opens into the minor duodenal papilla, while the duct of Wirsung opens into the papilla of Vater.

1.2.2 Cytogenesis

The cytogenesis of the pancreas corresponds to the period of cell differentiation leading to the formation of acinar, islet, and mature duct cells (McLean, 1979). The studies devoted to this subject are based on light and electron microscopy descriptions and histochemical analysis of the developing pancreas.

1.2.2-1 Exocrine pancreas

The early pancreatic buds are formed by one layer of cuboidal epithelium as it arises from the foregut endodermal epithelium (Pictet et al., 1972; Laitio et al., 1974). The cells are linked by junctional complexes and their apices form a narrow lumen. At their basal side, they are encompassed by a continuation of the gut basal lamina. The pancreatic buds soon increase in the number and the height of their cuboidal cells. As a consequence, the buds

appear as either a pseudostratified or a multistratified organization. The pancreatic canaliculi increase in length and become tubular structures that branch progressively into the surrounding mesoderm. Between the ducts, the mesodermal cells differentiate into fibroblasts and form the primitive pancreatic stroma.

At 12 weeks in man, numerous outgrowths from the tubules give rise to early lobules (Laitio et al., 1974; Mclean, 1979). The multibranching epithelial tubes terminate distally in solid cords or in small clumps of cells which are termed as cell buds. The primitive acinar cells are identified by their basophilic cytoplasm. They begin to appear as small clumps of cells along the lateral walls and at the distal ends of the ducts. They are pyramidal in shape and may be located immediately adjacent to duct cells from which they originate. The duct cells retain their light cytoplasm and cuboidal shape. The primitive acinar cells contain small zymogen-like granules in their apical cytoplasm which lack immunoreaction to serine proteinases (Fukayama et al., 1980). The Golgi apparatus begins to develop in these acinar cells.

At 14-20 weeks a lobular configuration becomes more distinct (Laitio et al., 1974). Well-formed exocrine acini begin to emerge and the interstitial connective tissue diminishes progressively with age. The mature zymogen granules increase gradually in number as the number of the small zymogen-like granules show progressive decrease with fetal development. No transitions are observed between the small granules and the mature zymogen granules at this stage. During acinar cell maturation, there is a concomitant increase in the numbers of the Golgi apparatus and the rough endoplasmic reticulum. Immunoreactions to serine proteinases (trypsin, chymotrypsin, and elastase I) are found in the acinar cells during the 14th week (Fukayama

et al., 1980). Nevertheless, there is no immunoreaction with amylase in fetal and neonatal pancreatic tissue.

Primitive duct-like cells are the precursors of the adult duct epithelium (Githens, 1989). In the early stage of development, each pancreatic bud as it arises from the foregut endoderm is formed by cuboidal epithelium arranged to form a main duct. Branches of the developing main duct become the interlobular ducts, which further branch and give rise to the intralobular ducts. A lumen develops progressively in the center of the acinus and is surrounded by centroacinar cells which are the terminals of intralobular ducts. As the pancreatic ducts multiply, numerous acini are formed and the lobulation becomes gradually organized thoughout the gland. The definitive structure of the exocrine pancreas is apparent between the 6th and 8th months of gestation in man.

1.2.2-2 Endocrine pancreas

At 8 and 8.5 weeks of gestation in the human, the embryonic pancreas is formed by simple branching epithelial-cell-lined tubules surrounded by mesenchymal cells. No endocrine cells can be identified. The first endocrine cells which are observed in the endocrine pancreas are the A cells at 9 weeks (Falin, 1967; Like & Orci, 1972). In the following week the D cells appear and finally, the B cells are identified at 10.5 weeks. In the rat, the A cells are first identified at 11 days of gestation, concomitant with the formation of the pancreatic primordia (Pictet & Rutter, 1972; Dubois, 1989). The B cells are detected on day 12.5 by immunohistochemistry and the D cells on day 15. By electron microscopy, the different endocrine cell types can be identified on the basis of the morphology of their secretory granules. They do not have a continuous belt of junctional complexes as do the protodifferentiated duct

cells and are not physiologically polarized. The Golgi apparatus, RER, and mitochondria have no specific orientation within the cells.

At the early developmental stage, the pancreatic endocrine cells either lie in small groups connected with the wall of the ducts, or are scattered as single elements throughout the epithelial lining of the ducts (Falin, 1967; Pictet & Rutter, 1972). The endocrine cells are then interdigitated above the surface of the duct walls as the paratubular cell buds and gradually separate. The process of mass formation of the islets is continued by increasing in size and number. In the rat, there is a rapid increase of the islet volume, particularly during the last four days of gestation (Freie et al., 1975). The percentage of islet tissue slowly increases from 0.7% at day 19 to 1.5% on day 22. A further and much more rapid rise occurs during the first few days after birth. At the 5th postnatal day, the islets comprise 3.6% of the pancreas in comparison with 1.1% in adult rats. The non-B-cells of the endocrine pancreas appear more numerous than the B cells at the early stage. The same experiment shows that the percentage of the B cells per islet is about 22% at day 19, increases rapidly to 71% on day 22, and remains stable after birth.

It is generally believed that protodifferentiated duct epithelia give rise to islet cells in embryos (Vinik, 1992). However, ontogenesis of the endocrine pancreas has been a controversial topic for many years. By using cytochemical and electron microscopical studies, Pearse demonstrated that a group of apparently unrelated endocrine cells shared a number of features (Pearse, 1969). All these cells are able to store amines, to take up precursors of monoamine (such as DOPA and 5-hydroxytyptophan) and to decarboxylate them. These common functions led to the concept of an APUD system (amine-precursor uptake and decarboxylation). The APUD system includes many types of endocrine cells: pancreatic A and B cells, thyroid and C cells,

pituitary corticotroph and melanotroph, and adenal medulla cells. The cytochemical and ultrastructural similarities of these cells inferred their origin from a common ancestor, the cell of the neural crest. However, there was no embryological proof of the neural crest origin of the endocrine pancreas (Pearse & Polak, 1971). The neural crest is the region of the embryo where future neural and epidermal ectoderm meet in the neural folds. One of the most striking features of behavior of the neural crest cells is their migration out of the neural tube and their differentiation into tissues known to be of neural crest origin. Isotopic grafts of quail neural primordium into host chick embryo at the same stage of development permits study of the migration of the neural crest cells (Andrew, 1976). This model showed that the pancreatic endocrine cells are not derived from the neural crest. In another experiment, the ectodermal component of 9 day rat embryos was removed completely and the mesoendoderm was cultured in vitro for 11 days (Pictet et al., 1976). Insulin and B cells in approximately normal proportions were present with the exocrine pancreas in every culture. Therefore, the neural crest as an origin for the pancreatic endocrine cells is eliminated.

All the pancreatic cell types originate from a multipotential precursor, the protodifferentiated duct cells during pancreatic development. However, the mechanisms that lead to the selective differentiation and maintenance of a proper balance among the different types of the pancreatic cells remain obscure. The term "intermediate cell" is used to describe pancreatic cells with the morphological characteristics of more than one cell type (Melmed et al., 1972; Melmed, 1979). The intermediate cells such as 'acinar-islet' cells or 'A-B' cells have been reported from different species under normal and pathological conditions. The studies of the intermediate cells would be of considerable biological interest, since they pose a problem on the mechanisms

for selective differentiation of cells. Using a double-labeling immunostaining procedure, Teitelman showed that all the subsequent insulin-producing cells were positively-stained for glucagon at 12 days of gestation in the mouse (Teitelman, 1991; Alpert et al., 1988). The converse was not true; only 11% of glucagon-producing cells contain immunoreactive insulin. The number of double-labeled cells decreased gradually with age. At birth, only a small percentage of islet cells contain both insulin and glucagon. Also, islet cell precursors expressed other hormones in addition to glucagon and insulin. At 17 days of gestation, 17% of the insulin-producing cells contained somatostatin and 20% of insulin-producing cells contained PP. After birth, very few cells co-express insulin and either somatostatin or PP. These mixed phenotypes expressed by islet cells during development can be explained by an "activation-inhibition model". The islet cells originate from precursors that co-express more than one hormone at a certain stage of development. During maturation, most of the cells retain the expression of only one hormone and inhibit the appearance of the others. In this model, intermediate precursor cells undergo a multistep process of maturation that is characterized by a gradual segregation of each phenotype to a different cell type.

1.2.3 Biochemical development of the pancreas

By biochemical and ultrastructural observation, there is a biphasic pattern of appearance in exocrine and endocrine B cells during the embryonic development of the rat pancreas (Pictet & Rutter, 1972; Wessells & Cohen, 1967). A low, relatively constant level of the specific hydrolytic enzymes and insulin occurs between days 12 and 14 of gestation. This is followed by a marked increase in the synthesis of the exocrine proteins up to day 20 or 21.

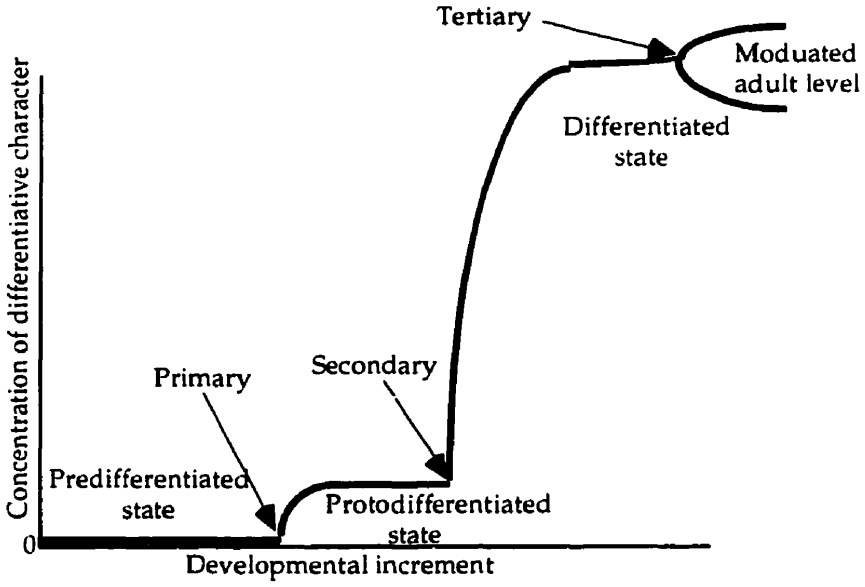

Figure 2-1 A model for cytodifferentiation of the rat pancreas (Rutter et al., 1968)

During this period of intense enzyme accumulation, there are concomitant increases in the content of RER, Golgi apparatus, and zymogen granules within the exocrine cells. At the same time, the concentration of insulin rises dramatically and differentiated B cells contain typical beta granules. Subsequently, the levels of enzymes and insulin may be altered, to some extent, to reach a final adult steady-state level. The coordinated accumulation of specific products and the morphologic changes of the differentiated pancreas suggest a multiphasic model of development with three regulator transitions and three steady states (Figure 2-1). This model implies that cytodifferentiation involves several discrete transitions at well-specified times during development.

1.2.3-1 The primary regulatory transition and the protodifferentiated state

The primary regulatory transition occurs between days 10 and 12 of embryonic development and involves the initial formation of the pancreatic diverticulum and the coincident synthesis of low levels of the digestive enzymes and insulin (Pictet & Rutter, 1972; Wessells & Cohen, 1967). A high level of glucagon and differentiated A cells are present at this time. However, there is no cytological correlation of the primary transition for the exocrine and B cells.

After the primary transition, the levels of the specific products produced by the exocrine and B cells remain rather low and constant for 2 to 3 days. The uniform appearance of the protodifferentiated cells is observed through the entire protodifferentiated state. No differentiated exocrine and B cells are detected by ultrastructural analysis. There is active cell division and continued formation of new lobules.

The primary regulatory transition appears to occur within one or two cell division cycles. Since there are several hundred cells in the presumptive pancreatic anlage, the primary transition would seem to have to occur in many cells at once. During this initial period, epithelial evagination of the foregut endoderm into the surrounding mesoderm results in a morphogenetic response followed by differentiation into acinar and islet cells. It has been proven in vitro that the mesenchymal-epithelial interaction is important for the primary transition and protodifferentiated state (Wessells & Cohen, 1967; Kathryn et al., 1977; Fell & Grobstein, 1968). Pancreatic epithelium was excised from the pancreatic rudiments of 10- or 11-day-old mice and cultured on one side of a transfilter with mesenchymal cells on the

other side. After several days in culture, normal development with the accumulation of differentiated-state enzymes and insulin ensued. In contrast, pancreatic epithelium cultured by itself failed to grow and contained no differentiated exocrine and B cells. These experiments demonstrate that some transmissible factor produced by mesodermal cells induces the differentiation of pancreatic cells. The experiments also show that the mesodermal requirement can be substituted by an embryo extract from mesodermal-rich tissues (Wessells & Cohen, 1966; Wessells & Cohen, 1967; Fell & Grobstein, 1968). It is of interest that the source of the mesenchyme is not crucial, in that mesenchyme from a variety of embryonic tissues would be sufficient. The mesenchymal extracts from older rat embryos or adult tissues such as muscle and liver are inactive (Ronzio & Rutter, 1973). The partially purified mesenchymal factor from chick embryos stimulates DNA synthesis and amylase accumulation in the protodifferentiated epithelium from rat pancreatic rudiments (Ronzio & Rutter, 1973). The mesenchymal factor is heat labile and destroyed by trypsin and periodate oxidation, but not by RNase or DNase (Wessells & Cohen, 1967). It is not a collagenous protein, since it is not sensitive to collagenase and collagen alone fails to stimulate pancreatic cell growth and differentiation (Wessells & Cohen, 1966; Ronzio & Rutter, 1973). It interacts with the cell surface and its action is mediated by cAMP (Filosa et al., 1975). The mesenchymal factor is presumably a glycoprotein and its chemical nature has not yet been defined.

1.2.3-2 The secondary regulatory transition and the differentiated state

The secondary regulatory transition occurs between days 14 and 18 of gestation following the protodifferentiated state. There is an abrupt and

dramatic increase in the levels of the exocrine enzymes and insulin during this period of time (Pictet & Rutter, 1972; Wessells & Cohen, 1967). The level of cellular proteins increases at least 10^4 -fold from the protodifferentiated to the differntiated state, while the concentration of RNAs increases several hundred-fold (Wessells & Cohen, 1967; Harding et al., 1977). The increased synthesis of cell specific proteins appears coupled with an increased formation of the RER and abundant appearance of zymogen granules by ultrastructure observation. Another prime feature of the secondary transition is the loss of cell proliferative capacity, in that differentiated pancreatic exocrine cells rarely divide (Wessells & Cohen, 1967). During the protodifferentiated state, ^{3}H-thymidine incorporation into DNA occurs in cells randomly distributed throughout the pancreatic epithelia, but at about the time of the secondary transition, dividing cells are restricted to the periphery of the epithelial tissue. Nondividing cells are observed in the central portions where zymogen granules are appearing. This indicates that the loss of proliferative capacity is linked to this phase of cytodifferentiation.

The mechanisms for the secondary transition are as yet unclear. It has been postulated that further changes at the genetic level result in different regulatory states at the transcriptional and translational levels (Wessells & Cohen, 1967). Studies of cDNA/mRNA hybridization show that between 14 and 20 days of gestation, the concentration of pancreas-specific mRNAs increases several hundred-fold, in parallel with the increased rate of synthesis of pancreas-specific proteins (Harding et al., 1977). This suggests that the increased production of these proteins is caused primarily by the increased transcription rather than the increased translation of mRNA. Furthermore, the thymidine analog 5-bromodeoxyuridine (BrdU), which is incorporated into DNA and subsequently inhibits functional RNA synthesis, dramatically

alters the pattern of pancreatic development in vitro. BrdU inhibited the cytodifferentiation of pancreatic rudiments derived from 14 day rat embryos in vitro (Wessells & Cohen, 1967; Harding et al., 1978; Van Nest et al., 1983). BrdU treated pancreata showed greatly reduced accumulation of digestive enzymes and of mRNAs for amylase and other specific proteins. Pancreatic rudiments grown in the presence of BrdU were healthy, but all the cells appeared with duct-like morphology (Githens et al., 1976). The use of two-dimensional gel electrophoresis demonstrated that BrdU treatment stimulated the secretion of a set of proteins apparently associated with duct cells (Van Nest et al., 1983). Recently, studies on tissue-specific gene expressions demonstrated that the early pancreatic rudiments express homeodomain transcription factor IPF1 (also known as IDX-1 or STF-1) (Ohlsson et al., 1993; Miller et al., 1994; Guz et al., 1995). IPF1 is a mammalian homologue of XIHbox8, which is expressed in *Xenopus* embryos in cells corresponding to endodermal cells that gives rise to the duodenum and developing pancreas (Wright et al., 1988). The expression of XIHbox8 in the adult *Xenopus* is mainly in the epithelial cells in the duodenum and the duct system of the pancreas. IPF1 probably has a determined role for the formation of the pancreas, since mice homozyous for this gene mutation show the complete lack of a pancreas (Jonsson, 1994).

Glucocorticoids that modulate the expression of pancreas specific genes have been shown to be important in pancreatic development (Harding et al., 1978; Van Nest et al., 1983; Rall et al., 1977). When the pancreatic rudiments at 13 or 14 days of gestation were cultured in the presence of dexamethasone, the specific activity of amylase increased 10-fold and the level of amylase mRNA increased 2.4 times. Dexamethasone-treated rudiments showed an enhanced number of zymogen granules and an increased amount of the RER.

However, glucocorticoids may play a modulatory role in pancreatic development, since the pancreas will develop normally in the absence of dexamethasone (Rall et al., 1977).

1.2.3-3 The tertiary regulatory transition

The secretory products of the differentiated cells present in late gestation are different both qualitatively and quantitatively from these in adulthood (Pictet & Rutter, 1972; Wessells & Cohen, 1967). The tertiary regulatory transition is characterized by adjustments of individual enzyme levels after birth. These adjustments may occur in response to certain specific changes in exogenous (nutritional) and endogenous (hormonal) factors. A carbohydrate-rich diet resulted in an increase in the specific activity of amylase with a concomitant decrease in the specific activity of trypsin and chymotrypsin in the rat pancreas (Schick et al., 1984). The converse is true for a protein-rich diet.

1.2.4 Functional development of the pancreas

1.2.4-1 The exocrine pancreas

The proteolytic activities of trypsin and chymotrypsin in the duodenal fluids of human infants are about 10% to 60% of the adult level (Lebenthal & Lee, 1980). The duodenal fluids of newborns and infants contain no amylase and only low levels of lipase. A refractoriness to CCK-PZ and secretin, which are potent secretagogues for the mature exocrine pancreas, has been observed in the pancreas of newborns and young infants. The degree of stimulated enzyme release shows an age-dependent increase postnatally and a full response of the pancreas to secretagogues is evident by age 2 years. Similar

results have been demonstrated in term-fetal and newborn rats (Doyle & Jamieson, 1978; Leung et al., 1986). The response of the exocrine pancreata to secretagogues is immature in spite of acquiring morphologic and biochemical characteristics of the adult gland several days before birth. The pancreas, at this age, fails to respond to CCK and carbachol but would respond to calcium ionophore A23187 and dibutyryl cAMP.

The lack of response of the exocrine pancreas to secretagogues may be due to the inadequacy of cell surface receptors. The acinar cells from the fetal and neonatal pancreas express about 10% of the number of CCK binding sites per cell in comparison with the adult gland (Rosenzweig et al., 1983). Also, binding of ^{125}I-CCK-8 to dispersed rat pancreatic acini at various perinatal ages shows that a low binding capacity of the high-affinity receptors to CCK is demonstrated (Leung et al., 1986). The binding capacity of the high-affinity receptors increases with age, whereas that of low-affinity receptors remains relatively constant throughout age. Similarly, the insensitivity of newborn pancreatic acini to cholinergic stimulation is related to a deficiency of muscarinic receptors (Dumont et al., 1981; Dumont et al., 1982).

The signaling mechanisms regulated by either cholecystokinin (CCK) or acetylcholine (ACh) involve mobilization of cellular calcium and activation of protein kinase C (Williams & Yule, 1993). The agonist-receptor interaction leads to a phospholipase C-mediated hydrolysis of phosphaidylinoditol 4, 5-bisphosphate to 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 induces release of calcium from the ER by binding to IP_3-gated Ca^{2+} -release channels in the ER membrane. The rise in cytosolic Ca^{2+} concentration activates calmodulin-dependent protein kinases. At the same time, DAG activates protein kinase C. Protein kinase C activation, calcium mobilization, and subsequent activation of calmodulin-dependent protein

kinase are regarded as important intracellular messages triggering exocytosis of pancreatic acinar cells.

Inability of the fetal pancreas in response to secretagogues may be also attributed to the immaturity of signal transduction events. A study in vitro shows that the rate of CCK-stimulated amylase released from the neonatal acinar cells (1 day after birth) increases at least 4 fold above that of the fetal acinar cells (1 day before birth) (Chang & Jamieson, 1986). Furthermore, the level of protein kinase C in the fetal and neonatal rat pancreas is very low and increases rapidly after birth, reaching to adult levels on postnatal day 2 (Shimizu et al., 1988). The calmodulin-dependent protein kinases are also deficient and increase 6 fold in concentration between 1 day before birth and 15 days after birth (Gorelick et al., 1987).

1.2.4-2 The endocrine pancreas

Although the B cells appear in the fetal pancreas at about 10.5 weeks gestation in man, plasma insulin has not been detected until 15 weeks of gestation (Thorell, 1970; Pronina & Sapronova, 1976). The level of plasma insulin in fetuses aged 15 to 28 weeks is low (range 2-8 mU/ml) and increases about fivefold from 28 to 32 weeks, up to 34 mU/ml. In the rat, the level of plasma insulin on day 19 of gestation is about 63 mU/ml and increases sharply to 101 mU/ml on day 21. It falls abruptly to 47 mU/ml on day 22 and continuously decreases after birth (Blazquez et al., 1974). Fetal plasma insulin is of fetal origin since insulin does not travel through the placenta (Adam et al., 1969). Unlike those in the adult pancreas, the B cells in the fetal and newborn human pancreas secrete little or no insulin in response to a glucose stimulus (Thorell, 1970; Grasso et al., 1968). Similar results have been reported in rodents and a low sensitivity of B cell response to glucose

stimulation remains until two days after birth (Asplund et al., 1968; Asplund, 1973). The insulin response to glucose shows a clear biphasic pattern in six day old rats. It has been postulated that the deficient insulin response to glucose could be due to an inadequate intracellular accumulation of cyclic AMP within B cells. In vitro studies with human fetal islet-like cell clusters demonstrate that theophylline and glucagon, both of which increase intracellular cAMP, stimulate insulin release (Otonkoski, 1988). The insulinotropic effects of glucagon and theophylline are independent of the ambient glucose level. Also, the potency of glucagon as a B cell stimulator indicates that glucagon may play a special role in the regulation of B-cell function during fetal development, probably by a paracrine effects.

Amino acids, such as arginine, are involved in regulating hormone production. The intravenous administration of a mixture of essential amino acids induces a striking increase in serum insulin in premature infants (Grasso et al., 1968). Culture of human fetal islet-like cell clusters show that arginine combined with glucose potentially induces insulin release (Otonkoski, 1988).

Glucagon has been detected by radioimmunoassay in the plasma of 105 day fetuses in the human and increases up to the 25th week of gestation (Assan & Boillot, 1973). As is the case with fetal insulin, glucagon in the plasma of the fetus does not cross the placenta (Girard et al., 1974). A full response of A cells to secretagogues or inhibition of glucagon secretion by glucose is not observed either in fetal mice or in fetal rat (Lernmark & Wenngern, 1972; Sodoyez-Goffaux et al., 1979). At birth, glucagon release can be stimulated by asphyxia (Johnston & Bloom, 1973). Arginine and alanine elicit a significant glucagon response while a glucose infusion suppresses it (Sperling et al., 1974; Grasso et al., 1983).

1.3 Cell differentiation in adult pancreas

Cell differentiation is a profound phenomenon involved in numerous physiological and pathological processes including embryological development, tissue regeneration, and carcinogenesis. By definition, cell differentiation is the process leading to the qualitatively and quantitatively selective realization of distinct parts of a given genetic material (Nover, 1982). These differential gene expressions result in the biochemical and morphological peculiarities characterizing the divergent cell types of an organism.

During the development of the pancreas, protodifferentiated duct epithelium gives rise to acinar, islet, and mature duct cells. Pancreatic cells retain a certain capacity of cell differentiation to replace cell loss and degeneration in the adult. The question of whether undifferentiated cells within the duct epithelium give rise to all cell types remains unclear. New secretory cells may arise 1) by differentiation from stem cells; 2) by differentiation of progenitor cells; 3) by transdifferentiation (Elsasser et al., 1986). A stem cell is a multipotential cell that is capable of rapid cell growth and division (Davila et al., 1990). In addition, a stem cell must have the potential to differentiate into two or more different cell phenotypes. A progenitor cell represents the progeny of a stem cell. It possesses more limited proliferation and differentiation potential and usually involves only a single cell lineage. Transdifferentiation is a change from one differentiated phenotype to another, involving morphological and functional phenotypic markers (Okada, 1986).

This part deals largely with islet and acinar cells and studies with rodents and human. It focuses on cell proliferation and differentiation in the adult pancreas under a variety of physiological and nonphysiological stimuli.

1.3.1 Differentiation of exocrine pancreas

1.3.1-1 Diet and hormones

Since the discovery that soybean trypsin inhibitor (SBTI) increased pancreatic weight and protease content of chicks in 1948, many factors inducing pancreatic growth have been recognized (Folsch, 1984). Such factors, which include cholecystokinin, secretin, carbachol, insulin, and epidermal growth factor, induce enzyme synthesis and cell enlargement (hypertrophy) or cell proliferation (hyperplasia). This part of the review will focus on factors regulating proliferation and differentiation of pancreatic cells closely related to my projects.

Soybean trypsin inhibitor (SBTI)

It is well known that feeding rats various trypsin inhibitors results in hypertrophy and hyperplasia of acinar cells with an increase in synthesis, content and secretion of pancreatic enzymes (Folsch, 1984). SBTI-fed rats showed an increase in pancreatic weight, DNA and RNA content (Oates & Morgan, 1982). The incorporation of ^{3}H-thymidine into total DNA was significantly higher during the first 4 weeks of SBTI feeding and then returned to the control value. Labeling indices of ducts increased over 10-fold in the first 2 weeks of feeding, dropped markedly thereafter and returned to normal by 4 weeks. The increase of acinar cell labeling was not observed until 2 weeks of feeding and remained significantly higher than control values for the next 10 weeks. These successive changes of labeling indices in the duct

and acinar cell population suggest that the cells with duct cell morphological characteristics may represent a precursor population, which divide and acquire the characteristics of acinar cells in hyperplastic pancreatic tissue.

Feeding rats SBTI also alters both the amount and composition of pancreatic enzymes. The specific activities of trypsin and chymotrypsin doubled after 10 days of feeding, while the specific activities of amylase and lipase were not affected (Temler et al., 1984). Northern blot analysis revealed that the levels of trypsinogen I and chymotrypsinogen B mRNAs increased five-fold after 2 days of intraduodenal perfusion with SBTI (Rosewicz et al., 1989). Also, SBTI perfusion induced a significant decrease in ribonuclease mRNA levels, but had no effect on amylase mRNA levels.

The effects of SBTI on pancreatic proliferation and differentiation are thought to be controlled by increasing circulatory levels of CCK. It has been postulated that removal of trypsin by its binding to a trypsin inhibitor leads to the release of CCK from the gut mucosa, indicating a negative feedback regulation by intestinal trypsin (Green & Lyman, 1972; Green et al., 1972). Experiments showed that repeated CCK administration mimicked the effect of SBTI on pancreatic weight, enzyme content and secretion (Temler et al., 1984; Rosewicz et al., 1989). Feeding rats SBTI resulted in an increase of plasma CCK levels and administration of the specific CCK receptor antagonist totally abolished the effect of SBTI treatment (Rosewicz et al., 1989).

Epidermal growth factor

Epidermal growth factor (EGF) is a single-chain polypeptide of 53 amino acid residues which stimulates the growth of a variety of tissues in vivo and in vitro (Carpenter & Cohen, 1979). EGF, originally isolated from the mouse submaxillary glands, is present in many types of human tissues, including thyroid gland, duodenum, jejunum, and kidney (Hirata & Orth, 1979). It has

been demonstrated that EGF is also produced in the human pancreas and excreted into the pancreatic juice (Hirata & Orth, 1979; Hirata et al., 1982). EGF is observed on the luminal surface of the columnar epithelial cells of the pancreatic ducts, but is not detected in the acinar cells and islet cells by immunofluorescent studies (Vaughan et al., 1991). The receptors for EGF in pancreatic tissue are mainly distributed in the acinar cells, intercalated duct cells and islet cells (Chabot et al., 1987).

The effect of EGF on the exocrine pancreas is quite controversial. Previous studies demonstrated that administration of EGF intraperitoneally stimulated DNA synthesis and caused an increase in pancreatic weight and the content of DNA and RNA in the rat pancreas (Dembinski et al., 1982). Recently, Morisset and colleagues have shown that injection of EGF subcutaneously does not alter pancreatic weight and protein content (Morisset et al., 1989). The same study also demonstrated that EGF inhibits ^{3}H-thymidine incorporation into DNA by 44% and decreases DNA content by 20% after 4 days of treatment, but increases the concentrations of amylase and chymotrypsinogen by 106% and 232%, respectively. In vitro studies with monolayer cultures of pancreatic acinar cells have revealed that EGF leads to a significant increase in DNA synthesis and the total content of DNA and protein (Logsdon, 1986). EGF also exerts trophic effects on cultured pancreatic duct cells. Studies on isolated interlobular and main duct cells from the guinea pig pancreas indicated that EGF significantly increases BrdU incorporation into DNA (Bhattacharyya et al., 1995).

1.3.1-2 Partial pancreatectomy

Unlike the liver, the pancreas after partial pancreatectomy appears to have a limited regenerative capacity to restore to the preoperative level (Lehv &

Fitzgerald, 1968; Pearson et al., 1977; Sommer, 1987). It has been postulated that the limited regeneration with partial pancreatectomy may be due to the lack of a connective tissue framework, since more rapid and extensive regeneration occurs after ethionine administration (Lehv & Fitzgerald, 1968).

A 55% pancreatic resection led to an increase in thickness and weight of the residual segments, which was 50-60% heavier than the corresponding control segments at 9 month after operation (Lehv & Fitzgerald, 1968). Similar results were observed by Pearson, in which 50, 70, and 90% removal of the pancreas brought on the corresponding increase in weight of the remaining gland by 21, 32, and 78% above the control segments (Pearson et al., 1977). The ^{3}H-thymidine uptake in the remnant pancreas after a 90% pancreatectomy increased 5-fold at postoperative day 3, decreased gradually thereafter, and returned to control level by day 14 (Oikawa et al., 1993).

The previous study showed that the labeling indices of acinar cells increased 6- to 7-fold at 3 days after 90% removal but lasted only 5 days following resection (Pearson et al., 1977). Recently, Bonner-Weir gave more details on the proliferation and differentiation of the exocrine and endocrine pancreas by labeling proliferating cells with BrdU in this model (Bonner-Weir et al., 1993). The elevated BrdU-positive cells were first seen in the common pancreatic duct at 24 hours after 90% partial pancreatectomy, then in the main ducts and interlobular ducts. The ductules started to proliferate at 48 hours after resection, when the labeling cells in the common ducts returned to control values. Sixty hours post surgery, elevated numbers of BrdU-positive cells were limited to the focal regions of small ductules surrounded by connective tissue and blood vessels, where all cell types were stained for BrdU. The small ductules in these focal regions differentiated into new lobules of the pancreas, containing new islets and acini within a few days.

This study suggested that the pancreatic ducts retain the capacity to give rise to both pancreatic endocrine and exocrine cells in adult rats.

1.3.1-3 Pancreatic necrosis after ethionine

Ethionine-induced pancreatitis has been shown to occur in different species of experimental animals, including the mouse, rat, hamster, and guinea pig (Lombardi et al., 1975; Fitzgerald et al., 1968a; Boquist, 1969; Wenk et al., 1974). Administration of ethionine with a protein-free diet to rats caused the progressive destruction of the pancreatic acinar parenchyma (Fitzgerald et al., 1968a). After 10 days of ethionine injection, there occurred marked cytoplasmic vacuolation, necrosis, or dissolution of the acinar cells. The acinar cells in the pancreatic remnant were less than 15-20% of the total number in the control animals. The pancreatic weight lost about 70% during the same period of ethionine treatment. However, the pancreas showed little morphologic alteration in the appearance of duct, ductular, vascular, or islet cell tissues. Regeneration of the acinar cells began on day 12 (the second day of standard diet feeding) and increased greatly afterwards. Within 2-3 weeks after standard diet feeding, the regenerated acinar tissue restored the pancreas to normal configuration. The pancreatic weight increased steadily after the cessation of ethionine treatment and reached the pre-experimental weight of the gland over a period of 3-4 weeks. Autoradiography showed that labeling indices of the acinar cells started to rise on day 12 and reached a peak value of about 10% on day 15, which was about 20 times over controls (Fitzgerald et al., 1968b). The labeling indices decreased slowly and were at control levels at day 36. The labeling indices of the duct and interstitial cells were significantly higher than the control values at day 12 through 15, with a peak value of about 3% (4-5 times over controls) occurring at day 13, 2 days early the labeling

peak of the acinar cells. Nevertheless, the increased nuclear labeling in the duct and interstitial cells was explained as a secondary response to pancreatic degeneration or regeneration.

1.3.2 Differentiation of endocrine pancreas

1.3.2-1 Hormones

The effects of hormones on the pancreatic islets have been extensively studied since the discovery in 1953 that cortisone induces a hyperplasia of the pancreatic islet cells (Hellerstrom et al., 1976). This review will focus on growth hormone (GH) and prolactin, which have been found to be potential growth and differentiation factors for the endocrine pancreas (Neilsen et al., 1993).

Growth hormone has long been thought to have an effect on islet cells. Administration of growth hormone repeatedly produces an increase of great magnitude in the concentration of serum insulin and induces diabetes in dog (Campbell & Rastogi, 1966). Hyperinsulinemia was also observed in rats bearing the MtT-W15 tumor secreting growth hormone and prolactin (Martin et al., 1968; Peake et al., 1969). Due to hypertrophy and hyperplasia of the islet cells, the tumor-bearing rats had a significantly increased islet/acinar ratio and an enlarged size of islets. Besides the increase in insulin content, the pancreas of these animals frequently contained immunoreactive insulin in close association with pancreatic ducts, where the insulin-positive cells appeared as an integral component of the duct epithelium (Parsons et al., 1983).

Ectopic anterior pituitary grafting provided a unique model in vivo for investigating the effects of hyperprolactinemia on the pancreas (Matsuda et

al., 1994). The serum prolactin level in the pituitary grafted mice elevated to more than 10 fold that in controls, whereas the serum GH level was only little affected. Pituitary grafting resulted in a significant increase in the weight of the pancreas and the DNA content as well as the protein content. The BrdU-labeling cell nuclei were mainly observed in B cells and acinar cells, but were rarely encountered in A cells.

In vitro studies demonstrated that both growth hormone and prolactin promoted proliferation of pancreatic B cells and increased the rates of insulin release in cultured islets isolated from rats (Nielsen et al., 1989; Brelje et al., 1994). In addition, long-term administration of growth hormone produced a significant rise in insulin biosynthesis of B cells (Nielsen et al., 1989).

1.3.2-2 Duct obstruction

Ligation of the pancreatic duct results in a rapid loss of acinar tissue, while the endocrine pancreas remains relatively intact (Jonsson, 1968). It is well known that Banting and Best used this model to make pancreatic extracts in the treatment of diabetes, which eventually led to the discovery of insulin (Samols, 1981). Morphological studies demonstrated that duct ligation of rat pancreas provoked progressive degeneration and atrophy of the acinar cells (Edstrom & Falkmer, 1968). The acinar parenchyma disappeared completely five days post operation and never reappeared. Due to extensive proliferation of duct cells, there was a marked increase in the number of tubular structures (Edstrom & Falkmer, 1968; Walker et al., 1992). Ductular transformation from the acinar structures has also been postulated by some authors (Mann et al., 1979). Outbudding of islet cells from ductular structures was observed one week after duct ligation (Edstrom & Falkmer, 1968). This neoformation of

islet tissue around ductules appeared as either ovoid or kidney shape, which ultimately transformed into the ordinary shape of islets.

Partial duct obstruction induced by wrapping of the hamster pancreas leads to the development of duct cell proliferation in association with new islet formation but in the absence of pancreatitis (Rosenberg et al., 1983). This model was developed by Rosenberg and Duguid in our laboratory when they tried to demonstrate the relationship between duct obstruction, hyperplasia, and carcinoma of the pancreas (Rosenberg et al., 1984). Five days after surgery, the epithelium of the duct in the head of the gland was hyperplastic and goblet cell metaplasia became increasingly evident. Newly formed islets were found in relation to hyperplasia and hypertrophy of the terminal ductules as early as two weeks post surgery. Autoradiography showed that the labeling index of ductular cells increased at 8 weeks, reached a maximum at 10 weeks, and then returned towards the initial value by 18 weeks, while the labeling index of islet cells elevated steadily from 10 to 18 weeks and declined thereafter (Rosenberg et al., 1989). This study suggested that proliferating endocrine tissue might be derived from cells located in the ductular epithelium.

1.3.2-3 Transgenic mice

A transgenic mouse strain bearing the interferon-gamma (IFN-γ) gene expressed in the pancreatic islets exhibits the progressive destruction of pancreatic islets, which eventually leads to diabetes (Sarvetnick et al., 1988; Sarvetnick et al., 1990). Pancreatic lesions were mild in neonatal mice and became more severe with age. Pancreata from transgenic mice revealed histologically an accumulation of a variable number of inflammatory cells within the parenchyma at one month of age. The widespread inflammation

of pancreas progressed by 10-20 weeks, when the disruption of islet and acinar structures became apparent. The pancreatic ducts enlarged and fused to form an array of ductal systems, in which the buds of variable cell mass occurred at different loci along the length of duct wall (Gu & Sarvetnick, 1993). In some instances, the islet-like structures were seen to protrude into the lumen of ducts, which was named 'reversed budding' as opposed to the normal budding that occurs in embryonic islet morphogenesis. Immunoreactive hormones including insulin, glucagon and somatostatin were present in the cells either as isolated single cells or clusters within the duct wall or the islet-like structures.

The intermediate cells have been observed in fetal islet neogenesis (see 1.2.2-2). The multiple hormone-containing cells represent an 'intermediate state' between newly formed endocrine cells and their mature phenotype. Transitional cells were also observed in this model, in which about 16% of the insulin-bearing cells within the duct walls and islet structures expressed an additional islet hormone (Gu et al., 1994). A high proportion of these double hormone-containing cells co-expressed insulin and glucagon. With double immunostaining, the ductal/acinar and exocrine/endocrine intermediate cells were also demonstrated in this model.

1.3.3 Maintenance of pancreatic cell differentiation in vitro

1.3.3-1 Duct cells

Although pancreatic ductal cells have been well characterized morphologically and physiologically by the studies of intact glands, an extensive characterization of the biochemical properties and cell behavior of ducts is required to retain their differentiated states in vitro. However, the

efforts to isolate and culture ductal cells were not successful until the seventies. Possible impediments to the isolation and study of ductal cells in vitro include: ductal cells are a minor cell type in comparison with acinar cells; the anatomical complexities make ducts more difficult to separate from surrounding connective and acinar tissue; and ductal cells do not produce obvious protein products.

Isolation of duct cells

The conventional methods to obtain ductal tissues are by microdissection and dissociation of the gland with collagenase. Microdissection is a mechanical method, which had been used for the isolation of ductal cells from the pancreas of cat, rat, and cow in the seventies (Wizemann et al., 1974; Singh et al., 1978; Stoner et al., 1978). With this technique, the common bile/main ducts were dissected from the gland and acinar tissue was trimmed away. Obviously, the procedure for isolation is tedious and considerable technological difficulties are encountered in the separation of ducts from acinar cells. In addition, the ducts isolated by this method are severely damaged by the mechanical trauma, which results in the low yield of viable cells (Sato et al., 1983).

Partial enzymatic digestion of the pancreas for isolating ducts was a modification of the procedure for pancreatic islet isolation, which was first introduced by Githens and collaborators in 1980 (Githens et al., 1980a). Pancreatic ducts obtained by this way have been reported from different species, including rat (Githens et al., 1980a; Chen et al., 1985; Tsao & Duguid, 1987), mice (Githens et al., 1992), hamster (Hubchak, 1990; Githen et al., 1987), guinea pig (Hootman & Logsdon, 1988), cow (Sato et al., 1983) and human (Trautmann et al., 1993). With this method, the pancreas was minced into small fragments and digested by collagenase (Githens et al., 1980a). The ductal

fragments were further purified either by hand picking (Hootman & Logsdon, 1988), or by sieving (Githens et al., 1980a; Hubchak,1990). In the seventies, Singh tried to separate duct cells from enzymatic digestion of the rat pancreas using discontinuous Ficoll gradients but was unable to get satisfactory results (Singh et al., 1978). Githens tried this technique to free the ducts from surrounding tissues with success (Githens et al., 1980a). However, he also found that this method for separation was less efficient than the sieve technique. Enzymatic digestion of the gland for isolating ducts yields a mixture of ducts ranging in size from the main duct to the intercalated ducts, although the majority are interlobular ducts. The maximal yield by this method is about 180 duct fragments/gram wet weight of the rat pancreas (Githens et al., 1980b). The major disadvantage of this technique is the difficulty in purifying significant quantities of small interlobular/intralobular and intercalated ducts. Also, it is a considerable problem to avoid contamination of ductal tissue with other tissues, especially acinar cells.

One way to increase the yield of isolated ducts and avoid the contamination of acinar cells is to use pancreatic tissue taken from copper-deficient rats (Arkle et al., 1986). In rats, feeding of a copper-deficient diet causes a noninflammatory atrophy of the acinar cells but leaves the ducts structurally intact (Smith et al., 1982).

Culture of duct cells

Stoner et al. were first to report the growth in vitro of isolated ducts in 1978 (Stoner et al., 1978). Ductal epithelial cells isolated from bovine pancreas by microdissection had been grown continuously in monolayer culture for 30 weeks. Since then, the ductal epithelial cells have been maintained and propagated in the different culture environments as monolayers from the rat, hamster, guinea pig and human (Tsao & Duguid, 1987; Hubchak, 1990;

Hootman & Logsdon, 1988; Trautmann et al., 1993). Cells spread rapidly from duct fragments and formed epithelial monolayers when they were cultured on uncoated plastic or on plastic coated with thin layers of rat tail collagen or human placental collagen (Hootman & Logsdon, 1988). However, these cells were squamous in appearance, devoid of the basolateral membrane amplification and apical microvilli. Cells did not spread from duct fragments when cultured on Matrigel, a reconstituted basement membrane. When ductal cells were cultured on either a thick layer of rat tail collagen or cellulose ester membranes, they formed confluent monolayers of cuboidal epithelial cells and retained the morphological properties of freshly-isolated duct cells. Interestingly, Tsao and Duguid showed that propagable ductal epithelial cells isolated from rat pancreas retained some capacity to differentiate into acinar-like cells during early passages, especially when cultured on a mixed ester cellulose membrane (Tsao & Duguid, 1987).

Ductal cells isolated from the pancreas of rodents were also cultured in either agarose or collagen gel as three-dimensional structures for up to 6 weeks (Githens et al., 1992; Githens et al., 1987; Githens et al., 1980b; Githens et al., 1989). During the early stages of culture, the cut ends of most ducts sealed to create lumina. The ductal cysts continued to increase in size due to fluid-electrolyte secretion by ductal cells. The walls of the ductal cysts consisted of a single layer of cuboidal epithelium surrounded by connective tissue. The particular advantage of culturing ducts in this system is that the cultured duct cells maintain a tubular structure as they do in vivo.

1.3.3-2 Acinar cells

In vitro studies of pancreatic acinar cells have been carried out using several systems such as an isolated perfused pancreas (Scratcherd, 1986),

pancreatic lobules (Scheele & Palade, 1975), and an organ explant culture (Resau et al., 1983). However, these systems contain mixed populations of islet, duct and acinar cells, which makes them unsuitable models for studies of acinar cell growth and differentiation. Like other exocrine gland cells, pancreatic acinar cells have been difficult to isolate and maintain in culture. Recently, some studies have reported that acinar cells have been maintained under culture conditions for several days and even up to one month.

The technique for preparation of dispersed pancreatic acinar cells was originally developed by Amsterdam and Jamieson in 1972 (Amsterdam & Jamieson, 1972). The gland is dissociated by repeated digestion with collagenase-hyaluronidase, with an interposed chelation of divalent cation using EDTA (Amsterdam & Jamieson, 1972; Amsterdam & Jamieson, 1974). After mild shearing, dispersed single acinar cells are separated by passing through a nylon mesh and by using a discontinuous Ficoll gradient (Oliver, 1980). Cell yields are 50-60%, based on recovered DNA (Amsterdam & Jamieson, 1972), and 40%, based on a dry weight basis (Schulz et al., 1988). This preparation contains about 95% acinar cells; the remainder consisting of islet, duct, and vascular endothelial cells (Amsterdam & Jamieson, 1974).

Oliver was first to demonstrate the maintenance of pancreatic acinar cells in vitro in 1980 (Oliver, 1980). Dispersed single acinar cells isolated from rats were cultured as suspension cultures at 35° C with the appropriate secretagogues. Under these conditions, the cells remained differentiated for up to 1 month in culture but no cell replication was observed. A monolayer culture of mouse pancreatic acinar cells has been described by Logsdon and Willams, who managed to retain acinar cells by culturing on collagen gel for up to 14 days (Logsdon & Williams, 1986). The acinar cells first underwent a period of adaptation, during which there was a decrease in the content of

DNA and protein. Then, the cells spread out, divided and subsequently formed confluent monolayers. Nevertheless, the cellular content of the digestive enzymes remained only about 4% of the initial specific activities by day 9. The cultured cells did not show stimulation of enzyme secretion with caerulein, a pancreatic secretagogue.

A reconstituted basement membrane gel is more suitable for the attachment and growth of acinar cells in vitro as compared to several types of substrata, including plastic, glass, laminin, and type 1 collagen (Oliver et al., 1987). When dispersed single acinar cells were seeded onto this reconstituted basement membrane gel, the cells reassociated themselves into acinar-like structures and retained their differentiated morphology (Bendayan et al., 1986).

1.3.3-3 Islet cells

Isolation and maintenance of islet cells in vitro are important for several reasons, including the development of an in vitro system to study metabolic characterization of the endocrine cells and preparation of islets intended for eventual transplantation. Since the first method was described by Moskalewski in 1965 (Moskalewski, 1965), a variety of in vitro preparations have been developed. These techniques have been employed to study islet tissue from different species.

Isolation of islet cells

The conventional method for the isolation of islets from rodent pancreas was adapted from the original procedure reported by Lacy and Kostianovsky (Lacy & Kostinovsky, 1967). The pancreas is minced into small pieces and dissociated by collagenase digestion (Wollheim et al., 1990). The islets are separated from exocrine tissue by either discontinuous Ficoll gradients or

hand-picking. For the preparation of single islet cells, the purified islets are further dispersed by trypsin-EDTA treatment.

Due to the fibrous, densely packed texture of the human pancreas, human islets have been difficult to isolate. Attempts at applying the technique for rodent islet isolation to the preparation of human islet tissue yielded unsatisfactory results, with only a small part of pancreas used for the isolation (Adersson et al., 1976; Weber et al., 1977). Both the number and the purity of islets were low (Sutherland et al., 1974). In 1984, Gray et al. introduced a method for the isolation of islets from the whole human pancreas (Gray et al., 1984). The gland was distended by intraductal injection of collagenase and incubated at 39° C. After dissociating the gland by a process of teasing and shaking, the islets were purified by sieving and Ficoll gradients. In spite of this successful effort, the yield of the islets was about 1000 islets/g pancreas and the average purity of the islets was about 30%.

A method for the reproducible high-yield isolation of human islets has recently been developed by Ricordi and collaborators (Ricordi et al., 1988). This automated method minimized traumatic action on the islets and avoided overdigestion of the islets during the isolation processes. After purification on Ficoll gradients, an average of 2279 islets/g of pancreas was obtained with a purity greater than 60%. The islets were well preserved morphologically and responded to glucose stimulation with a fivefold increase from the basal insulin secretion after an overnight culture.

Culture of islet cells

A free-floating culture is one of the most frequently used methods to maintain islet cells in vitro. With islets kept afloat, their intact structures were preserved and a better response to glucose stimulation was found (Andersson, 1978). Another advantage of culturing in suspension is to avoid

fibroblast outgrowth (Wollheim et al., 1990). However, there are several disadvantages, including the difficulty to perform a complete medium change and the larger islets tend to develop central necrosis. Adult human islets have been maintained by culturing in such a way for up to 9 months, with an intact insulin production (Nielsen et al., 1979). The constituents of the culture medium, especially the glucose concentration, are important for retaining the endocrine function of the islet cells. Among different media, RPMI with 11 mmol/l glucose was found to be superior in response to glucose stimulation for mouse islets after one week in culture (Andersson, 1978). A long-lasting exposure of islets to high glucose concentrations would impair the function of pancreatic islets from human and rat (Eizirik et al., 1992; Svensson & Hellerstrom, 1991; Davalli et al., 1991). In addition, islets cultured at high glucose concentrations showed a decrease in insulin content, a deficiency in glucose-induced insulin release and a deficiency in glucose oxidation. Nevertheless, these functional impairments could be partially reversed by an additional culture in a normal glucose medium.

Dispersed single cells from rat islets were also cultured in suspension, which may provide a unique model for studying cell interactions between islet cells in vitro (Takaki et al., 1975; Ono et al., 1979). The free-floating single cells adhered to each other and formed small cell aggregates consisting of two to three cells after a few hours in culture. These aggregates became larger as the culture continued and appeared spherical in shape, composed mainly of B-cells and a few A- and D-cells, within 1 week. They were similar to the undissociated islets in appearance, but not as compact as native islets. Hormone secretion by the cultured islet cells remained at constant levels for 50 days and declined during the subsequent 30 days.

Monolayer culture is another commonly used technique to maintain morphologically and functionally intact islet cells in vitro. This can be achieved by seeding either a single cell suspension (Weir et al., 1984; Weir et al., 1986) or intact undissociated islets (Beattie et al., 1991; Jain et al., 1985; Montesano et al., 1983a). Monolayer formation was apparent on uncoated plastic or on coated plastic with poly-L-lysine, gelatin, Matrigel, collagen, bovine corneal endothelial cell matrix (BCEM) and disrupted fibroblasts (Weir et al., 1984; Beattie et al., 1991; Montesano et al., 1983a). BCEM was found to be superior to other substrata in response to glucose stimulation after 1 week of culture of human islet cells (Beattie et al., 1991). It is noteworthy to point out that dispersed single cells formed islet-like organoids when they were entrapped in collagen (Montesano et al., 1983a). Single cells seeded on the surface of collagen gel became flattened clusters of endocrine cells. These flattened clusters sandwiched between the two collagen layers gradually changed into cell aggregates, 60 to 250 mm in diameter. The topographical distribution of endocrine cells within these aggregates was similar to the native islets, in which B cells were usually concentrated in the central portion of the aggregates with non-B cells at their periphery. However, it is not known whether the formation of the islet-like organoids arises from a redistribution of pre-existing cells or from cell proliferation and differentiation.

1.3.4 Transdifferentiation

The phenomenon of transformation of one differentiated cell type to another type of cell is known as transdifferentiation or metaplasia (Okada, 1986; Kodama & Eguchi, 1993; Slack, 1986). During this process, cells completely lose their original morphological and biochemical characteristics

and acquire features of an entirely different cell type. This phenomena has been observed in different cell types of amphibia and mammalia under physiological and pathological conditions. Transdifferentiation can occur between cell types belonging to the same cell class, the same cell lineage, or different cell lineages. This review will focus on cell transdifferentiation in the adult pancreas.

1.3.4-1 Pancreatic hepatocytes

Pancreatic hepatocytes were originally reported in 1981 by Scarpelli and Rao in the regenerative pancreas following ethionine administration (Scarpelli & Rao, 1981). Pancreatic injury was induced by feeding hamsters a methionine-deficient diet combined with simultaneous injections of ethionine for 8 days. Regeneration of pancreatic cells was initiated on the 9th day by giving a large dose of methionine and returning to the full amino acid diet. Numerous foci of hepatocytes developed after a single injection of the pancreatic carcinogen N-nitrosobis(2-oxopropyl)amine (NBOP) to hamsters during the S phase of pancreatic cell regeneration.

Feeding of copper-deficient diet for 8-10 weeks in rats caused extensive pancreatic atrophy with a loss of 85-90% of acinar cells (Rao et al., 1986). Multiple clusters of hepatocytes were randomly distributed throughout the pancreas after placing on a normal diet for another 12 to 15 weeks following a copper depletion regimen. In long-term cultured organ explants from hamster pancreata, hepatocytes appeared more frequently in the carcinogen-treated explants than that in the controls (Resau et al., 1985).

The hepatocytes in the hamster and rat pancreata are indistinguishable from hepatic parenchymal cells, both morphologically and functionally (Scarpelli & Rao, 1981; Rao et al., 1986). They are polyhedral with a centrally

placed nucleus and a granular eosinophilic cytoplasm. The cytoplasm contains abundant peroxisomes and glycogen. In the pancreas of rats fed methyl-deficient, ethionine-supplemented diets, hepatocytes always lay immediately adjacent to the islets of Langerhans (Hoove & Poirier, 1986), while they were present in continuity with intercalated ducts or in the fatty stroma in the pancreas of hamsters with copper depletion-repletion regimes (Rao et al., 1988). Albumin was localized in all hepatocytes in these pancreata by immunofluorescence methods. Pancreatic hepatocytes expressed several liver-specific genes including carbamoyl phosphate synthetase, a mitochondrial enzyme and urate oxidase, a peroxisomal enzyme (Makino et al., 1990). Administration of ciprodibrate, a potent hepatic peroxisome proliferator, caused an increase in peroxisome population of pancreatic hepatocytes (Rao et al., 1986; Reddy et al., 1984).

Two fundamental questions that are pertinent to the transdifferentiation of the pancreatic cells to hepatocytes are the mechanisms for triggering this change and the histogenesis of these cells. Phenotypic transformation is effected through a process in which the original expression of pancreas-specific genes is blocked with simultaneous activation of liver-specific genes that are normally blocked in pancreatic cells. In the ethionine model, it had been postulated that the interaction of the pancreatic carcinogen NBOP with DNA at the peak of acinar DNA synthesis might activate a battery of liver-specific genes in the pancreas (Scarpelli & Rao, 1981). However, a recent study suggested that such a change could not only be initiated but could continue during acinar cell regeneration without any mutagenic or carcinogenic stimulus (Kitazawa et al., 1990). Administration of NBOP to hamsters before the occurrence of pancreatic regeneration would inhibit the appearance of pancreatic hepatocytes in a dose-dependent manner. It was also speculated

that the hepatization of pancreatic acinar cells in ethionine-treated hamsters might be the consequence of hypomethylated DNA (Hoove & Poirier, 1986).

The histogenetic pathways leading to the differentiation of hepatocytes in the pancreas are not fully understood. Scarpelli and Rao originally suggested that the hepatocytes in the pancreas of the ethionine-treated hamster were derived from acinar cells, since hepatocytes appeared during acinar cell regeneration (Scarpelli & Rao, 1981). However, morphological studies demonstrated that the hepatocytes were found within the epithelial cell lining of ductules during the early stages of pancreatic regeneration (Makino et al., 1990). It appeared that the hepatocytes arose from duct cells in the apparent absence of cell proliferation. Using in situ hybridization, albumin mRNA was localized in interstitial cells and ductular cells in the pancreas of rats at the end of seven weeks on a copper-deficient diet (Rao et al., 1989). Pancreatic hepatocytes were identified within the ductules and the interstitium immediately after the rats were returned to a normal diet. Therefore, both the ductular cells and interstitial cells might be considered as stem cells, which were capable of differentiating into pancreatic hepatocytes. Furthermore, Chen and colleagues demonstrated that cultured ductal epithelial cells from rat pancreas differentiated into hepatocytes after six weeks following in vivo implantation (Chen et al., 1995). The pancreatic hepatocytes expressed mixed phenotypes, both hepatocytes and ductal cells, including the expression of α-fetoprotein, tyrosine aminotransferase, carbonic anhydrase II, and cytokeratin 19.

1.3.4-2 Transdifferentiation of acinar cells to ductal cells

The phenotypic changes of cultured acinar cells to ductal cells were first observed by De Lisle and Logsdon, in which the acinar cells of an adult mouse

pancreas were cultured on type 1 collagen (De Lisle & Logsdon, 1990). In this study, monoclonal antibodies specific for acinar cells (mAb Acinar-1) and ductal cells (mAb Duct-1) were developed to investigate the cell type-specific antigen expression in the primary cultures (De Lisle et al., 1988). The starting material for the monolayer cultures consisted of predominantly Acinar-1 positive cells, and a few Duct-1 positive cells were also observed. The cultured acinar cells rapidly lost both their typical morphological appearances and Acinar-1 antigen during the cell growth, while acquiring duct-like phenotypes and expressing Duct-1 antigen. The cells progressively regained the acinar antigen with a concomitant loss of the duct antigen as the growth rate of cells declined.

When human acinar cells were cultured on plastic, the cells lost the acinar cell phenotype within 4 days and subsequently transdifferentiated to a ductal phenotype (Hall & Lemoine, 1992). Furthermore, isolated acinar cells from rat and guinea pig pancreas also changed their morphologic appearance to a duct-like phenotype when the cells were embedded into the Matrigel basement membrane in the presence of dimethylsulfoxide (DMSO) (Arias & Bendayan, 1993). Within Matrigel, the single acinar cells reaggregated and formed branched tubular structures lined by a single cell layer, which could be maintained in culture for a period of 21 days. These cells progressively lost intracellular amylase and further expressed the duct cell marker carbonic anhydrase II.

1.4 General objectives

The pancreas is composed of two unique tissues: (1) the exocrine compartment comprising the acinar and duct cells and (2) the endocrine compartment comprising the islets of Langerhans. All these cell types are derived from common precursor cells within the duct system during pancreatic development. However, cell differentiation in the adult pancreas remains unclear. Resolution of this question would be of importance to the understanding of the development of two diseases: pancreatic cancer and diabetes mellitus. The presence of stem cells in the adult pancreas is supported by several in vivo models. Studies of stem cells in vivo, nevertheless, are difficult because of the intricate anatomical relationship among different types of pancreatic cells. Direct proof will require the isolation and culture of adult pancreatic cells.

The fundamental work for my Ph.D. program is to study how adult pancreatic cells differentiate when cultured in type 1 collagen. The overall objectives are:

(1) To establish a method for the isolation and culture of intralobular ducts from the hamster pancreas.

(2) To study the effect of different culture conditions on the morphological phenotype of cultured acinar cells from hamster pancreas.

(3) To study the differentiation of purified human islets when cultured in collagen matrix.

Preface of Chapter 2

Studies of cell differentiation in the adult pancreas require the isolation and culture of populations of intralobular duct cells, which putatively have the ability to differentiate into islet and / or acinar cells.

A number of methods for adult pancreatic duct epithelial isolation and culture from cow, human, rat, hamster and guinea pig have been reported. However, the conventional methods yield populations consisting primarily of main and interlobular duct cells. Reliable methods for the large scale isolation and culture of the terminal segments of the pancreatic duct system are not available. Based on the complicated relationship between the intralobular ducts and acini, I hypothesized that a technique of tissue digestion and separation would produce acinar fragments of a particular size, as it was predicted that these fragments would contain small intralobular duct cells.

Chapter 2

Isolation and Culture of Intralobular Ducts from the Hamster Pancreas

(In Vitro Cell Dev Biol, 31: 77-80, 1995)

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2.1 Abstract

Intralobular and terminal ducts of the hamster pancreas were isolated by partial digestion with collagenase, embedded in a matrix of rat-tail collagen, and cultured in DME:F12 media and 10% NuSerum supplemented with cholera toxin (CT, 100 ng/ml) and epidermal growth factor (EGF, 10 ng/ml). The material plated on day 0 consisted of clumps of acinar cells and a few fragments of larger ducts. From 1-3 days, a few cystic structures appeared, usually without any connection to the acinar fragments. These cysts appeared to be derived from the fragments of interlobular ducts that were observed at the time of plating. After 3-4 days, numerous small cystic structures appeared in the midst of clumps of acinar cells. Between days 7-10 there was progressive widespread necrosis of the acinar tissue and a continued increase in the number of cystic areas. On day 12, approximately 3200 cysts per g tissue were harvested from the primary culture. The question of the cellular origin of the newly formed ducts was addressed by autoradiography. The cells were exposed to tritiated thymidine on the first day of culture. The cultures were sampled at 2, 4, 6 and 9 days. On day 2, very few cells had incorporated the radiolabel, and these labeled cells were primarily in the acinar units. After 6 days, duct cysts were well formed, and most of the duct epithelial cells were labeled. These findings suggest that the cysts originated from either progenitor cells of ducts or those of acini, but not from transformed acinar cells, because acinar cells underwent progressive degenerative changes and necrosis under our culture conditions.

2.2 Introduction

The ductal epithelium is a minor component of pancreatic tissue, comprising only 3-4 % of the rodent and guinea pig pancreas by volume (Githens, 1988; Bolender, 1974). The duct system, however, serves a major function as the site of bicarbonate and fluid secretion from the pancreas (Schulz, 1987; Case & Argent, 1993). Cells in the interlobular and main ducts are believed to play a major role in carcinogenesis, and several pancreatic cancer models have been developed in the rodent based on this belief (Pour et al., 1974; Mohr et al., 1977). Cells in the intralobular ducts are important because they may have the ability to differentiate into islet and/or acinar cells (Parsa & Marsh, 1976).

Our laboratory has reported an experimental model of nesidioblastosis, induced by cellophane wrapping of the head of the hamster pancreas in order to create partial obstruction of the pancreatic duct (Rosenberg et al., 1983). Nesidioblastosis is defined as a condition in which proliferating endocrine cells arise directly from the ductal epithelium, and islet hormone-containing cells are present in the epithelium of the smaller ductules (Laidlaw, 1938).

In order to study these mechanisms, it is imperative to develop methods for isolation and culture of intralobular pancreatic ductules. A number of methods for adult pancreatic duct epithelial isolation and culture have been reported, either as monolayers from cow, human, rat, hamster and guinea pig (Stoner et al., 1980; Jones et al., 1980; Tsao & Duguid, 1987; Hubchak et al., 1990; Hootman & Logsdon, 1988), or as intact duct fragments from rat and hamster (Githens et al., 1980b; Githens et al., 1987; Githens et al., 1989). However, the conventional methods yield populations of main and interlobular duct epithelium. Reliable methods for the large scale isolation

and culture of the terminal elements of the pancreatic duct system are not available.

In this paper, we describe the isolation and culture of the terminal elements of the pancreatic duct system of the Syrian golden hamster as epithelial cysts suspended in a collagen matrix. The ductules in this culture system arise from within partially digested fragments of acinar tissue, and a very high yield of ductular epithelium is achieved.

2.3 Materials and methods

2.3.1 Isolation of pancreatic ducts

Ducts were isolated from outbred female Syrian golden hamsters (CBL, St. Constant, Quebec, Canada) weighing between 85 to 95 g. For each isolation, one hamster was anaesthetized with pentobarbital sodium administered intra-peritoneally. The pancreas was excised completely and carefully trimmed of fat and mesentery under microscopic guidance. The pancreas was injected with 2-3 ml of a digestive medium (pH 7.4) enriched with Hanks' balanced salt solution (HBSS, GIBCO, Burlington, Ontario), 1.0 mg/ml collagenase (type XI, Sigma, St. Louis, MO) and 0.1 mg/ml α-chymotrypsin (Sigma). The HBSS was supplemented with 0.2 mg/ml of bovine serum albumin (fraction V, GIBCO), 0.1 mg/ml of soybean trypsin inhibitor (Sigma), 100 u/ml of penicillin (GIBCO), 100 µg/ml of streptomycin (GIBCO) and 0.25 µg/ml of amphotericin B (GIBCO).

The pancreas was transferred to a 15 ml centrifuge tube containing 5 ml of HBSS and incubated in a 37°C water bath for 30 min. The digestion process was halted by the addition of 5 ml of ice-cold HBSS and the tissue was then dispersed by vortex at top speed for 10 seconds. Since, in this technique, the

pancreatic ductules arise from within acinar tissue, it is very important to maintain a certain size (about 2-3 mm in diameter) of pancreatic fragments. The suspension was centrifuged at 4°C for 1 min. at 210 g. The supernatant was discarded and the tissue pellet was then washed three times in cold HBSS, reversing the tube gently to resuspend the fragments after each wash.

The fragments were resuspended in 10 ml of HBSS and pipetted on to a prewetted No. 100 mesh stainless steel sieve (pore size 140 μm, Bellco Glass, Vineland, NJ). After the fluid and small fragments passed through the filter, the large fragments remaining on the sieve were harvested by rinsing the inverted sieve with 20 ml of HBSS and collecting the retentate into four 15 ml centrifuge tubes. The fragments were spun down at 210 g for 1 min. and the supernatant discarded.

2.3.2 Duct culture

The pancreatic fragments were resuspended in 6 ml of rat tail collagen solution. Crude collagen solutions were prepared according to Richards (Richards et al., 1983a). No further purification of the crude collagen solution was carried out. The collagen containing the pancreatic fragments was then neutralized at room temperature with 1 ml of a mixture of 0.34 N NaOH and 10X Waymouth's medium (ratio: 4: 6) and separated into three 60-mm Petri dishes. To each plate was added 2 ml of DME:F12 medium (Sigma) supplemented with 10% NuSerum (Collaborative Research, Bedford, MA), insulin (1μg/ml, Sigma), dexamethasone (1μM, Sigma), soybean trypsin inhibitor (0.1 mg/ml, Sigma), penicillin (100 U/ml, GIBCO), streptomycin (100 μg/ml, GIBCO), amphotericin B (0.25 μg/ml, GIBCO), and cholera toxin (100 ng/ml, Sigma) and epidermal growth factor (10 ng/ml, Sigma). The culture

medium was changed every other day. The cultures were incubated in an atmosphere of 5% carbon dioxide in air at a temperature of 37°C.

2.3.3 Passaging of ducts

After 9 to 12 days of culture, the collagen gel was dissolved with 0.25 mg/ml of collagenase in HBSS medium at 37°C for 30 min. Duct epithelial cysts were harvested into 50 ml centrifuge tubes. After dispersion by vortex, the fragments were pipetted onto No.150 mesh stainless steel sieve (pour size 94 μm, Bellco glass), in order to remove fibroblasts, necrotic debris and dead cells. The duct fragments remaining on the sieve were collected into a 100 mm Petri dish by rinsing the inverted sieve with 20 ml of HBSS medium. The duct fragments were hand-picked under an inverted microscope, washed, and then re-embedded in collagen using the same culture conditions as described above. Cysts were passaged twice producing a culture of intralobular ductules by 3 weeks.

2.3.4 Microscopy

Cultures were observed using a Nikon inverted light microscope. Fields of tissue chosen at random were photographed using a time-lapse technique at 0, 2, 4, 6, 8, 10, and 12 days. Dishes were sampled at the same time points and fixed in 10% formalin. The collagen containing the tissue was embedded in paraffin and submitted for routine light microscopy. Deparaffinized sections cut at 5 μm were stained with hematoxylin and eosin. Other dishes were fixed in phosphate buffered glutaraldehyde, postfixed in 1% osmium tetroxide, and processed for electron microscopy. Ultrastructural analysis was performed on epon embedded tissue using a Phillips 200 electron microscope.

2.3.5 *Autoradiography*

After one day in primary culture, the medium was replaced by medium supplemented with 1 μCi/ml of tritiated thymidine (deoxribose-6[^{3}H]thymidine; specific activity: 35 Ci/mmol, ICN, St. Laurent, Montreal, Canada). After a 2 hour incubation, the labeled medium was replaced with unlabeled medium. The dishes were sampled on days 2, 4, 6, 9, fixed in 10% formalin and processed as above for light microscopy. Deparaffinized slides were covered with a nuclear tract emulsion (Kodak NTB-2, Kodak Canada, Toronto, Canada) diluted 1:1 with 0.6 M ammonium acetate at 43°C. Air-dried slides were exposed at 4°C for 15 days, then developed and stained (Rosenberg et al., 1989).

2.4 Results

From the stage of acinar fragments through to duct epithelial cyst formation, the primary cultures were observed through an inverted microscope and photographed serially over the first 10 days.

The material plated into primary culture on **day 0**, consisted of clumps of acinar cells, the occasional islet, and a few fragments of larger ducts or blood vessels (**Fig. 2-1a**). Light microscopy (**Fig. 2-1b**) confirmed that the cell aggregates consisted mostly of acinar tissue with intercalated or intralobular ducts. In addition there were fragments of larger interlobular ducts and islets, that were completely devoid of adjacent acinar tissue. At the electron microscopy level, freshly isolated acinar cells displayed a massive amount of rough endoplasmic reticulum (RER) and numerous zymogen granules (**Fig. 2-2**). The junctional complexes between adjacent acinar cells were well preserved during the isolation process.

From 1-3 days, only a few cystic structures appeared either on the periphery of large clumps of acinar tissue, or as separate cysts without any linkage to acinar fragments. These cysts appeared to be derived from the fragments of interlobular ducts that were observed at the time of plating, and tended to form very distended spherical structures on longer culture. Light microscopic examination of these structures demonstrated that the cyst wall was composed of a single layer of columnar or pseudostratified columnar epithelium. At the ultrastructural level, some of these cells were noted to contain secretory vacuoles near the apical plasma membrane that were reminiscent of mucin. Acinar cells showed evidence of degranulation and intracellular vacuolation.

Small cysts were present in the midst of clumps of acinar tissue **after 3-4 days** (**Figs. 2-1c, 2-1d**). Histologic examination showed that the wall of these cysts consisted of a single layer of cubical or flattened epithelium.

From 4-6 days , there was progressive necrosis of acinar cells adjacent to the areas in which cysts were forming (**Figs. 2-1d, 2-1e**). The number of small cystic areas surrounded by surviving acinar cells increased. Fibroblasts now appeared, but remained few in number and were preferentially located at the periphery of the forming cysts. **Between days 7-10** there was widespread necrosis of acinar tissue, a continued increase in the number of cystic areas and enlargement of cysts already formed (**Figs. 2-1e, 2-1f**). On **day 12,** approximately 3200 cysts/g tissue were harvested from the primary culture. The secondary cultures consisted of mixtures of small cysts with flattened or cuboidal epithelial cells and large cysts with columnar epithelium. There was progressive loss of the remaining acinar tissue, and very little fibroblastic contamination. Tertiary cultures contained pure duct epithelial cysts of both types.

The ultrastructural appearance of the cysts resembled that of duct epithelium in cultured duct fragments and in vivo. Apical microvilli and lateral interdigitations of the plasma membrane were present, cells were interconnected by tight junctions, and there was no basal lamina (**Fig. 2-3**).

The epithelial cells appeared to adapt to their new environment by eliminating unneeded or injured organelles. With longer time in culture, epithelial cells in the smaller cysts that formed within the acinar tissue assumed a more primitive appearance, with few cytoplasmic organelles and little apical membrane specialization.

Autoradiographic analysis of DNA synthesis with tritiated thymidine showed that on **the second day,** very few cells had incorporated the radiolabel (**Fig. 2-4**), and these were found predominantly within the acinar units. **After 6 days**, when duct cysts were well formed (**Fig. 2-5**), well over 85% of the cells that comprised the newly formed ductular epithelium were labeled, demonstrating a significant proliferative capability.

2.5 Discussion

In this paper, we describe a method of isolation and culture of pancreatic duct epithelium from Syrian golden hamsters. In comparison with previous reports (Githens et al., 1989), our method resulted in a high yield of epithelial structures that are similar to intralobular ducts.

The duct system of the pancreas has been extensively reviewed (Kern, 1993). The organization of intralobular ducts within the lobule is quite complex (Bockman, 1993). Many acini may be formed on the sides of a duct as it courses through a lobule. A duct may lead to an acinus, and another duct may continue on from the opposite side of the acinus. Based on this

complicated relationship between the intralobular ducts and acini, our intention was to develop a technique of tissue digestion and separation that would produce acinar fragments of a certain size, which it was hoped, would contain small intralobular ductules. This approach is similar to the one taken by Tsao and Duguid (Tsao & Duguid, 1987), who also dissociated the whole rat pancreas to produce propagatable cell lines that were presumed to be of ductal origin.

Githens was the first to report on the isolation of ductal fragments from the rodent pancreas (Githens et al., 1980b; Githens et al., 1989). The gland was digested non-specifically by collagenase. The acinar tissue was destroyed by the digestion and later was removed by filtration, so that partially purified ductal fragments could be obtained. Most intralobular ducts, however, were also destroyed and eliminated concomitantly with the acinar units during the isolation procedure. This resulted in a low yield of ductal cysts (about 200 cysts/g), most of which appeared to be interlobular ducts.

With the method that we present here, the acinar units, which include intralobular ductular cells, are retained, hence, more ductular cells are preserved. Our results demonstrate that these intralobular ductular cells can survive and proliferate to form epithelial cysts within areas of degenerating acinar tissue. The number of epithelial cysts retrieved is comparable to that of acinar fragments saved. Normally, more than 3200 cysts/g tissue can be recovered.

Support for the contention that our isolation and culture conditions favor the emergence of intralobular ducts comes from the following findings: (i) very few ductules are seen on day 1 of culture. Where duct-like epithelium is identified, it is in small fragments of acinar tissue and has a flattened epithelium characteristic of small intralobular or intercalated ducts in vivo;

(ii) unlike systems in which interlobular ducts are cultured, cysts are not visible immediately after the cultures are established, and finally, (iii) the large number of cysts per gram tissue is consistent with the conclusion that these structures are derived from the more terminal elements of the duct system.

The cysts derived from primary culture can be maintained for over two months. Nevertheless, in order to get a more purified culture, the cysts need to be passaged at least twice to eliminate acinar debris and remaining fibroblasts. Because serum strongly favors fibroblastic growth (Ham, 1984), and this growth advantage over other cell types is in large measure due to PDGF and EGF, as well as other serum factors, we also used serum-free media to inhibit their growth, as recommended by Scarpelli (Hubchak et al., 1990). The concentration of EGF was even lowered below their recommendation of 20 ng/ml to 10 ng/ml. These modifications, together with passage, retarded fibroblast growth.

The use of collagen gel as an extracellular matrix material was also an integral part of our culture system. Extracellular matrix is known to increase the proliferation of a variety of epithelial cells (Gospodarowicz et al., 1980; Yang et al., 1982b; Wicha et al., 1982; Richards et al., 1983b), as well as preserve 3-dimensional tissue-like architecture. In addition collagen gel matrix can help to promote or maintain differentiation of several related cell types in culture, including liver cells (Guguen-Guilouzo et al., 1982; Rubin et al., 1981), mammary epithelial cells (Yang et al., 1982b; Foster et al., 1983) and submandibular gland cells (Yang et al., 1982a). The use of rat-tail collagen to culture pancreatic cells, as recommended by Githens, was based on the successful culture of mammary and other epithelia under similar conditions (Githens et al., 1989).

In our culture system, two populations of duct epithelial cysts were formed. The initial cysts to appear were derived from the fragments of larger intralobular ducts that were also plated into primary culture. These are lined by a columnar or pseudostratified columnar epithelium and become greatly distended. The other type of cysts are the smaller ones that appear later and contain a flattened or cuboidal epithelium. One of the important questions that needed to be answered was how these duct epithelial cysts developed from what appeared to be fragments of acinar tissue. We have tried to address this question using autoradiography. The cells were exposed to tritiated thymidine on the first day of culture. The cultures were sampled at 2, 4, 6 and 9 days. On the second day, very few cells had incorporated the radiolabel, and these labeled cells were found primarily in the acinar units. After 6 days, epithelial cysts were well formed, and most of the duct epithelial cells were labeled. These findings suggest that the ductal cysts originated from either progenitor cells of ducts or those of acini. The newly formed ductules were not derived from transformed acinar cells, as may have been suggested by other studies (Githens et al., 1989), because acinar cells underwent progressive degenerative changes and necrosis under our culture conditions. These findings also suggest, that in this model, cyst formation and the increase in the size of cysts, can be attributed in large part to cell proliferation as demonstrated by the relatively large number of cells engaging in DNA synthesis, as well as the continued ability to secrete fluids and electrolytes.

In conclusion, our technique of tissue processing, combined with the culture conditions and the use of three-dimensional collagen gel matrix offers adult hamster pancreatic cells in culture an environment conducive to cellular differentiation and reorganization into epithelial cystic structures. The isolation and culture of ductal structures that arise from acinar cell

fragments has not previously been reported in rodents. In this system, ductular cytodifferentiation from ductal cells in the acinar fragments occurs in vitro resulting in a culture of pure intralobular ductules that can be maintained for over two months.

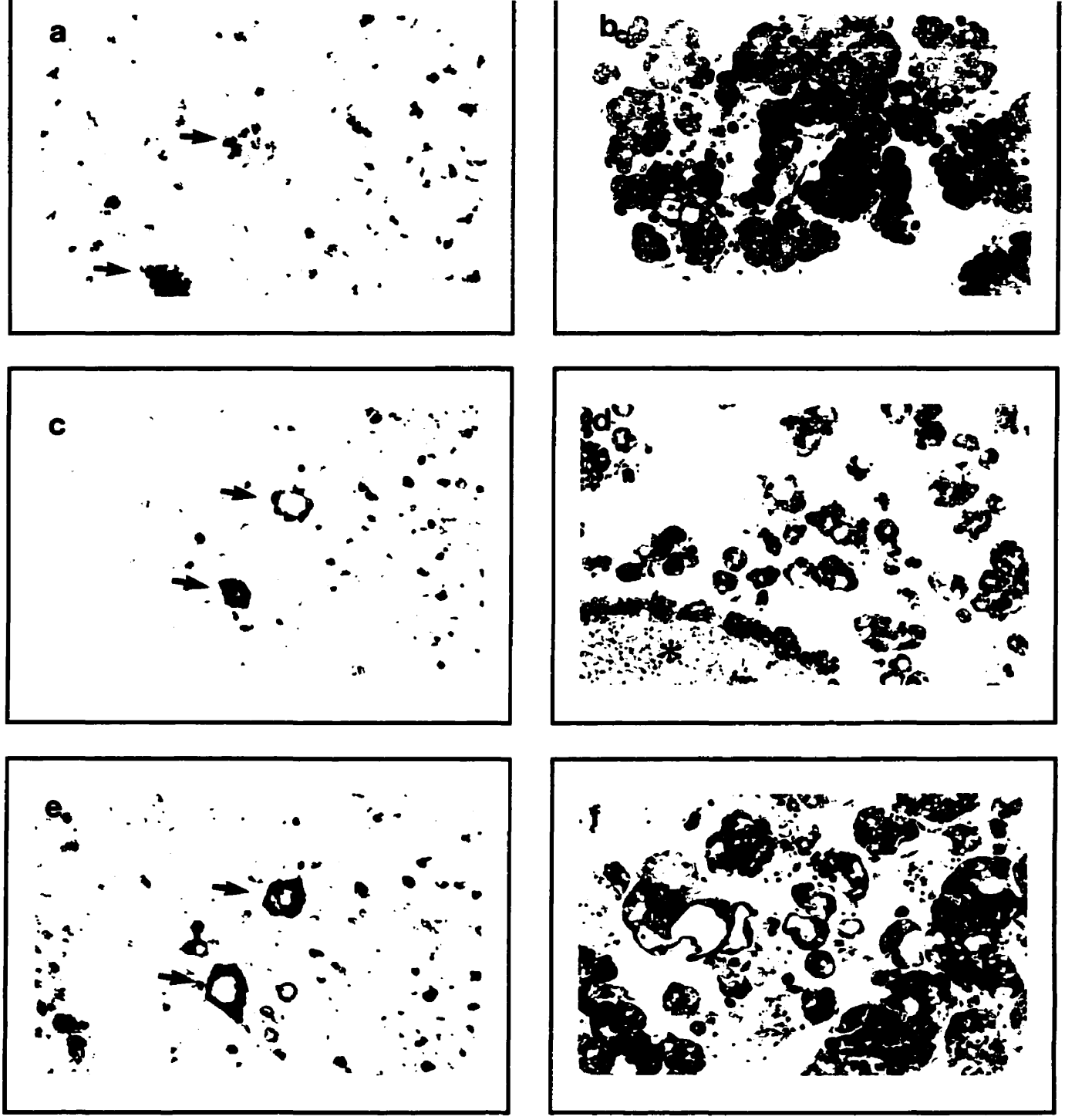

Figure 2-1. **a**, Time-lapsed photograph of collagenase digest of pancreas embedded in collagen in primary culture. Day 0. Cellular debris with fragments of acinar tissue (arrows). (64x). **b**, Pancreatic acinar fragments showing minimal vacuolation. Day 0. (H&E 201x). **c**, Time-lapsed photograph of collagenase digest of pancreas embedded in collagen on Day 4. The two clumps of acinar tissue (arrows) begin to demonstrate marked cystic changes. (64x). **d**, Pancreatic acinar fragments. There is central necrosis in the larger fragment (*). Some smaller fragments show increasing cytoplasmic vacuolation, while cystic structures appear to be developing from within other areas. Day 4. (H&E 128x). **e**, Time-lapsed photograph of collagenase digest of pancreas embedded in collagen on Day 8. (64x). **f**, Ductular formation. Well formed ductules now present and are lined by a single layer of flattened ductal epithelium. Day 8. (H&E 128x).

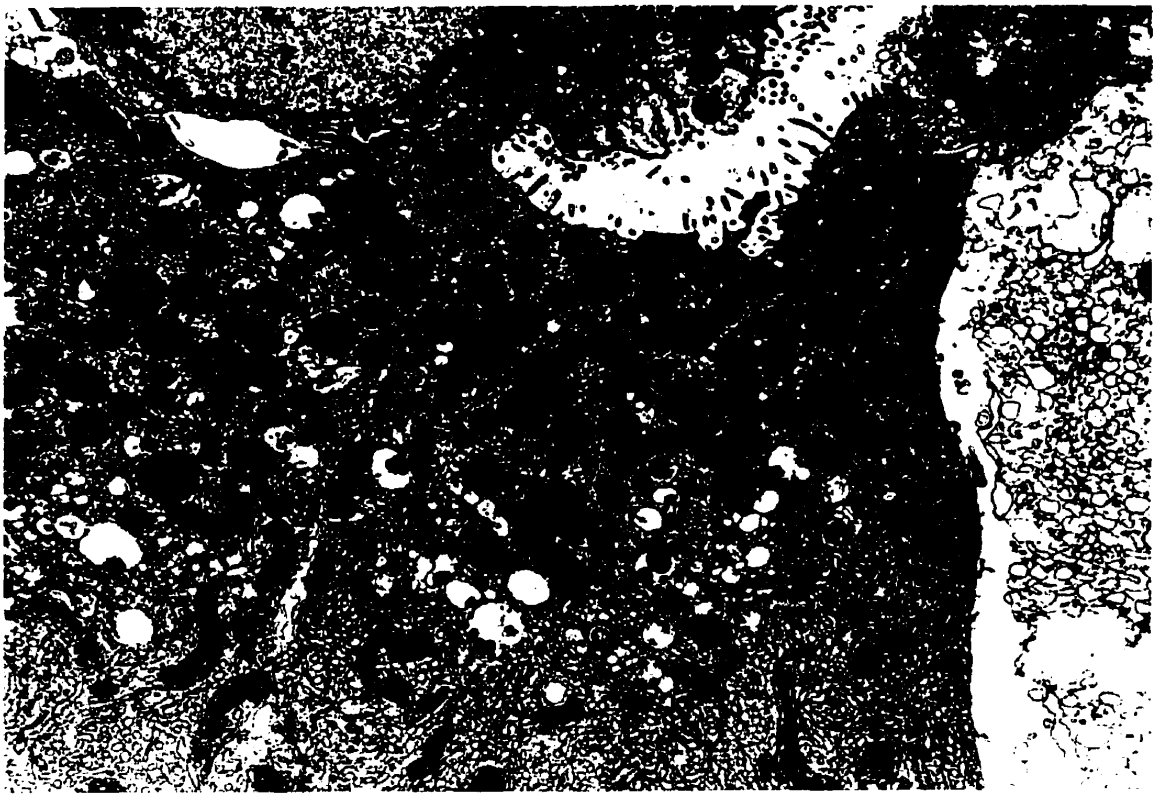

Figure 2-2. Electron micrograph of pancreatic acinar cells at Day 0. Microvilli were present at the apical surface of the cells and project into the lumen. A centroacinar cell is seen at the top left hand corner. uranyl acetate/lead citrate (8418x).

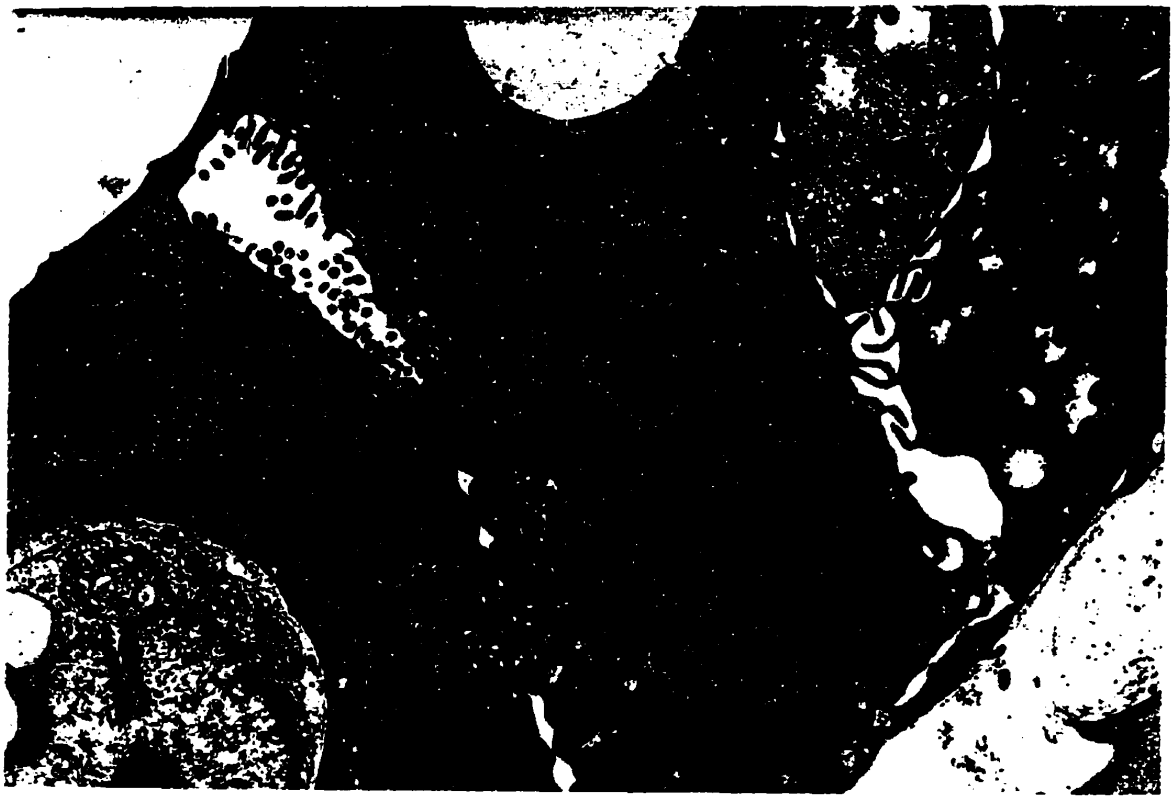

Figure 2-3. Electron micrograph of cells in culture at Day 10. These cells are now definitely ductal with prominent villi, tight junctions and few subcellular organelles. uranyl acetate/lead citrate (14,707x).

Figure 2-4. Clumps of cells on second day of culture showing degenerated acinar cells and two non-defined tritiated thymidine-labeled cells at arrow. (1140x).

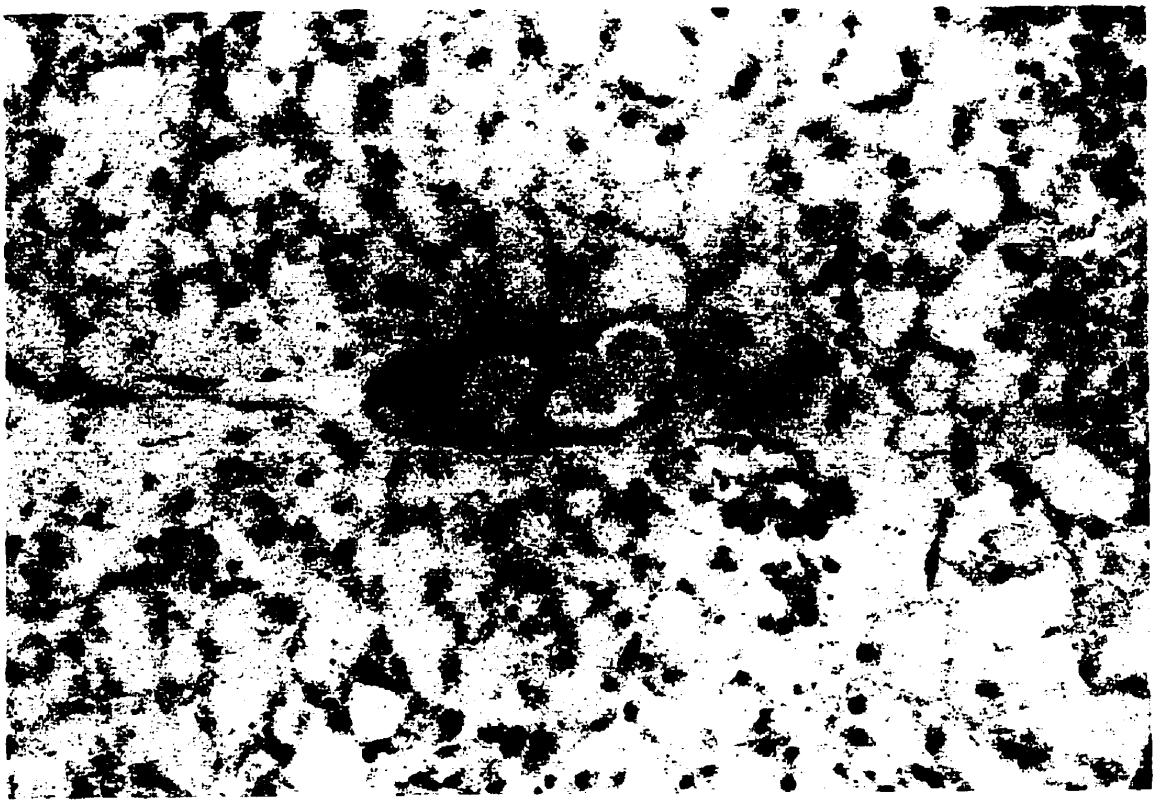

Figure 2-5. Developing duct (arrows) within necrotic zone at 6 days of culture. Note the tritiated thymidine labeling of all cells. (529x).

Preface of Chapter 3

In chapter 2, it was observed that cystic structures developed from acinar fragments cultured in collagen. It was postulated that these duct epithelial cysts originated from proliferation of the intralobular ducts, while the acinar cells underwent progressive degeneration and necrosis. However, in vitro studies have demonstrated that pancreatic acinar cells isolated from human, rat and guinea pig changed their phenotype into ductal cells (Hall & Lemoine, 1992; Arias & Bendayan, 1993). Therefore, the destiny of acinar cells in our culture system needs to be re-evaluated.

The aim of this study was to assess the effect of different culture conditions on the survival and morphological phenotype of cultured acinar cells.

Chapter 3

Phenotypic Modulation of Hamster Acinar Cells by Culture in Collagen Matrix

(Submitted to Exp. Cell Res.)

S Yuan, L Rosenberg, WP Duguid

3.1 Abstract

The aim of this study was to assess the effect of different culture conditions on the survival and morphological phenotype of cultured acinar cells. Acinar fragments isolated from hamster pancreas were embedded in rat-tail collagen. Four groups were established: Medium 1 - 5% NuSerum+basic medium (basic medium = DMEM/F12 supplemented with dexamethasone, IBMX and antibiotics); Medium 2 - 10% NuSerum+basic medium; Medium 3 - Medium 2 supplemented with EGF and cholera toxin; and Medium 4 - Medium 3 supplemented with SBTI. Freshly isolated acinar cells were retrieved morphologically intact. In Medium 1, over 80% of cells retained a normal histological appearance at 34 days in culture. Immunostaining for amylase was observed at the apical pole of the cells. The remaining cells showed variable degrees of degeneration. In Medium 2, approx. 50% of acinar cells appeared normal at 34 days in culture, while the remainder were severely degenerated. A few cystic structures were also observed. Positive immunostaining for amylase was limited to the cells with a normal histological appearance. The cells grown in Media 3 and 4 had similar courses of morphological changes. After 8 days in culture, most acinar fragments disappeared and were replaced by cystic structures, lined by a single layer of cuboidal cells. Some amylase positive immuno-reactive cells were integral components of the cystic wall. Cellular amylase activity was a function of the different culture media, a more rapid decrease in amylase activity being observed in Media 3 and 4. Uptake of ^{3}H-thymidine did not show any significant differences between the media. It was also found that the duct-like cells cultured in Medium 4 had a limited capacity to redifferentiate into acinar

cells. This study showed that the acinar cell phenotype can be maintained in vitro over a 1 month period. This study also suggested that ductal-like epithelial structures may arise from transformation of acinar cells and /or proliferation of ductal cells.

3.2 Introduction

Acinar cells are the major tissue component of the pancreas and are specialized for synthesizing and secreting a variety of digestive enzymes. A stereological analysis of the guinea pig pancreas indicated that acinar cells comprise 82% of pancreatic volume, a 21-fold difference over duct cells (Bolender, 1974). It is of interest to note, however, that adenocarcinomas of the duct system make up over 90% of non-endocrine pancreatic tumors in man, while acinar cell carcinomas account for only 1% (Kloppel, 1993). It is generally believed that adenocarcinomas originate from the pancreatic ducts, largely on the basis of morphological similarities (Cubilla & Fitzgerald, 1975; Githens, 1988). Studies on chemical carcinogenesis have suggested that acinar cells may also participate in the histogenesis of adenocarcinomas, during which acinar cells undergo dedifferentiation and give rise to duct-like cells (Bockerman, 1981; Flaks, 1984; Scarpelli et al., 1991). Recently, in vitro studies revealed that normal pancreatic acinar cells can change their differentiation commitment pattern and subsequently transform into a ductal phenotype (Hall & Lemoine, 1992; Arias & Bendayan, 1993). It would therefore be important to understand the growth and differentiation of acinar and ductal cells in the normal pancreas.

We have previously reported on a method to isolate and culture intralobular ducts from the hamster pancreas. In this system, duct epithelial

cysts arose from partially digested fragments of acinar tissue cultured in collagen matrix (Yuan et al., 1995). Githens et al. observed similar findings by culturing mouse and monkey acinar cells either in collagen or on other extracellular matrices (Githens et al., 1994a; Githens et al., 1994b). The ductal cells derived from acinar fragments are phenotypically indistinguishable from cultured interlobular ducts (Githens et al., 1994b). It has been postulated that these duct epithelial cysts originated from the proliferation of intralobular ductules and / or transdifferentiation of acinar cells (Yuan et al., 1995; Githens et al., 1994a; Githens et al., 1994b).

The present communication shows that acinar cell phenotype can be maintained in rat tail collagen for over 1 month, and that a phenotypic change of acinar cells to ductal cells can be obtained by adding EGF and cholera toxin to the culture medium.

3.3 Material and methods

3.3.1 Isolation of pancreatic acinar fragments

Outbred female Syrian golden hamsters, weighing between 85 to 95 g, were obtained from CBL (St. Constant, Quebec, Canada). Acinar cells were isolated according to the procedure in chapter 2 (see 2. 3. 1).

3.3.2 Primary cell culture

The pancreatic fragments were resuspended in 6 ml of rat tail collagen solution. Crude collagen solutions were prepared according to Richards (Richards, 1983). No further purification of the crude collagen solution was carried out. The collagen containing the pancreatic fragments was then neutralized at room temperature with 1 ml of a mixture of 0.34 N NaOH and

10X Waymouth's medium (ratio: 4: 6) and separated into three 60-mm Petri dishes. According to the different medium components, four groups were established: Medium 1 was basic medium + 5% NuSerum (Collaborative Research, Bedford, MA); Medium 2 was basic medium + 10% NuSerum; Medium 3 was Medium 2 supplemented with cholera toxin (100 ng/ml, Sigma) and epidermal growth factor (EGF) (10 ng/ml, Sigma); Medium 4 was Medium 3 supplemented with soybean trypsin inhibitor (0.1 mg/ml, Sigma). The basal medium consisted of DMEM/F12 medium (GIBCO) containing dexamethasone (1μM, Sigma), 3-isobutyl-1-methylxanthine (IBMX) (0.1 mM, Sigma), penicillin (100 U/ml, GIBCO), streptomycin (100 μg/ml, GIBCO) and amphotericin B (0.25 μg/ml, GIBCO). Cultures were incubated in an atmosphere of 5% carbon dioxide in air at a temperature of 37°C and the culture media was changed every other day.

3.3.3 Secondary cell culture

The cells cultured in medium 4 were passaged to establish a secondary cell culture. After 9 to 12 days of culture, the collagen gel was dissolved with 0.25 mg/ml of collagenase in HBSS medium at 37°C for 30 min. Duct epithelial cysts were harvested into 50 ml centrifuge tubes. After dispersion by vortex, the fragments were pipetted onto No.150 mesh stainless steel sieve (pour size 94 μm, Bellco glass), in order to remove fibroblasts, necrotic debris and dead cells. The duct fragments remaining on the sieve were collected into a 100 mm Petri dish by rinsing the inverted sieve with 20 ml of HBSS medium. The duct fragments were hand-picked under an inverted microscope, washed, and then re-embedded in collagen. The culture flasks were divided into two groups. The cells in Group 1 were incubated in Medium 1, while the cells in Group 2 were incubated in medium 4.

3.3.4 *Microscopy*

Cultures were observed using a Nikon inverted light microscope. Dishes were fixed in 10% formalin. The collagen containing the tissue was embedded in paraffin and submitted for routine light microscopy. Deparaffinized sections cut at 5 μm were stained with hematoxylin and eosin. Other dishes were fixed in phosphate buffered glutaraldehyde, postfixed in 1% osmium tetroxide, and processed for electron microscopy. Ultrastructural analysis was performed on epon embedded tissue using a Phillips 200 electron microscope.

3.3.5 *Tritiated thymidine incorporation and DNA assay*

At 0, 1, 3, 5, 7, 9, and 11 days in culture, the media was replaced by medium supplemented with 1 μCi/ml of tritiated thymidine. After a 24 hour incubation, the collagen matrix was dissolved by 0.25 mg/ml collagenase. The tissue was washed three times in 1X PBS and sonicated for 30 seconds. Tritiated thymidine incorporation into trichloroacetic acid precipitated material was determined (Struhl, 1994). DNA was measured using a fluorometric technique using bisbenzimidazole (Hoechst 33258, Sigma) as described (Labarca & Pagen, 1980). Tritiated thymidine incorporation was expressed as dpm/μg DNA (mean ± SD).

3.3.6 *Immunocytochemistry*

Immunoperoxidase staining of tissues was performed for amylase using kits purchased from Dako Corp. (Santa Barbara, CA). The final reaction product used was ethylaminocarbazole (EAC). The polyclonal rabbit anti-porcine a-amylase was provide by Dr. D. G. Scarpelli (Northwestern

University, Chicago, IL). Nonspecific staining was excluded by using the second antibody without the primary antibody, and specificity was determined by neutralization with excess antigen.

3.3.7 Amylase activity

At 2, 4, 6, 8, 10, and 12 days in culture, the collagen matrix was dissolved by 0.25 mg/ml collagenase. The tissue was washed three times in 1X PBS and sonicated for 30 seconds. Intracellular amylase activity were determined by the Phadebas reagent (Ceska et al., 1969). At the same time, the DNA content of the samples was determined. Amylase content was expressed as U/μg DNA (mean ± SD).

3.3.8 Statistical Analysis

All data are expressed as mean ± standard deviation with n=4 (flasks) at each time point. The difference between time points with respect to thymidine incorporation and intracellular amylase content was evaluated by unpaired Student's t-test. Differences were considered significant at the 5% level ($p<0.05$).

3.4 Results

The tissue preparation consisted mainly of one to several acinar units (**Fig. 3-1**). Ductal fragments and islets of Langerhans were rarely seen. Acinar cells retained their in situ polarity with their narrow apical poles abutting the acinar lumen. Immunocytochemistry for amylase showed that the zymogen granules were accumulated in the apical cytoplasm of the cell (**Fig. 3-2**).

In Media 1 and 2, some acinar cells, especially those in the larger acinar fragments, underwent necrotic changes during the first 6 days in culture (**Fig. 3-3**). The remaining cells were relatively normal in appearance. Intracellular vacuolation was observed in only a few acinar cells. In Medium 1, over 80% of acinar cells maintained a normal appearance at 34 days in culture, whereas the remaining cells showed different degrees of vacuolation in the cytoplasm (**Fig. 3-4**). The normal-appearing acinar cells varied in size and shape. The nuclei were usually rounded and were located toward the basal end of the cell. The cytoplasm between the nucleus and the apex of each cell contained acidophilic clusters. Positive immunostaining for amylase was observed at the apical pole of the cells (**Fig. 3-5**). Very few cystic structures were identified.

In Medium 2, more than 50% of the acinar cells appeared normal at 34 days in culture (**Fig. 3-6**). Cyst formation and cell degeneration were more often observed in comparison with the acini cultured in Medium 1. Positive immunostaining for amylase was limited to the cells with a normal morphological appearance (**Fig. 3-7**).

The cells grown in Media 3 and 4 had similar courses of morphological progression. During the first 3 days in culture, some acinar cells showed a normal phenotype, while others showed intracellular vacuolation and necrosis (**Fig. 3-8**). Small cysts were present in the midst of acinar fragments after 4 days. These cystic structures progressively increased in size and number when the cultures were continued, with a concomitant loss of acinar tissue. Histological examination showed that the wall of these cysts was lined by a single layer of cubical or flattened epithelium (**Figs. 3-9, 3-10**). Amylase positive immuno-reactive cells were an integral component of the cyst wall (**Fig. 3-11**).

The cellular amylase content declined rapidly within the first 8 days of culture regardless of medium (**Fig. 3-12**). In Media 1 and 2, cellular amylase content remained at about the same level for the subsequent 4 days. By day 12, cells grown in Media 1 and 2 contained about 15% and 18%, respectively, of their initial specific amylase activity. In Media 3 and 4, cellular amylase content declined slightly during the subsequent 4 days. By day 12, cells grown in Media 3 and 4 contained about 7% and 5%, respectively, of the initial specific amylase activity.

The uptake of ^{3}H-thymidine for all groups increased markedly during the first six days of culture, then was maintained at about the same level for the subsequent 6 days (**Fig. 3-13**). The level of thymidine incorporation was not significantly different between each group at 6 days of culture. By day 2, the value of thymidine incorporation for Medium 1 was significantly higher than that in Media 2 and 4, but was not statistically different in comparison with that in Medium 3. By day 12, the value of thymidine incorporation for Medium 2 was significantly lower than that in Media 1 and 4, but was not statistically different in comparison with that in Medium 3.

The secondary culture in Medium 4 consisted mainly of different sized cystic structures (**Fig. 3-14**). A few cells with positive immunostaining for amylase were observed within duct-like cells (**Fig. 3-15**). After 10 days of culture, the cellular amylase activity was only about 0.7% of the specific activity of freshly isolated acinar cells (**Fig. 3-16**). When cells from secondary cultures were incubated in medium 1, the cellular amylase activity increased 3.6 times compared to being incubated in Medium 4. Cells in Medium 1, however, retained cystic structures in spite of the increased amylase activity (**Fig. 3-17**). In addition, immunostaining for amylase did not show any difference in comparison with cells cultured in Medium 4. Cystic structures

observed in Medium 1, although evident, were generally of a smaller size than those in Medium 4.

3.5 Discussion

In the present study, we demonstrate that pancreatic acinar cells can be maintained in long term culture. While the uptake of ^{3}H-thymidine by these cells increased with time, the intracellular amylase activity was not well preserved- 85% of activity being lost after only 12 days of culture. The question that needs to be addressed is whether this loss of amylase activity results from a dedifferentiation of the cultured acinar tissue (Arias & Bendayan, 1993; De Lisle & Logsdon, 1990) or is due to some other cause. Based on our observations, it seems likely that the decrease of intracellular amylase activity does not represent dedifferentiation since very few duct epithelial-like cystic structures were observed in culture. Moreover, immunocytochemistry for amylase still demonstrated the presence of amylase in the apical cytoplasm of acinar cells at 34 days in culture. Therefore the decrease of intracellular amylase is more likely to suggest that manipulation of the micro-environment caused a degranulation and degeneration of acinar cells, and this is consistent with the observation of intracellular vacuolation.

Collagen and other extracellular matrices can profoundly influence the morphology and growth of epithelial cells in culture (Yang & Nandi, 1983; Gospodarowicz et al., 1978). Epithelial cells sustained in a collagen gel form three-dimensional tissue-like structures (Chambard et al., 1981; Hall et al., 1982; Montesano et al., 1983b; Yang et al., 1986). When pancreatic acinar cells were cultured on a reconstituted basement membrane gel, the cells

reassociated into acini-like structures, reassuming a polarity that resembled the natural situation (Bendayan et al., 1986). Growth and differentiation of mouse pancreatic acinar cells have also been promoted by culture on a collagen gel (Logsdon & Williams, 1986). The present study shows that isolated acini have the ability to retain a normal morphological phenotype for over 1 month when cells are cultured in collagen.

Previous reports demonstrated that the presence of secretagogues and the extracellular matrix itself were essential for the maintenance of differentiated acinar cell aggregates in vitro (Bendayan et al., 1986; Arias & Bendayan, 1991). Our findings suggest, however, that secretagogues are not necessary for the preservation of normal acinar morphology when cells are cultured in collagen. Moreover, the culture medium used in the present study was less complex, making it easier to elucidate factors that might exert regulatory effects on the differentiation of pancreatic acinar cells.

The present study also demonstrated that cultured acini underwent phenotypic changes when EGF and cholera toxin were added to their micro-environment. Under these circumstances, acinar cells gradually disappeared and were replaced by duct-like structures. These duct-like structures were lined by a single layer of cuboidal or flattened epithelium, and at least some of the constituent cells demonstrated amylase immunoreactivity. The mechanisms involved in this phenotypic transformation are presently unclear and remain to be fully elucidated.

One possibility is that acinar cells undergo transdifferentiation and become duct-like in appearance. Transformation of acinar cells to a duct-like phenotype has been reported in vitro in the rat, guinea pig and human (Hall & Lemoine, 1992; Arias & Bendayan, 1993). Another possibility is that stem

cells within the ductules proliferate and form cystic structures, whereas the acinar cells die.

During the development of the pancreas, the protodifferentiated duct epithelium gives rise to acinar, islet, and mature duct cells (Githens, 1993). The question of whether undifferentiated cells within the duct epithelium of the adult pancreas gives rise to all cell types remains controversial. The presence of stem cells in the adult pancreas is supported by in vivo models, such as partial duct obstruction, transgenic mice and partial pancreatectomy (Rosenberg et al., 1983; Gu & Sarvetnick, 1993; Bonner-Weir et al., 1993). In vitro, propagable ductal epithelial cells isolated from rat pancreas were shown to retain some capacity to differentiate into acinar-like cells during early passages (Tsao & Duguid, 1987). In order to elucidate the mechanism, markers specific to ductal cells will be needed, but they are not available to us presently.

EGF, together with cholera toxin, showed a synergistic effect on the growth and tubular formation of mammary epithelial cells in a collagen gel culture (Yang et al., 1980; Richards et al., 1982; Ormerod & Rudland, 1988). The effect of EGF is mediated through specific receptors in the cell membrane, which results in the activation of protein kinase C and an increase in the intracellular Ca^{2+} concentration (Boonstra et al., 1995). Cholera toxin acts through the activation of adenylate cyclase, leading to an increase in the intracellular concentration of c-AMP (Holmgren, 1981). Githens et al. showed that the addition of EGF+cholera toxin to the medium caused a more rapid distention of cultured ducts and a higher yield of duct fragments (Githens et al., 1989). In his studies, the labeling index of the ductal cells was significantly reduced by the omission of EGF+cholera toxin. Our results demonstrate that the addition of EGF+cholera toxin to the media was important for cystic

transformation from acinar fragments, but did not increase the uptake of ^{3}H-thymidine by cultured cells.

It is well known that feeding rats soybean trypsin inhibitors (SBTI) results in acinar cell hypertrophy and hyperplasia (Folsch, 1984; Oates & Morgan, 1982). The effects of SBTI on pancreatic proliferation and differentiation are thought to be mediated by increased circulating levels of cholecystokinin (CCK). It has been postulated that removal of trypsin by its binding to a trypsin inhibitor leads to the release of CCK from the gut mucosa (Green & Lyman, 1972; Green et al., 1972). As predicted, SBTI did not show any trophic activity on cultured acinar cells. In contrast, it appeared that SBTI caused a more rapid decline in cellular amylase content.

It is of interest to know whether the newly formed duct-like cells can fully redifferentiate into acinar cells. When the duct-like cells in the secondary culture were maintained in Medium 1, the cellular amylase activity significantly increased after 10 days of culture. However, the cystic structure was retained and the cellular amylase activity was far below that of cells in primary culture. These duct-like cells, therefore, appear to have a limited capacity to redifferentiate into acinar cells.

In conclusion, the present study attempted to determine the effect of different medium components on the maintenance, cystic transformation and redifferentiation of cultured acinar cells. The culture of acinar cell fragments in collagen matrix has not previously been reported. In this system, the normal acinar phenotype can be maintained for over one month. The cystic formation from acinar fragments can be obtained when EGF and cholera toxin are added to the medium. Although the mechanisms for the cystic transformation are unknown, transdifferentiation of cultured acinar cells and/or proliferation of putative stem cells within the ductules should be

considered. We also provide an in vitro system to study the redifferentiation of acinar cells. The duct-like cells that arise from the acinar fragments may contain acinar cell precursors, which, under appropriate conditions, will redifferentiate into acinar cells. The understanding of the differentiation, dedifferentiation and redifferentiation of acinar cells will have important implications in the early stage of human carcinogenesis.

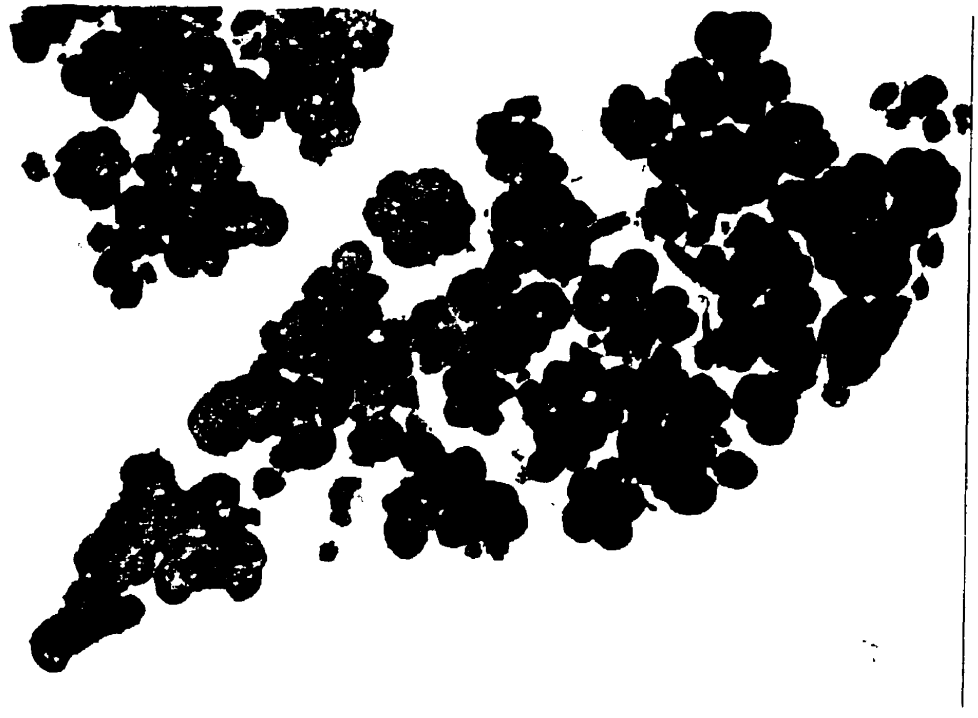

Figure 3-1. Freshly isolated pancreatic acini (H&E stain, 312x).

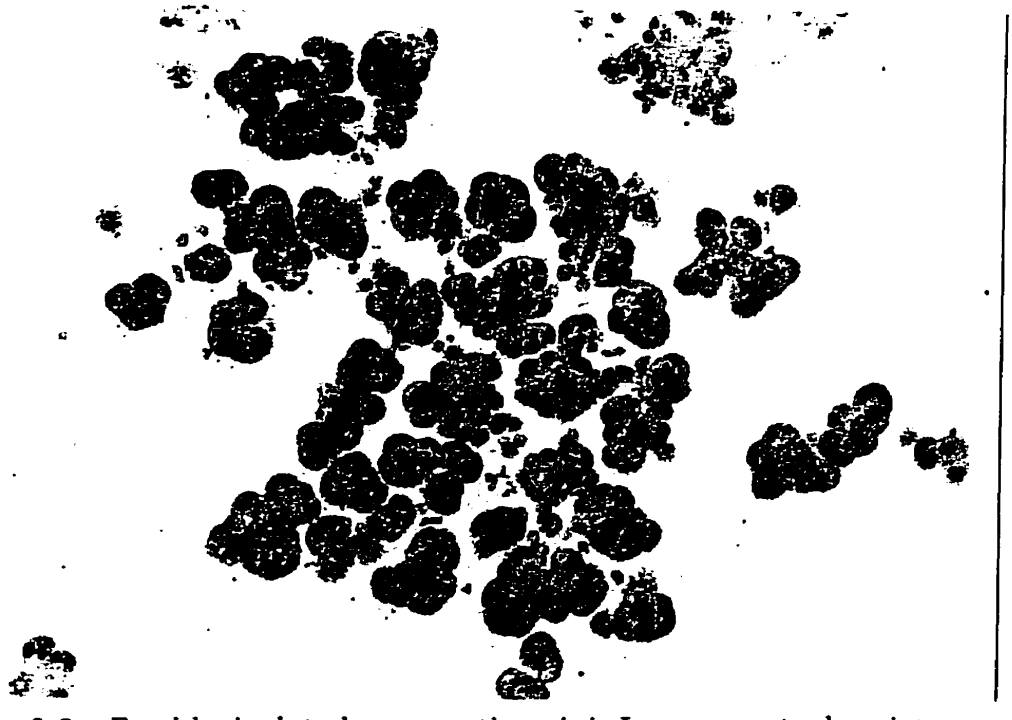

Figure 3-2. Freshly isolated pancreatic acini. Immunocytochemistry - acinar cells showing positive staining for amylase (312x).

Figure 3-3. Day 4. Acinar cells cultured in Medium 2. Foci of viable acinar cells within areas of necrosis. Some acinar cells showing intracellular vacuolation (arrow) (H&E stain, 390x).

Figure 3-4. Day 34. Acinar cells cultured in Medium 1. Most acinar cells have a normal appearance, while some cells show different degrees of intracellular vacuolation (arrow). (H&E stain, 312x).

Figure 3-5. Day 34. Acinar cells cultured in Medium 1. Immunocytochemistry for amylase - some acinar cells showing positive staining at the apical pole of cells (arrow) (500x).

Figure 3-6. Day 34. Acinar cells cultured in Medium 2. Normal acinar cells together with cells showing intracellular vacuolation (arrow). (H&E stain, 312x).

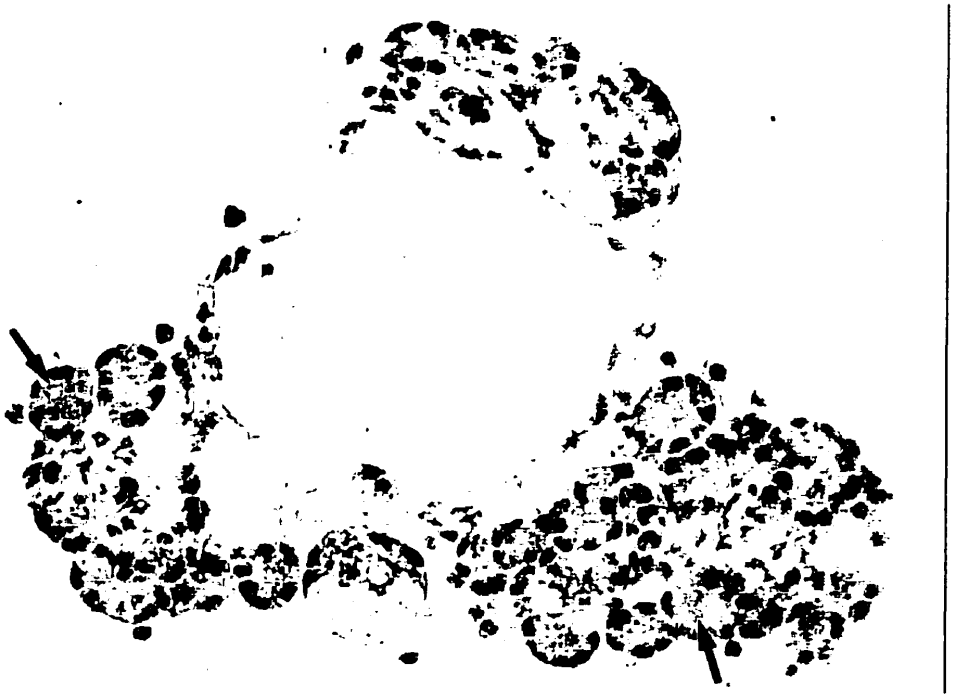

Figure 3-7. Day 34. Acinar cells cultured in Medium 2. Immunocytochemistry-positive immunostaining for amylase limited to the cells with a normal acinar appearance (arrow) and duct-like cells showing negative staining (390x).

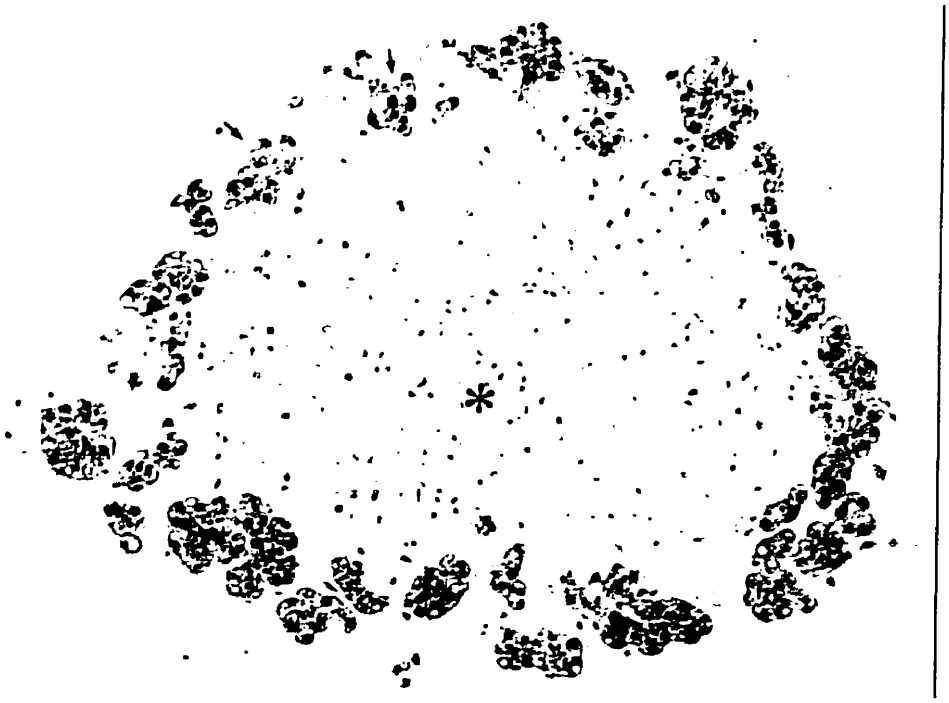

Figure 3-8. Day 2. Cells cultured in Medium 3. There is central necrosis in the acinar fragment (*). Immunocytochemistry for amylase - some acinar cells showing degranulation (arrow) (195x).

Figure 3-9. Day 12. Cells cultured in Medium 3. A well formed cyst lined by a single layer of flattened epithelium. (H&E stain, 156x).

Figure 3-10. Day 12. Cells cultured in Medium 4. A well formed cyst lined by a single layer of cuboidal epithelium. (H&E stain, 195x).

Figure 3-11. Day 8. Cells cultured in Medium 3. Immunocytochemistry for amylase- Some cells in the cystic wall showing positive staining at the apical cytoplasm (arrow) (312x).

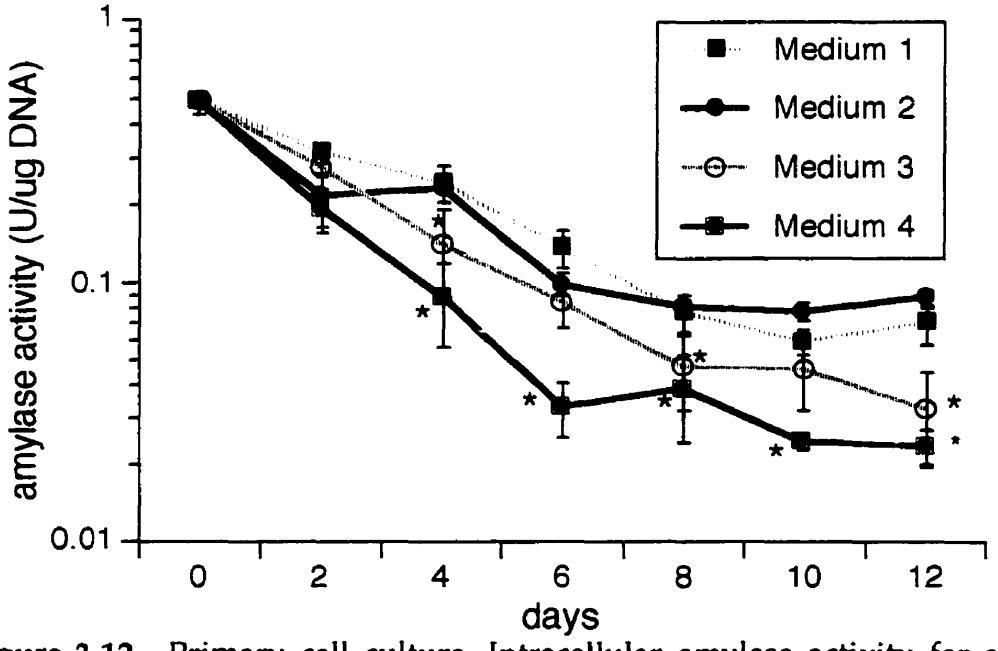

Figure 3-12. Primary cell culture. Intracellular amylase activity for all Media (*: $p<0.05$ vs. other media).

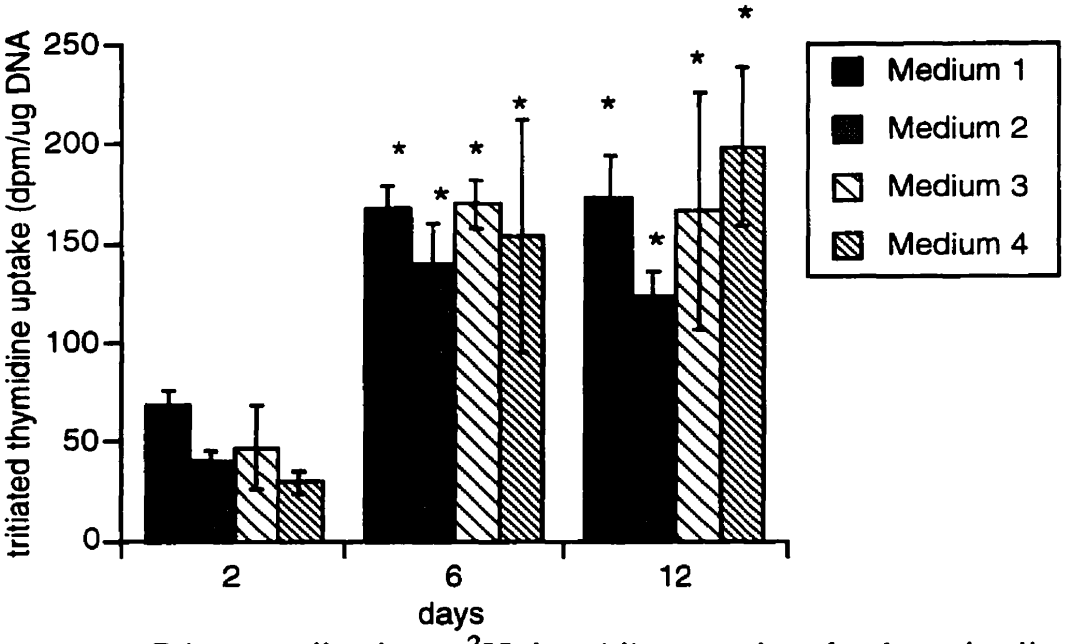

Figure 3-13. Primary cell culture. ^{3}H-thymidine uptake of cultured cells for all Media (*: $p<0.05$ vs. day 2).

Figure 3-14. Day 10. An inverted microscopic photograph of secondary cell culture in Medium 4 (64x).

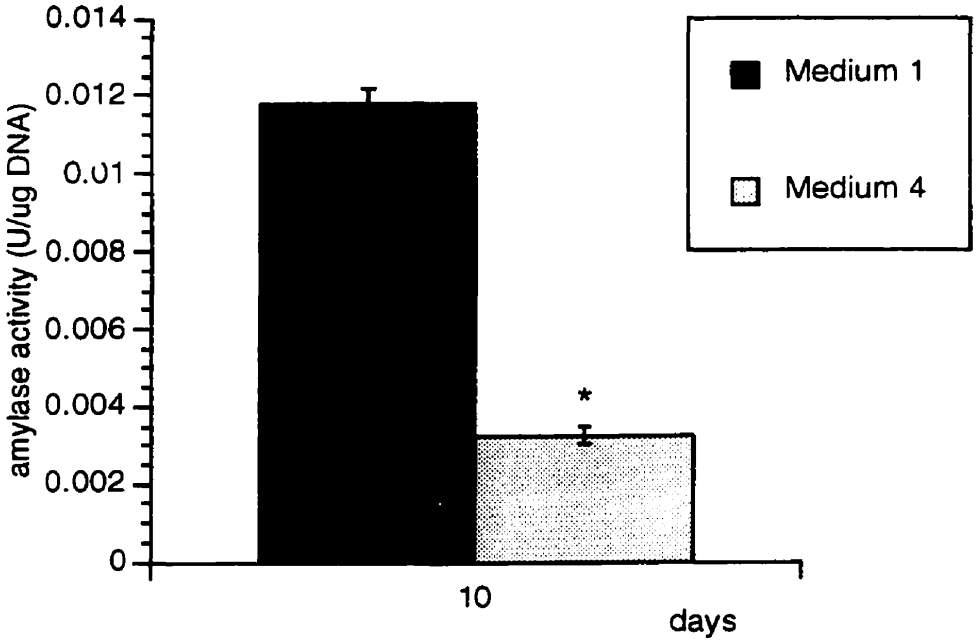

Figure 3-15. Secondary cell culture. Intracellular amylase activity for Media 1 and 4 (*: $p<0.05$).

Figure 3-16. Secondary cell culture on day 10. Immunocytochemistry for amylase - A single cell in the cystic wall showing positive staining (arrow) (195x).

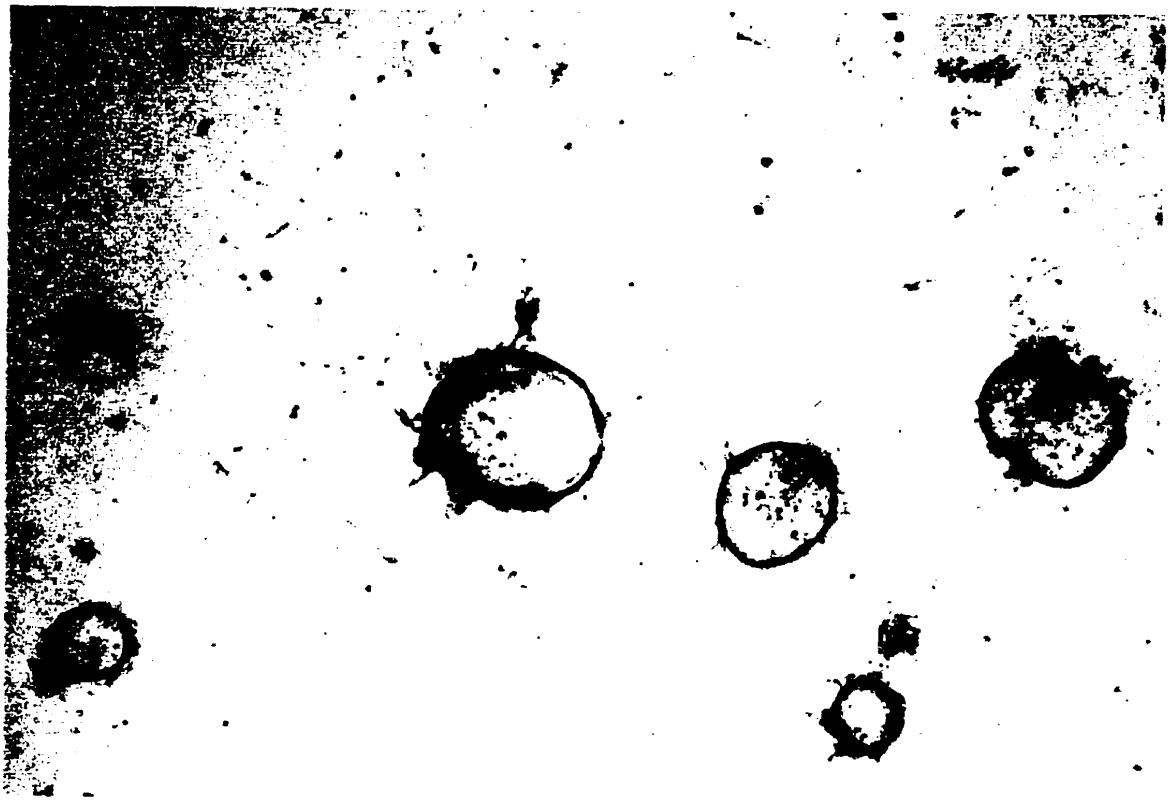

Figure 3-17. Day 10. An inverted microscopic photograph of secondary cell culture in Medium 1 (64x).

Preface of Chapter 4

In a previous report, we tried to determine whether there is intercellular communication between the ducts and islets of adult pancreas (Metrakos P, 1993). Purified terminal ducts and islets from hamster pancreas were co-cultured in rat tail collagen. The results demonstrated that pancreatic duct epithelium could stimulate islet cell proliferation in a paracrine manner. It would be of interest to examine whether hamster pancreatic ducts could act on the islets from different species. The islets isolated from human pancreas were co-cultured with hamster terminal ducts in rat tail collagen. However, human islets became duct-like structures after several days in culture.

In this chapter, human islets were isolated by a semi-automated method and cultured in a collagen matrix with the same medium used for the culture of intralobular ducts. This study showed that cultured human islet cells transdifferentiated into ductal cells.

Chapter 4

Transdifferentiation of Human Islets to Pancreatic Ductal Cells in Collagen Matrix Culture

(Differentiation, 61: 67-75, 1996)

S Yuan, L Rosenberg, S Paraskevas, D Agapitos and WP Duguid

4.1 Abstract

Transdifferentiation is a change from one differentiated phenotype to another, involving morphological and functional phenotypic markers (Okada, T. S. 1986. Transdifferentiation in Animal Cells: Fact or Artifact? *Dev Growth Differ.* 28(3):213-221). Stability of the cellular phenotype is probably related to the extracellular milieu, as well as cytoplasmic and nuclear components that interact to control gene expression, and the conversion of cell phenotype is likely to be accomplished by selective enhancement of gene expression which controls the terminal developmental commitment of cells. In this paper, we show the induction of cultured human islets cells to alter their usual phenotypic expression and attain morphological and functional characteristics of duct cells.

Islets were isolated by collagenase digestion of pancreata that were removed from cadaveric organ donors. The islets were purified on a two-step density gradient of bovine serum albumin and were then placed into a three-dimensional rat-tail collagen gel matrix supplemented with NuSerum, EGF and cholera toxin.

During the initial 96 hours of culture, the islets underwent a cystic transformation that was associated with (i) the maintenance of immunoreactivity for neuron specific enolase, an endocrine cell marker, but a progressive loss of insulin gene expression, (ii) a loss of immunoreactivity for insulin protein, and (iii) the appearance of CK-19, a marker for ductal cells. After the transformation was complete, the cells had the ultrastructural appearance of primitive duct-like cells. Cyst enlargement after the initial 96

hours was associated, at least in part, with cell replication, as reflected in the 1500% increase in the incorporation of tritiated thymidine.

These experiments are consistent with the transdifferentiation of an islet cell to a ductal cell. The exact mechanisms involved remained to be fully elucidated.

4.2 Introduction

Transdifferentiation is a change from one differentiated phenotype to another, involving morphological and functional phenotypic markers (Okada, 1986). The best studied example of this process is the change of amphibian iridial pigment cells to lens fibers, which proceeds through a sequence of cellular dedifferentiation, proliferation and finally redifferentiation (Okada, 1983; Okada & Kondoh, 1986; Yamada, 1977). Direct transdifferentiation without cell division has also been reported, although it is much less common (Beresford, 1990).

Stability of the cellular phenotype in adult organisms is probably related to the extracellular milieu, as well as cytoplasmic and nuclear components that interact to control gene expression. The conversion of cell phenotype is likely to be accomplished by selective enhancement of gene expression which controls the terminal developmental commitment of cells.

The pancreas is composed of several types of endocrine and exocrine cells, each responding to a variety of trophic influences. The ability of these cells to undergo a change in phenotype has been extensively investigated because of the implications for the understanding of pancreatic diseases such as cancer and diabetes mellitus. Transdifferentiation of pancreatic cells was first noted nearly a decade ago. Hepatocyte-like cells, which are normally not present in

the pancreas, were observed following the administration of carcinogen (Rao et al., 1983; Scarpelli & Rao, 1981) to hamsters and the feeding of copper depleted-diets to rats (Rao et al., 1986). Recently, transdifferentiation of isolated acinar cells into duct-like cells has been observed by several groups (Arias & Bendayan, 1993; Hall & Lemoine , 1992; Tsao & Duguid, 1987). In view of these observations it is probably germane that during embryonic development, the hepatic and pancreatic anlagen are derived from a common endodermal evagination of the foregut and that acinar cells arise from progenitor cells in the pancreatic ducts (Githens, 1993). It remains to be clarified whether these examples of transdifferentiation result by a direct or indirect conversion of developmentally committed cells, or by conversion of an intermediate cell such as a pluripotent precursor cell.

In this paper, we show the induction of cultured human islets cells to alter their usual phenotypic expression and attain morphological and functional characteristics of duct cells. It is demonstrated that this process of transdifferentiation occurs by means of both direct and indirect cell conversion.

4.3 Materials and methods

4.3.1 Human islet isolation

Human pancreata were procured from cadaveric donors (age 18-50 years) following in-situ flush with UW Solution (Dupont Pharma, Montreal, Que.). Prior consent for organ donation was obtained by the local organ procurement agency, Quebec-Transplant. Cold ischemia time ranged from 1 to 5 hours. Islets were separated from the surrounding exocrine tissue by enzymatic digestion with Collagenase P (Boehringer Mannheim, Montreal,

Que.) at 37°C using the semi-automated technique described by Ricordi et. al. (Ricordi et al., 1988). Briefly, the pancreatic duct was cannulated and the gland distended with a cold solution of Collagenase P (1.2 mg/ml) in Hank's buffered salt solution (HBSS) (GIBCO, Montreal, Que.) with penicillin and fungizone (GIBCO, Montreal, Que.). The pancreas was placed in a sterilized steel and glass digestion chamber through which HBSS was recirculated at 37°C. The final concentration of the enzyme was 0.45 mg/ml. The extent of digestion was assessed by visualizing the islets under an inverted microscope after staining aliquots of digestate with dithizone (33.3 mg/ml in 95% ethanol) (Sigma, Montreal, Que.). The digestion process was arrested by cooling the circuit to between 5°C and 10°C when islets were observed to be free of surrounding acinar tissue. The digestate was washed three times in HBSS and islets were purified in a two step discontinuous BSA gradient (Sigma). Islets were collected from the interface between 1.000 and 1.080 g/ml, and were cultured overnight in suspension in RPMI 1640 (GIBCO, Montreal, Que.) supplemented with 4% NuSerum (Collaborative Research, Bedford, MA).

4.3.2 Culture of human islets

Crude rat-tail (type I) collagen solutions were prepared according to Richards (Richards et al., 1983a). Approximately 3,000 islets were resuspended in 6 ml of rat tail collagen. The collagen was then neutralized with 1 ml of a solution of 0.34N NaOH and 10X Waymouth's medium in a 4:6 ratio and separated into three 60-mm Petri dishes for polymerization. Islets were fed with a medium consisting of DMEM/F12 (GIBCO) supplemented with dexamethasone (1 μM, Sigma), NuSerum (10%, Collaborative Research, Bedford MA), cholera toxin (100 ng/ml, Sigma), epidermal growth factor (10

ng/ml, Sigma), soybean trypsin inhibitor, penicillin (100 U/ml, GIBCO), streptomycin (100 μg/ml, GIBCO) and amphotericin B (0.25 μg/ml, GIBCO). The culture medium was changed on alternate days. The cultures were incubated in an atmosphere of 5% carbon dioxide in air and at a temperature of 37°C.

4.3.3 Microscopy and tissue processing

Cultures were observed using a Nikon inverted light microscope. Fields of tissue were chosen at random, then photographed at 0, 2, 4, 6, 8, 10, and 12 days. Dishes were sampled on days 0, 1, 2, 3, 4, 6, 8, 10 and 12 and the tissue was fixed in 10% formalin. The collagen containing the tissue was embedded in paraffin and submitted for routine light microscopy. Deparaffinized sections cut at 5 μm were stained with hematoxylin and eosin. Other dishes were fixed in phosphate buffered glutaraldehyde, postfixed in 1% osmium tetroxide, and processed for electron microscopy. Ultrastructural analysis was performed on epon embedded tissue using a Phillips 200 electron microscope.

4.3.4 Autoradiography

On days 1, 4, 7, 9 and 13, the medium was replaced by medium supplemented with 1 μCi/ml of tritiated thymidine (deoxyribose-6[^{3}H]thymidine; specific activity: 35 Ci/mmol, ICN, Montreal, Que.). After a 24 hour incubation, the labeled medium was replaced with unlabeled medium and incubation was continued for a further 3 hours. Tissue from these plates was sampled on days 2, 5, 8, 11 and 14, fixed in 10% formalin and processed as above for light microscopy. Deparaffinized slides were covered with a nuclear tract emulsion (Kodak NTB-2, Kodak Canada, Toronto, Ont.)

diluted 1:1 with 0.6 M ammonium acetate at 43°C. Air-dried slides were exposed at 4°C for 15 days, then developed and stained (Rosenberg et al., 1989).

4.3.5 Tritiated thymidine incorporation and DNA assay

At 0, 1, 3, 5, 7, 9, 11 and 13 days in culture, the medium was replaced by medium supplemented with 1 μCi/ml of tritiated thymidine. After a 24 hour incubation, the collagen matrix was dissolved by 0.25 mg/ml collagenase. The tissue was washed three times in 1X PBS and sonicated for 30 seconds. Tritiated thymidine incorporation into trichloroacetic acid precipitated material was determined (Struhl, 1994). DNA was measured using a fluorometric technique using bisbenzimidazole (Hoechst 33258, Sigma, Montreal, Que.) as described (Labarca & Pagen, 1980). Tritiated thymidine incorporation was expressed as dpm/μg DNA (mean ± SD).

4.3.6 Total RNA extraction and Northern blots

RNA was extracted from cells in culture at 0, 1, 2, 4, 6, 9, and 12 days with guanidine thiocyanate as described (Chomczynski & Sacchi, 1987). Total RNA (30 μg) was separated by electrophoresis in 1% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond-N, Amersham, Oakville, Ont.). Prehybridization was performed in 50% formamide, 5x SSC, 5x Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 0.1% bovine serum albumin), 25 mM KPO_4 (pH 7.4), 50 μg/ml salmon sperm DNA at 42°C for 2-3 hours. A human insulin 40 mer oligonucleotide probe (Oncogene Science, Richmond Hill, Ont.) was ^{32}P-labeled using a 5'-end labeling system (Promega, Montreal, Que.). A human ß-actin cDNA probe (Clontech, Mississauga, Ont.) was labeled using a random primer method (Promega).

Hybridization was performed at 42°C for 20 hours in the same solution as was used for prehybridization.

After hybridization, the filters were washed three times at high stringency with 2x SSC/0.1% SDS at room temperature for 20 min., 0.1x SSC/0.1% SDS once at 50°C for 20 min. and 0.1x SSC/0.1% SDS once at 55°C for 20 min. The hybridized filters were exposed to X-ray film at -70°C overnight.

4.3.7 Quantification of insulin mRNA

The exposed autoradiographs were analyzed using a Hoefer GS-300 densitometer in transmittance mode. For each sample, both the insulin and ß-actin bands were scanned and the area under each peak calculated using the Hoefer GS 365W Electrophoresis Data System software. The result was expressed as the ratio of insulin/ß-actin.

4.3.8 Immunocytochemistry

Immunoperoxidase staining of tissues was performed by using kits purchased from Dako Corp. (Santa Barbara, CA). The final reaction product used was ethylaminocarbazole (EAC). B cells were stained with a guinea pig polyclonal anti-porcine insulin antibody (Dako Corp.). A cells were stained with a rabbit polyclonal anti-porcine glucagon antibody (Dako Corp.). To identify duct cells expressing cytokeratin 19 (CK19), a mouse monoclonal anti-human CK 19 antibody (Dako Corp.) was used. A rabbit polyclonal anti-neuron specific enolase (NSE) (Zymed Lab., San Francisco, CA) was used to identify endocrine cells. Nonspecific staining was excluded by using the second antibody without the primary antibody, and specificity was determined by neutralization with excess antigen.

4.3.9 *Insulin assay*

At 2, 4, 6, 8, 10, 12, and 14 days in culture, the collagen matrix was dissolved by 0.25 mg/ml collagenase. The tissue was washed three times in 1X PBS and sonicated for 30 seconds. The insulin contents of the samples were measured in duplicate by RIA using porcine insulin as a standard (Immunocorp, Montreal, Que.). At the same time, the DNA content of the samples was determined. Insulin content was expressed as pmol/μg DNA (mean ± SD).

4.3.10 *Statistical analysis*

All data are expressed as mean ± standard deviation with n=4 (flasks) at each time point. The difference between time points with respect to thymidine incorporation and intracellular insulin content was evaluated by unpaired Student's t-test. Differences were considered significant at the 5% level ($p<0.05$).

4.4 Results

4.4.1 *Microscopy*

Day 0. Initially, the islets maintained their solid spheroid appearance under the inverted microscope (**Fig. 4-1a**). Histologically, there were some cells with pyknotic nuclei observed in the center of many islets, but otherwise the tissue appeared normal (**Fig. 4-2b**). No other cell types were observed. *Day 1*: Small cystic structures appeared at the periphery of islets examined under the inverted microscope. By light microscopy, single or multiple small cystic spaces were seen in both the center and the periphery of islets. *Days 2-3*:

Progressive cystic transformation of islets was observed, accompanied by a concomitant loss of the normal islet tissue component (**Figs. 4-1b, 4-2c**). *Days 4-5.* More than 90% of islets showed partial or complete cystic transformation under the inverted microscope (**Fig. 4-1c**). Histologic examination demonstrated the cyst wall to be composed of a single layer of cuboidal cells (**Figs. 4-2d, 4-2e**). *Days 6-14.* The size of cysts increased and there was now complete replacement of the normal islet morphology (**Figs. 4-2e, 4-2f**). The lining epithelial cells were flattened and by electron microscopy, they had the appearance of a primitive ductal epithelium with nuclear polarity and surface microvilli (**Fig. 4-3**). When the cysts were punctured, the cells assumed a tall columnar appearance.

4.4.2 Tritiated thymidine uptake and autoradiography

The incorporation of tritiated thymidine (dpm/μg DNA) remained low during the initial 48 hours of culture - 2.52 ± 0.23 on day 1 and 10.93 ± 3.07 on day 2 (**Fig. 4-4**). Thereafter, the level of activity increased by more than 1500% ($p<0.01$) to plateau at day 10 (177.30 ± 16.73), before declining. In support of these data, there were very few cells labeled by autoradiography on days 2 and 5, but by day 11, approximately 40% of cells showed nuclear labeling (data not shown).

4.4.3 Insulin synthesis and storage

The cellular content of insulin was highest in freshly isolated islets immediately following pancreatic digestion, 1731.73 ± 223.88 pmol/μg DNA. After 24 hours in culture, the insulin content had declined 58% to 727.11 ± 109.05 pmol/μg DNA. Thereafter, it continued to decrease to 136.45 ± 30.93 pmol/μg DNA by Day 8, a level that was subsequently maintained (**Fig. 4-5**).

The transcriptional level of insulin mRNA in islet cells during the initial 24 hours in culture was comparable to that of freshly isolated islets, and it declined slightly, from day 2 to day 4. Thereafter, insulin mRNA levels decreased significantly (**Figs. 4-6, 4-7**).

4.4.4 Immunocytochemistry

Freshly isolated islets demonstrated positive immunoreactivity to insulin and glucagon (**Figs. 4-8b, 4-9b**). Some islet cells showed marked degranulation after the initial 24 hours of culture (**Figs. 4-8c, 4-9c**). From day 2 to day 7, positively stained cells for insulin and glucagon were observed within the cystic wall (**Figs. 4-8d, 4-8e, 4-9d, 4-9e**). From day 7 to day 10, single positive cells within some of the cysts could be identified (**Figs. 4-8f, 4-9f**). Insulin and glucagon immunoreactive cells disappeared after 10 days in culture.

NSE immunoreactivity was demonstrated in freshly isolated islets and its presence was maintained throughout the period of observation from day 1 to day 12. NSE was present in all cells (**Fig. 4-10**).

Weak positive staining for CK-19 first appeared on day 2 as cystic structures developed in the islets. CK-19 positive immunoreactivity continued to increase throughout the experiment and by day 12, 100% of cells were found to express this marker (**Fig. 4-11**).

4.5 Discussion

Endocrine cells in the pancreas develop by budding from embryonic duct-like cells (Githens, 1993). This process leads to the formation of primitive islets in the mesenchyme adjacent to the ducts and requires the presence of factors derived from embryonic mesenchyme (Golosow & Grobstein, 1962).

During this transition, an orderly sequence of gene activation and expression produces cells that synthesize, store and secrete islet cell hormones. The process can be reproduced in vitro, with the pancreatic duct being induced to differentiate into both exocrine and endocrine cells (Dudek & Lawrence, 1988; Teitelman et al., 1987).

The ability of cells in the *adult* pancreas to undergo proliferation and differentiation toward an endocrine cell phenotype has been investigated since the beginning of this century using different models. In a series of studies (Shaw & Latimer, 1926) from 1902-1914 the observation was made that islet and acinar regeneration occurred following complete pancreatic duct obstruction. In 1924, Fisher described islet and acinar cell regeneration from the ductal stump following near total pancreatectomy (Shaw & Latimer, 1926). The ductal epithelium was also implicated as the source of new islet tissue in 1926 by Shaw and Latimer, who reported islet regeneration from pancreatic duct fragments transplanted to the submucosal space of the canine duodenum (Shaw & Latimer, 1926). In 1978, Pour described the appearance of duct-like cells in islets during BOP-induced pancreatic carcinogenesis in hamsters (Pour, 1978). More recently, we reported that partial obstruction of the pancreatic duct in the adult hamster leads to the induction of islet cell differentiation from cells in the intralobular ducts, followed by the formation of new islets (Rosenberg et al., 1983).

The question arises as to whether the converse situation is possible. Can the endocrine cells of the islet transform into another distinct cell type? In this study, we report that adult islets of Langerhans cultured in a collagen gel matrix undergo a transformation to cystic structures whose constituent cells express both ductal and endocrine phenotypic markers.

Should this finding be totally unanticipated? The possibility is suggested by numerous reports of the presence of so-called intermediate cells in the pancreas (Cossel, 1984; Gu et al., 1994; Melmed, 1979; Melmed et al., 1972). The term intermediate cell is used to describe pancreatic cells which exhibit the characteristic morphologic features of both exocrine (acinar or duct cells) and endocrine cells (Melmed, 1979). Intermediate cells have been observed in various animal species under normal and pathological conditions, and the subject has been extensively reviewed by Cossel (Cossel, 1984). The predominant intermediate cell type in adult rats is the acinar-ß cell, but acinar-α and duct-ß cells have also been reported (Melmed et al., 1972). Acinar-α and acinar-ß cells have been found in the pancreas of type I diabetics (Cossel, 1984). In transgenic mice, Gu et. al. demonstrated the presence of numerous intermediate cell types including cells that simultaneously exhibited both ductal and acinar cell phenotypes, endocrine and exocrine features, as well as single endocrine cells that contained multiple islet hormones (Gu et al., 1994). The occurrence of intermediate cells demonstrates the intimate relationship between genes coding for pancreatic endocrine and exocrine phenotypic expression.

In our experiments, the results are consistent with the transdifferentiation of an islet cell to a ductal cell. CK-19, a duct cell marker was not demonstrated prior to cyst formation, whereas insulin mRNA was present during this same period and gradually diminished thereafter. At 10 days, the insulin protein and message were absent and all cells expressed both the duct cell marker CK-19 as well as the endocrine marker NSE. Finally, cystic transformation of the islet occurred prior to proliferation. From the foregoing, pre-existing ductal cells at the periphery of the islet cannot be the precursors of the epithelial cells lining the cysts.

Is it possible that acinar cells could be the cells of origin for the duct-like cells observed in our culture system? The physical presence of acinar cells in the islet preparation was excluded by repeating the separation gradient step a second time at 24 hours after the initial isolation. By waiting this additional period, any remaining acinar cells would be expected to have died under the prevailing culture conditions. Even allowing for some survival, they would have been removed by the second density gradient purification. To further confirm the absence of acinar cells, an extensive ultrastructural study was performed of the isolated islet tissue to identify cells that contained zymogen granules or that had other features of acinar cells, e.g. stacked RER, large basally located nucleus and many abundant mitochondria. This imaging method for identifying acinar cells was used, because the cells may degranulate during the islet isolation and purification procedures and immunocytochemical staining for amylase could have produced a falsely negative result. Thus we ruled out the possibility that acinar cells were the source of the duct-like cells that arise from the islets in these experiments.

In many instances, transdifferentiation involves a sequence of steps. Early in the process, intermediate cells appear that express neither the phenotype of the original nor the subsequent differentiated cell types, and therefore they have been termed *dedifferentiated.* The whole process is accompanied by DNA replication and cell proliferation (Okada, 1983). The immunocytochemical evidence provided by these experiments supports the indirect route for transdifferentiation in this system, i.e. through an intermediate or pluripotential cell type, rather than a direct conversion from endocrine to ductal cell.

Little is known regarding the molecular events of transdifferentiation. The differentiated state of a cell may be regulated at all the generally

recognized levels, i.e., DNA methylation, transcription of RNA and translation of protein. Transfection of the chicken crystalline gene into mouse cells indicates that the level of crystalline synthesis is controlled mainly at the transcription level (Kondoh et al., 1987). Insulin is a terminal product of islet cell differentiation. Utilization of this marker in our study does not directly address the regulatory mechanisms underlying the transdifferentiation process. Nevertheless, information gained from a comparison of immunoassay and Northern blot for insulin is useful for analyzing specific gene expression in relation to transdifferentiation. The results of similar changes for insulin suggest that the differentiated state during transdifferentiation is mainly controlled at the transcriptional level. Therefore, the process may involve two distinct steps: repression of islet genes and upregulation of ductal cell genes, for example, CK-19.

Differentiated, functional cells usually maintain their cellular specificities in adult organisms. Change of cellular specificities can frequently be observed under some pathological conditions, such as reparative regeneration and carcinogenesis. Stability of the cellular phenotype is probably related to cell-cell and cell-substrate interaction in vivo. A perturbation or loss of stabilizing factors may induce cells to change their commitment. We cultured human islets in collagen, which leads islet cells to change their phenotype to ductal cells. However, using similar culture conditions Lucas-Clerc et. al. maintained human islets in collagen for more than 8 weeks (Lucas-Clerc et al., 1993). These contrary findings imply that the stability of islets may have been disrupted during our isolation and culture procedure. An understanding of how to manipulate human islets *in* vitro may shed light on the difficulties facing islet cell transplantation, i.e. qualitative control of islets and optimal implantation site conditions.

The presence of basement membrane around islets of Langerhans has been demonstrated by van Deijnen et al. (Deijnen van et al., 1994). Immunocytochemistry has shown that major components of basement membrane are type IV collagen and laminin. Histochemical analysis of the process of pancreatic digestion showed that protease enhanced the degradation of all four major components of the extracellular matrix: collagen, proteoglycans, glycoproteins and elastin (Wolters et al., 1992). Enzymes which degrade the extracellular matrix could be a key factor in destabilization of differentiated cells. Previous studies have confirmed that destabilization by collagenase or hyaluronidase is a necessary step in the initiation of transdifferentiation of striated muscle cells isolated as tissue fragments (Schmid, 1978). Our experiments indicate that during the isolation process, islets lose their basement membrane after 30 minutes of digestion by Collagenase-P (data not shown). Further digestion may damage the outer layers of islets. Therefore, ECM may play an important role in stabilizing the differentiated state of islet cells.

The use of collagen gel as an extracellular matrix material is an important part of the culture system, allowing the reconstruction of 3-dimensional tissue architecture. In addition, collagen gel matrix can help to promote or maintain the differentiated state of variable cell types in culture, including liver cells (Guguen-Guillouzo et al., 1983; Rubin et al., 1981) and mammary epithelial cells (Foster et al., 1983; Yang et al., 1982b). On the other hand, extracellular matrix may promote the process of transdifferentiation. This point is highlighted by isolated pancreatic acinar cells which transdifferentiate to duct-like structures when entrapped in Matrigel basement membrane (Arias & Bendayan, 1993), and by retinal pigmented epithelial cells, which transdifferentiate into neurons when plated on laminin-containing substrates

(Reh et al., 1987). Most recently, Gittes et al. (Gittes et al., 1996) demonstrated, using 11-day embryonic mouse pancreas, that the default path for growth of embryonic pancreatic epithelium is to form islets. In the presence of basement membrane constituents, however, the pancreatic anlage epithelium appears to programmed to form ducts. This finding again emphasizes the interrelationship between ducts and islets and highlights the important role of the extracellular matrix.

Growth factors may also play an important role in the induction of transdifferentiation. Fibroblast growth factor (FGF) has been found to initiate transdifferentiation of the retinal pigment epithelium to neural retinal tissues in chick embryo in vivo and in vitro (Hyuga et al., 1993; Park & Hollenberg, 1991; Pittack et al., 1991). Transforming growth factor-beta (TGF-ß) has been demonstrated to induce transdifferentiation of mouse mammary epithelial cells to fibroblast cells (Miettinen et al., 1994). Similarly, we have used epithelial growth factor (EGF) and cholera toxin to enhance duct epithelial cyst formation from among acinar cell fragments (Yuan et al., 1995). In the present study, the role of EGF and cholera toxin, as well as the constituent components of NuSerum remained to be investigated.

In summary, islets have been isolated from cadaveric organ donors, purified and then placed into a three-dimensional rat-tail collagen gel matrix. During the initial 96 hours of culture, the islets underwent a cystic transformation that was associated with (i) the maintenance of immunoreactivity for neuron specific enolase, an endocrine cell marker, but a progressive loss of insulin gene expression, (ii) a loss of immunoreactivity for insulin protein, and (iii) the appearance of CK-19, a marker for ductal cells. After the transformation was complete, the cells had the ultrastructural appearance of primitive duct-like cells. Cyst enlargement after the initial 96

hours was associated, at least in part, with a tremendous increase in cell replication. These findings are consistent with the transdifferentiation of an islet cell to a ductal cell. The reasons for this phenotypic change, as with the exact mechanisms involved, remain to be fully elucidated.

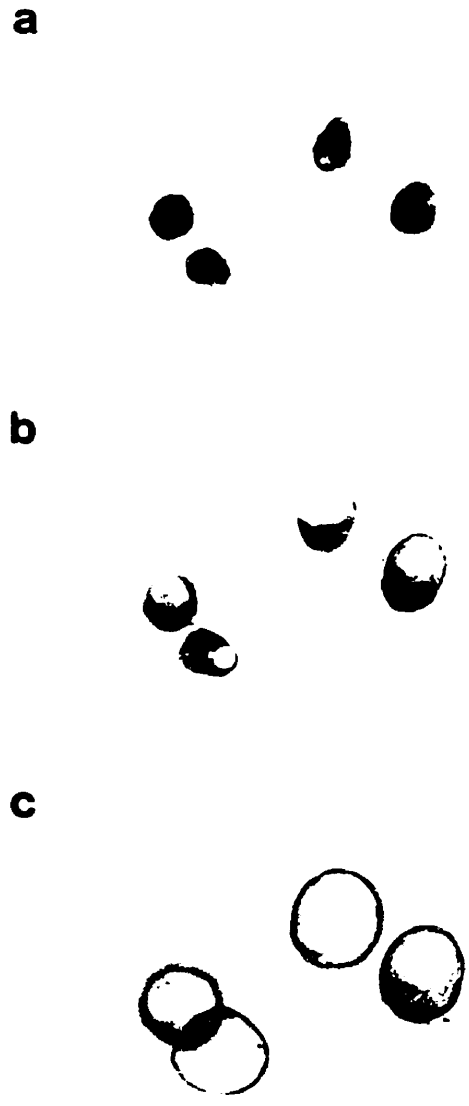

Figure 4-1. Inverted microscopic photographs of cystic transformation from cultured human islets. **a**, Day 0. Purified human islets in collagen matrix (200x). **b**, Day 3. Progressive cystic transformation of the islets has occurred. There is a concomitant loss of normal islet tissue, which forms a crescent at the periphery of the cyst (200x). **c**, Day 4. Cystic transformation of the islets is virtually complete. These structures continue to enlarge until days 7 - 10 (200x).

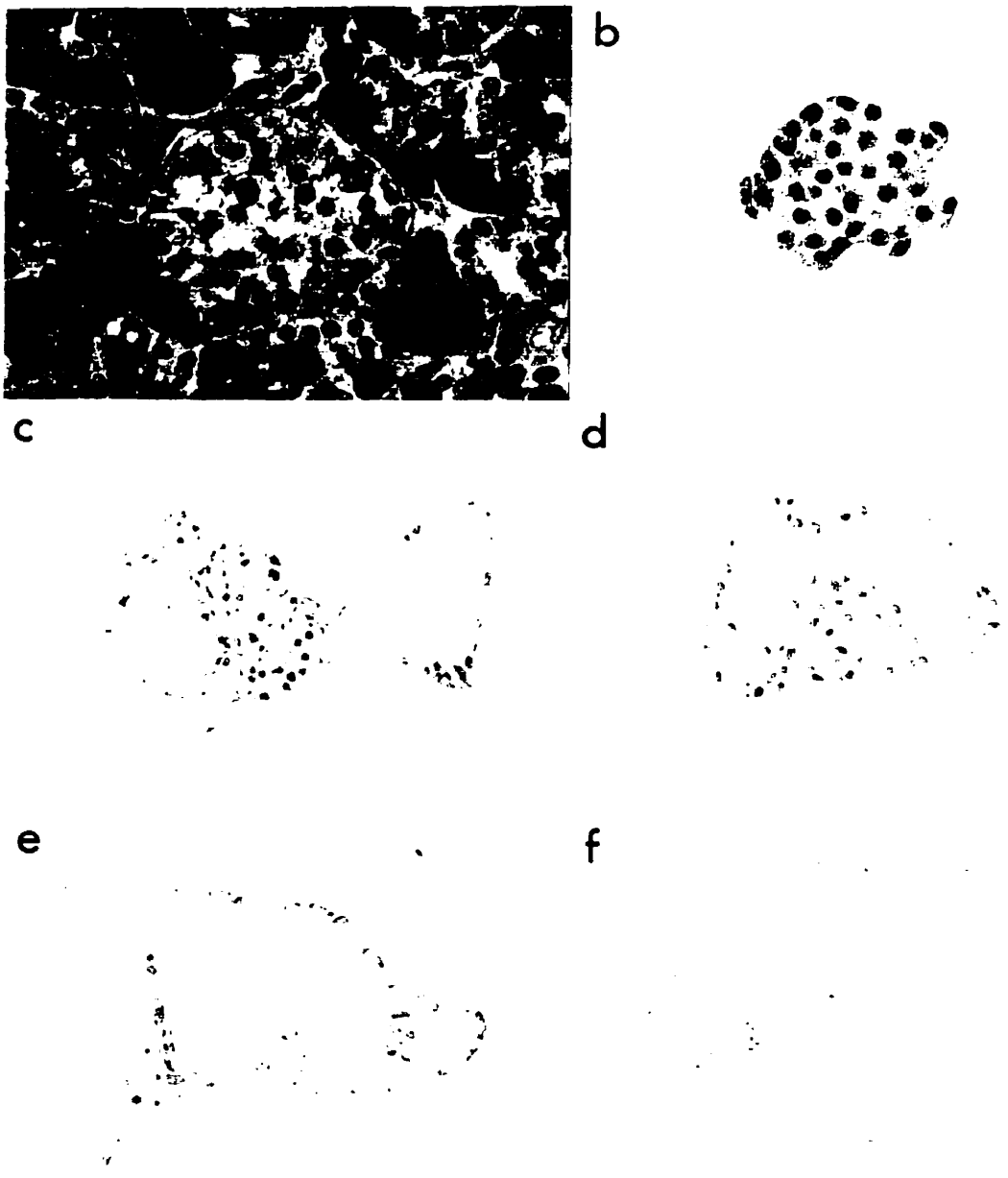

Figure 4-2. Light microscopic photographs of cystic transformation from cultured human islets (H&E stain). **a**, Normal human pancreas (440x). **b**, Day 0 (440x). **c**, Day 3 (176x). **d**, Day 4 (440x). **e**, Day 5 (141x). **f**, Day 9 (88x).

Figure 4-3. Day 10 - Electron micrograph of cystic structures derived from human islets. The cells have the appearance typical of a primitive ductal epithelium, with nuclear polarity and apical microvilli. (3185x)

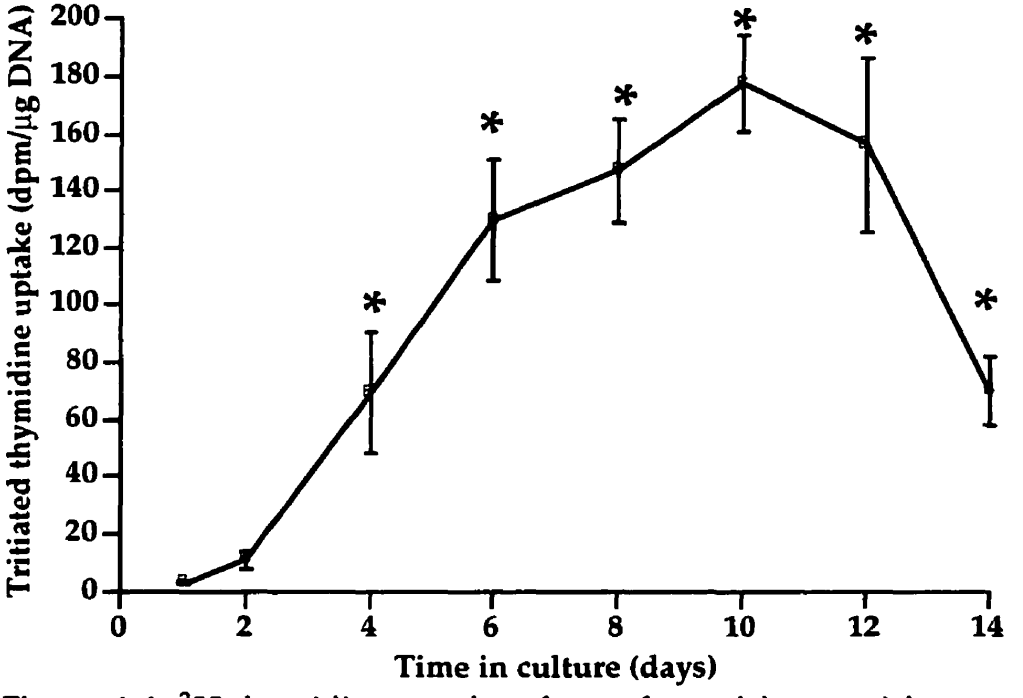

Figure 4-4. ^{3}H-thymidine uptake of transformed human islets, expressed as dpm/µg DNA vs time in culture. From day 2 to day 10, an increase of >1500% is observed (*: $p<0.05$ vs. day 0)

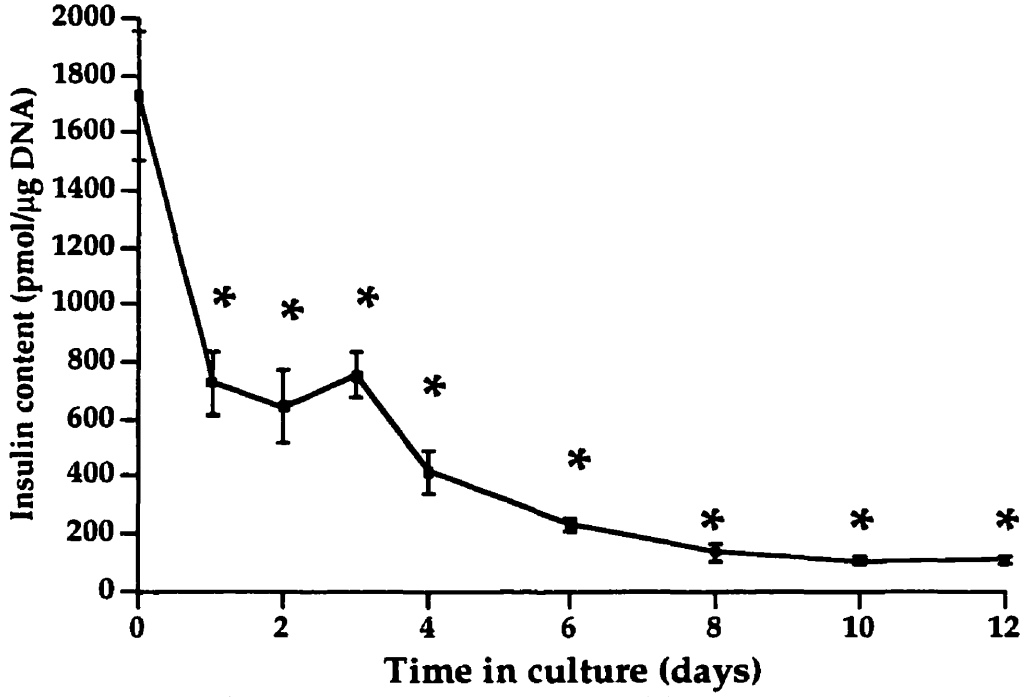

Figure 4-5. Cellular insulin content in pmol/µg DNA was observed to decline over time in human islets, as cystic transformation took place. After 24 hours in culture, insulin content decreased 58% (*: $p<0.05$ vs. day 0).

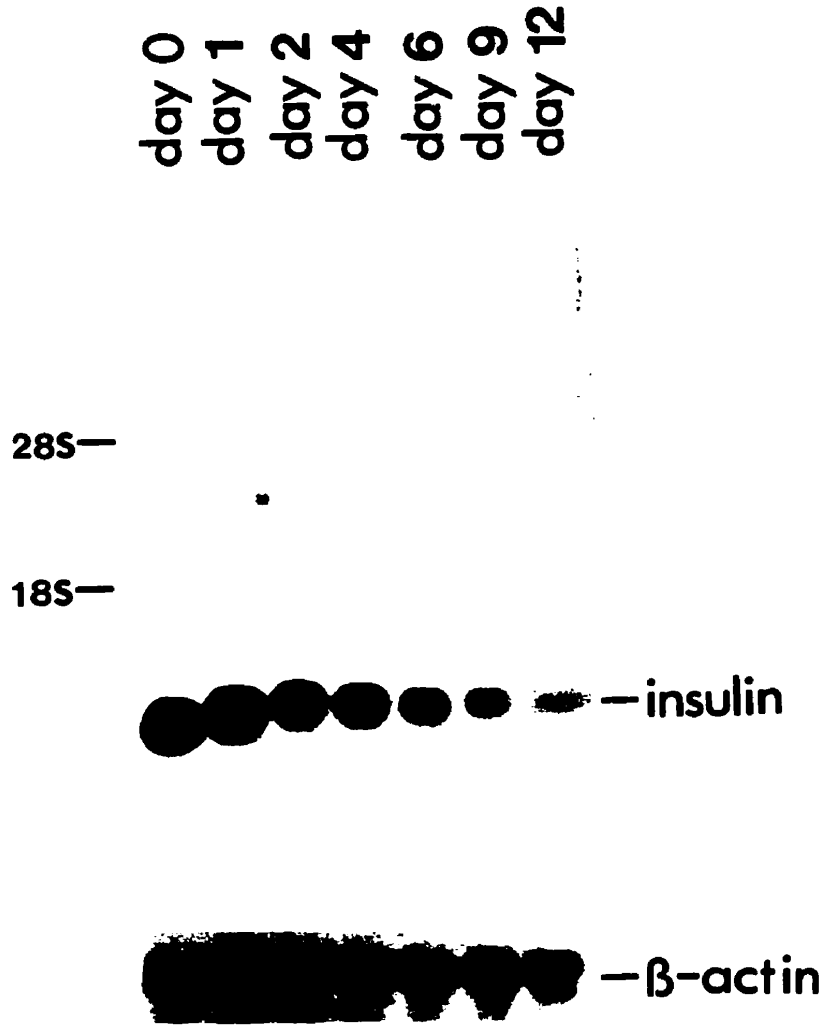

Figure 4-6. Northern blot of insulin and ß-actin mRNA in human islets cultured in collagen gel.

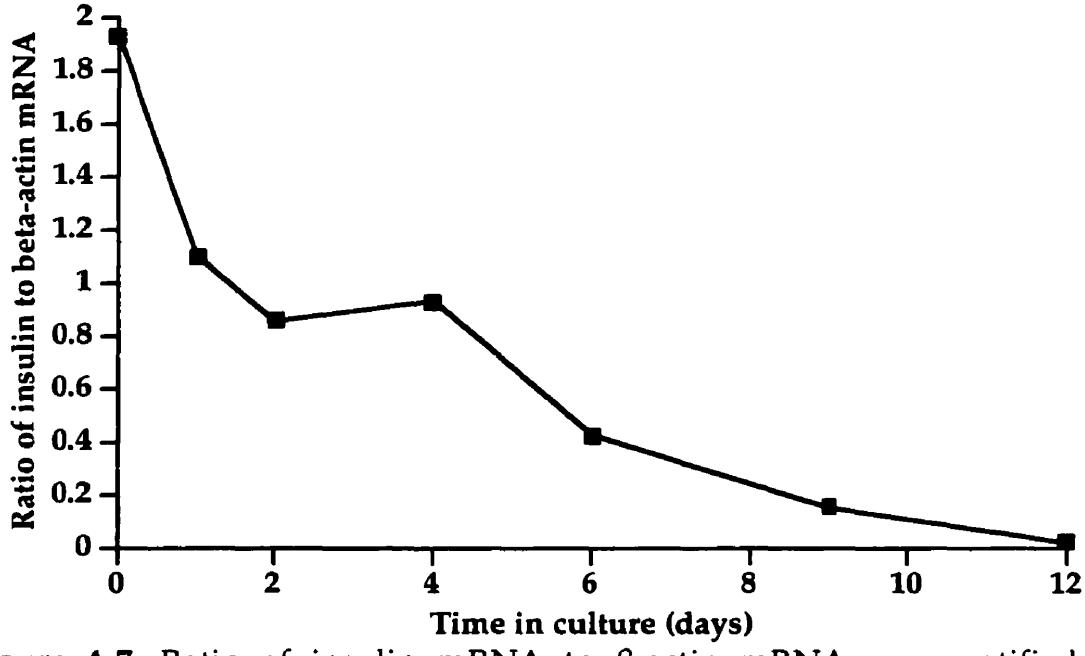

Figure 4-7. Ratio of insulin mRNA to ß-actin mRNA as quantified by densitometric analysis of Northern blot. The relative abundance of insulin mRNA declines with time in culture.

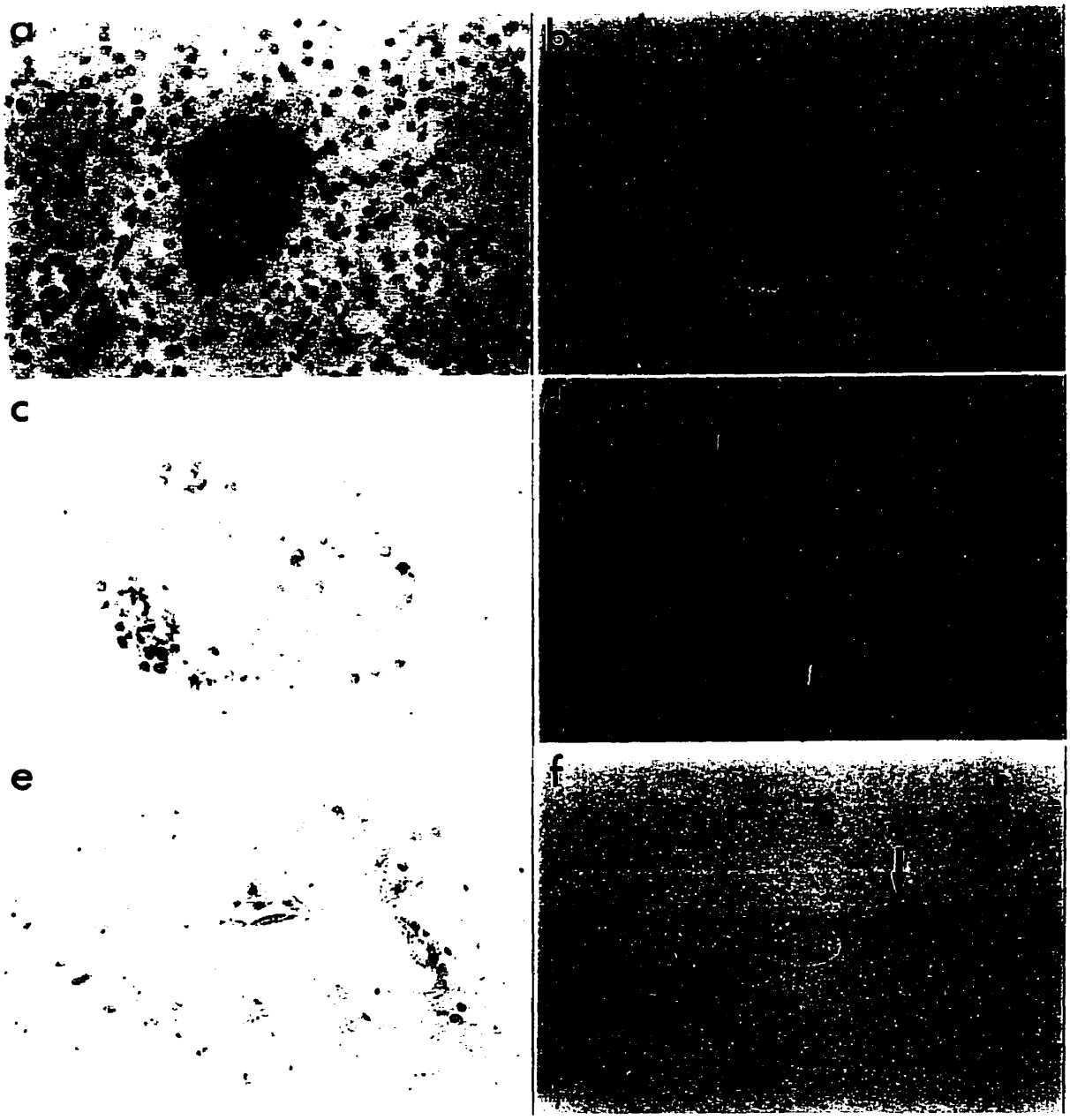

Figure 4-8. Immunocytochemistry for insulin (immunoperoxidase stain). **a,** Normal human pancreas (282x). **b,** Day 0 (282x). **c,** Day 1 (282x). **d,** Day 3 (352x). **e,** Day 4 (176x). **f,** Day 7 (352x).

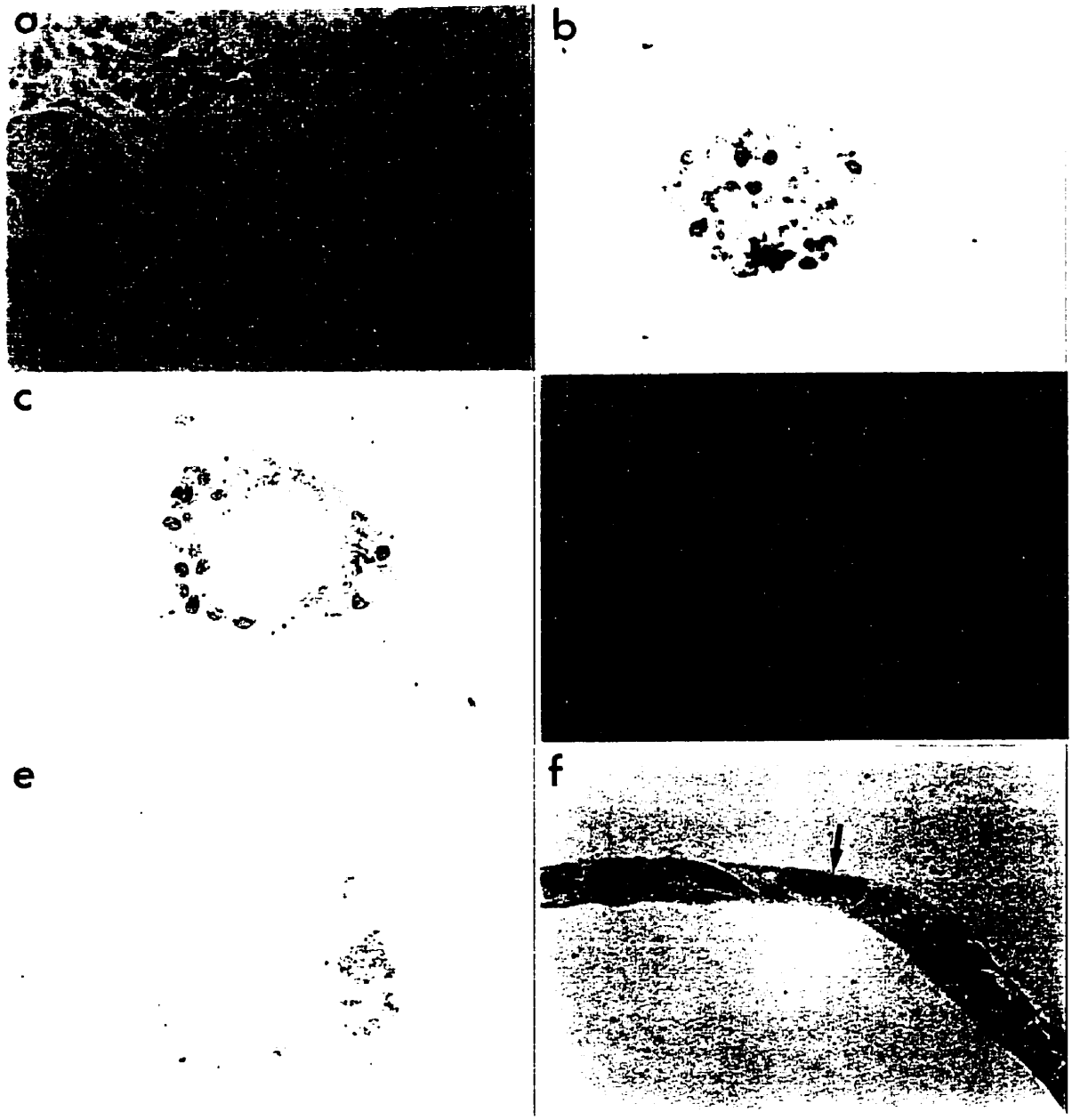

Figure 4-9. Immunocytochemistry for glucagon (immunoperoxidase stain). **a**, Normal human pancreas (220x). **b**, Day 0 (220x). **c**, Day 1 (440x). **d**, Day 3 (352x). **e**, Day 7 (220x). **f**, Day 7 (440x).

Figure 4-10. Day 12 - Fully transformed cysts stain strongly for NSE (Immunoperoxidase stain, 250x)

Figure 4-11. Day 12 - Fully transformed cysts stain strongly for CK-19 (Immunoperoxidase stain, 250x).

Chapter 5

Final Discussion and Conclusions

The pancreas is composed of two unique tissues: (1) the exocrine compartment comprising the acinar and duct cells and (2) the endocrine compartment comprising the islets of Langerhans. It is generally believed that the protodifferentiated duct epithelium gives rise to acinar, islet, and mature duct cells during the development of the pancreas (Githens, 1993; Slack, 1995). All three cell types show a high rate of proliferation in the prenatal pancreas (Githens, 1993). The rapid proliferation continues throughout the neonatal period and declines shortly after weaning. During the adult life, the mitotic index of acinar, ductal and islet cells is very low (Githens, 1988). A fraction of the islet cells, which are capable of entering the cell cycle and undergoing mitosis, comprise less than 3% in the adult pancreas, but are about 10% in the fetal pancreas (Swenne, 1983).

Despite a large numbers of studies in recent years, cell differentiation in the adult pancreas is largely unknown. Resolution of this question would be of importance to the understanding of the development of two diseases: diabetes mellitus and pancreatic cancer. It is postulated that new secretory cells may arise by 1) replication of existing differentiated cells; 2) differentiation from stem cells within the ductal epithelium; 3) transdifferentiation of one differentiated cell type to another type (Elsasser et al., 1986). Figure 5-1 is a hypothetical model of the possible relationships among different cell types in the adult pancreas and has been modified from one originally proposed by Githens (Githens, 1993).

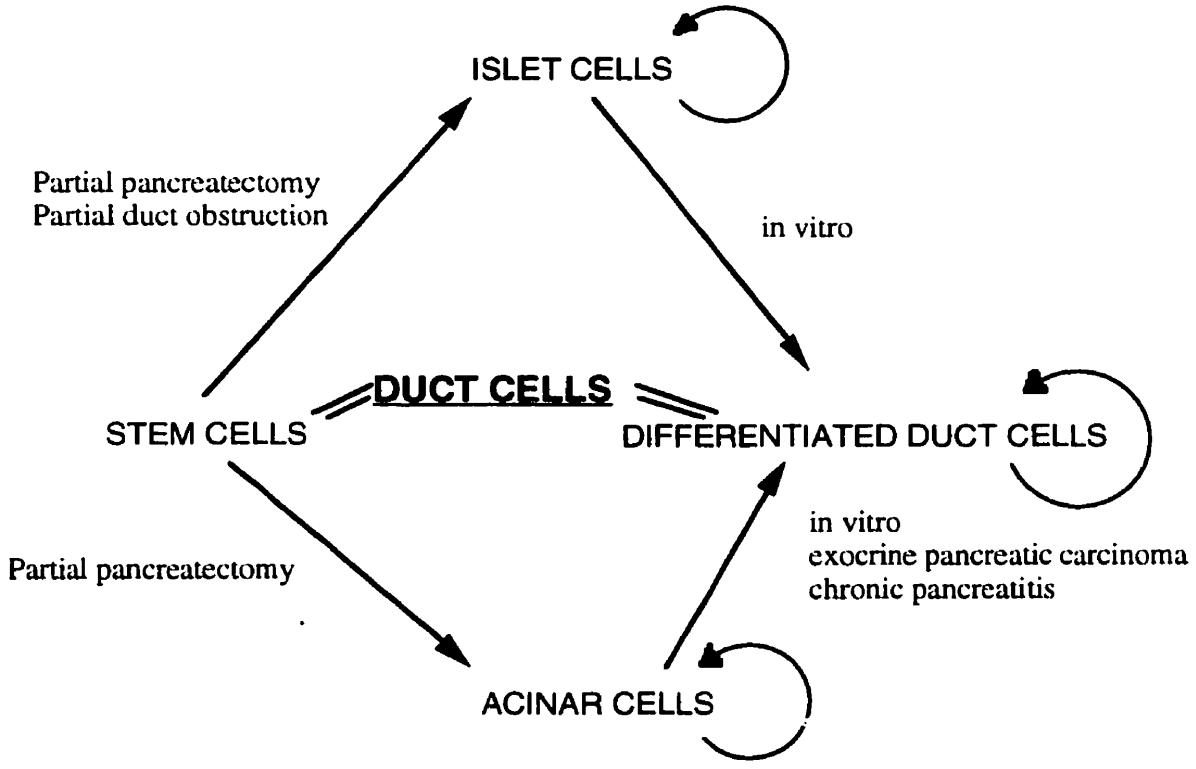

Figure 5-1. A model of relationships among adult pancreatic cells

Evidence has accumulated that differentiated acinar cells, ductal cells and islet cells are capable of entering the cell cycle and increasing their respective cell populations. Application of autoradiography to measure incorporation of ^{3}H-thymidine into the DNA of individual cell populations has demonstrated that differentiated pancreatic cells could undergo mitosis at a high rate under certain conditions (Solomon et al., 1983; Lutcke et al., 1987; Matsuda et al., 1994; Nielsen et al., 1989). Replication of these cells was further confirmed by the subsequent ultrastructural identification of acinar cells, intralobular duct cells and B-cells in mitosis (Lutcke et al., 1987; Chick & Like, 1969). However, these observations can not resolve the controversy regarding the existence of stem cells in the adult pancreas, which have the potential to differentiate into acinar, duct, and islet cells.

The hypothesis that the stem cells within ductal epithelium develop into acinar and islet cells has been investigated with different models. Adler et al. showed that centroacinar cells differentiated into acinar cells after severe damage to the exocrine pancreatic cells caused by supramaximal hormonal stimulation (Adler et al., 1979). The centroacinar cells were seen to divide and then undergo a morphological transformation as shown by the accumulation of secretory granules. Rosenberg and Duguid observed that partial duct obstruction induced by wrapping of the hamster pancreas leads to the formation of new islets (Rosenberg et al., 1983; Rosenberg et al., 1989). Newly formed islets were found in relation to hyperplasia and hypertrophy of the terminal ductules, a process is known as nesidioblastosis. Bonner-Weir et al. reported some regeneration of both exocrine and endocrine pancreas following a 90% partial pancreatectomy (Bonner-Weir et al., 1993). The small ductules in the focal regions proliferated and then differentiated into new lobules of the pancreas, containing new islets and acini within a few days after resection. Gu and Sarvetnick demonstrated that transgenic mice bearing the interferon-gamma gene exhibited progressive destruction of pancreatic islets, which eventually led to diabetes (Gu & Sarvetnick, 1993). In this model, the pancreatic ducts enlarged and fused to form an array of ductal systems, in which the buds of islet-like structures occurred at different loci along the length of the duct walls. In some instances, the islet-like structures were seen to protrude into the lumen of ducts. Dudek and colleagues combined adult pancreatic ductal epithelium with fetal mesenchymal tissue and then transplanted them in nude mice (Dudek et al., 1991). Islet tissue, but no acinar tissue, developed in 20% of the recombinants.

Studies of stem cells in vivo, however, are difficult because of the intricate anatomical relationship among different types of pancreatic cells. Direct proof

will require the isolation and culture of populations of duct cells, particularly intralobular duct cells, which putatively have the ability to differentiate into islet and/or acinar cells (Parsa & Marsh, 1976). A number of methods for adult pancreatic duct epithelial isolation and culture from cow, human, rat, hamster and guinea pig have been reported (Githens, 1988). Despite these efforts, the conventional methods yield populations consisting mainly of main and interlobular duct cells. Reliable methods for the large scale isolation and culture of the terminal segments of the pancreatic duct system are not available.

A three-dimensional reconstruction of exocrine pancreas demonstrated that the organization of intralobular ducts and acini within the lobule is quite complex (Adao et al., 1986). In addition to ductules ending in a terminal acinus, many acini may be formed on the sides of a duct as it courses through a lobule. A duct may lead to an acinus, and another duct may continue on the opposite side of the acinus. Based on the complicated relationship between the intralobular ducts and acini, I formulated a method of tissue digestion and separation that would produce acinar fragments of a particular size. These fragments would contain small intralobular duct cells as predicted.

The acinar fragments of the hamster pancreas were isolated by partial digestion with collagenase, embedded in a matrix of rat-tail collagen, and cultured in DME:F12 media and 10% NuSerum supplemented with cholera toxin, epidermal growth factor and additives. The starting material consisted of clumps of acinar tissue fragments and a few pieces of larger ducts. Ductal cysts appeared within the areas of degenerated acinar tissue after a few days of culture. The number and size of cystic structures increased with time with a progressive loss of the acinar tissue. Histological examination showed that

the wall of these cysts consisted of a single layer of cubical or flattened epithelium. The ultrastructural appearance of the cysts resembled that of duct epithelium in vivo. Apical microvilli and lateral interdigitations of the plasma membrane were present and cells were interconnected by tight junctions. This method gave a high yield of ductal cysts, which could be maintained in vitro for over two months.

The question of the cellular origin of the newly formed ducts was addressed by autoradiography. The cells were exposed to tritiated thymidine on the first day of culture. On day 2, very few cells had incorporated the radiolabel, and the labeled cells were primarily in the centroacinar unit. After 6 days, duct cysts were well formed, and most of the duct epithelial cells were labeled. These findings suggested that the cysts originated from the proliferation of intralobular duct cells within the acinar fragments. However, transdifferentiation of acinar cells into ductal cells could not be ruled out.

The conversion of one differentiated cell type to another in postnatal life is well documented and encountered in a variety of pathological conditions (Slack, 1986). In pathology textbooks, such a change in cell differentiation is referred to as metaplasia. Conventional biological wisdom has held that metaplasia refers to an alteration of tissue specificity rather than a change at the cellular level. Transdifferentiation, therefore, is a more appropriate term for the process of cell type conversion (Okada, 1986). The phenomenon of transdifferentiation can occur between cell types belonging to the same cell lineage or different cell lineages. The best known example of cell type conversion in the adult pancreas is the development of pancreatic hepatocytes in hamsters and rats treated with carcinogens or maintained on diets deficient in copper or methionine (Scarpelli & Rao, 1981; McDonald & Boorman, 1989; Rao et al., 1986; Hoover & Poirier, 1986).

Transdifferentiation of acinar cells into the morphological appearance of ductules has been documented in experimental pancreatic models and human pancreatic lesions. Sequential analysis of pancreas of carcinogen-treated hamsters showed the earliest changes in acinar cells, characterized by degeneration of acinar cells, loss of zymogen granules and apical cytoplasm, and the formation of pseudoductules and cystic complexes (Scarpelli et al., 1983; Flaks et al., 1982). Based on these findings, it has been postulated that acinar cells might play a role in the pathogenesis of neoplastic ducts (Scarpelli et al., 1983; Flaks et al., 1982, Scarpelli et al., 1984; Flaks, 1984). Tubular complexes in human chronic pancreatitis have been shown to originate from acinar cells by a transitional process, involving cessation of zymogen granule synthesis and storage, and a decrease in cell height (Bockman et al., 1982; Bockman et al., 1983). The duct ligation of the pancreas gave rise to extensive atrophy of its acini, which lost their zymogen granules and transformed into a duct-like morphology (Graves et al., 1986). In transgenic mice, the expression of an elastase promoter-TGFα construction of acinar cells led to multifocal ductular metaplasia, in which acinar cells appeared to degranulate, dedifferentiate and form tubular complexes (Sandgrean et al., 1990; Jhappan et al., 1990).

Direct experimental evidence for the transdifferentiation of acinar cells to a ductal phenotype has been provided by in vitro observations. Dissociated neoplastic pancreatic cells derived from a azaserine-induced transplantable acinar carcinoma underwent reorganization into duct-like structures when cells were maintained on a basement membrane scaffolding (Reddy et al., 1986). The duct-like cells retained the acinar cell-specific antigen and continued to synthesize and secrete amylase in spite of the absence of differentiated characteristics of acinar cells. When acinar cell clusters were

isolated from normal human pancreas and cultured on plastic substrata (Hall & Lemoine, 1992), they lost the differentiated phenotype within 4 days and subsequently transdifferentiated into a ductal phenotype as assessed with specific immunological markers. Furthermore, isolated acinar cells from rat and guinea pig pancreas also changed their morphological appearance to a duct-like phenotype when the cells were embedded into the Matrigel basement membrane in the presence of dimethylsulfoxide (Arias & Bendayan, 1993). These cells progressively lost intracellular amylase and increasingly expressed the duct cell marker carbonic anhydrase II.

I examined the effect of different factors in the culture media on the differentiated phenotype of cultured hamster acini. In these experiments, dissociated acini were sustained in a collagen gel and maintained in a variety of culture media. When isolated pancreatic acini were cultured in DMEM/F12 medium supplemented with 5% NuSerum and additives, some of the acini underwent necrosis during the first 6 days in culture. The remainder of cells were relatively normal in appearance. The intracellular amylase activity of the cultured acinar cells decreased with time and there was an 85% loss of the initial specific activity after 12 days in culture. Nevertheless, the normal acinar phenotype could be maintained for over 1 month in this culture system and very few cystic structures were found. However, pancreatic acini had a completely different course of morphological changes when they were grown in DMEM/F12 supplemented with 10% NuSerum, cholera toxin, EGF, and additives. After 8 days in culture, most acinar cells disappeared and were replaced by cystic structures lined by a single layer of cuboidal cells. Some amylase positive immuno-reactive cells became an integral component of the cystic wall. The level of intracellular amylase activity was correlated with the morphological observations. The cells

contained only 5% of the initial specific activity after 12 days in culture. This result indicates that EGF and cholera toxin are important factors for cystic transformation from cultured acini.

The mechanisms involved in this phenotypic transformation remain unclear. One possible explanation is that the acinar cells undergo transdifferentiation and become duct-like in appearance. Another possibility is that stem cells within ductules proliferate and form the cystic structures, as the acinar cells die. One of major impediments to the further study of this phenomenon is the anatomical complexity of the pancreas, which make acinar cells more difficult to separate from terminal ducts. A number of methods for isolation of pancreatic acinar cells have been reported since Amsterdam and Jamieson first developed the technique for preparation of dispersed pancreatic acinar cells in 1972 (Amsterdam & Jamieson, 1972; Oliver, 1980; Schulz et al., 1988; Logsdon & Williams, 1986). These methods largely focused on the isolation of a high yield of viable acinar cells. A technique to completely separate acinar cells from terminal ductal cells is not available at the present time. A possible way for acinar cell purification is to dissociate pancreas into individual cells and then subsequently separate acinar cells from other cells by using density gradient centrifugation. A discontinuous Ficoll gradient has been used for the purification of pancreatic islets (Wollheim et al., 1990) and ductular fragments (Githens, et al., 1994a), since the density of pancreatic cells are different.

The lack of specific markers for pancreatic ductular and acinar cells of hamsters is another obstacle in the evaluation of the mechanisms involved in this phenotypic transformation. Biochemical markers such as amylase, insulin and carbonic anhydrase often represent the terminally differentiated pancreatic cells. I have used amylase as a differentiation marker to follow

acinar cells during the process of phenotypic change. However, cell transdifferentiation cannot be determined using biochemical markers when the starting material contains a mixed cell population. Cytokeratins (CKs), which are intermediate filament proteins, are other commonly used markers to identify differentiated pancreatic cells. Pancreatic duct epithelium show a presence of CKs 7, 8, 18, 19 and 20, while acinar cells are positive for CKs 8 and 18 (Santini et al., 1994; Schussler et al., 1992; Bouwens et al., 1995). Nevertheless, most of the commercially available antibodies to CKs are human specific and were found not to react with hamster pancreatic tissue. De Lisle et al. have developed monoclonal antibodies as probes for the apical membranes of mouse pancreatic acinar and duct cells (De Lisle et al., 1988), but these antibodies do not label rat and hamster pancreatic tissues (personal communication). In order to elucidate the mechanisms for the phenotypic changes of cultured acini, it is necessary to generate new markers, specific to dedifferentiated and transdifferentiated acinar cells.

Isolation and maintenance of islet cells in vitro are important for several reasons. These include the development of an in vitro system to study the proliferation and differentiation of the endocrine cells and the preparation of islets intended for transplantation. Nielsen et al. reported that human islets could be kept in a suspension culture for more than 9 months with preservation of the ability to release insulin in response to glucose stimulation (Nielsen et al., 1979). Lucas-Clerc et al. showed that human islets could maintain their spherical structure for more than 8 weeks when the islets were cultured within collagen gel (Lucas-Clerc et al., 1993). They also noticed that some vacuolization formed at the islet periphery within the first 10 days of culture. Some of these vacuoles enlarged progressively and disappeared in consecutive days. However, the evidence for vacuolization in

this report was merely from the observance of cultures by inverted microscopy and not further characterized.

In my study, human islets were isolated by collagenase digestion of pancreata that were removed from cadaveric organ donors. The islets were purified on a discontinuous density gradient and were then placed into a three-dimensional rat-tail collagen gel matrix. These islets were cultured in the same medium that was used for culturing intralobular ducts. The results show that the cultured islets underwent a cystic transformation that was associated with (i) a progressive loss of insulin gene expression, (ii) a loss of immunoreactivity for insulin protein, and (iii) the appearance of CK-19, a marker for ductal cells. After the transformation was complete, the cells had the ultrastructural appearance of primitive duct-like cells. Cysts showed a progressive enlargement with cell replication, as reflected in a 15-fold increase in the incorporation of tritiated thymidine. These findings are consistent with the transdifferentiation of an islet cell to a ductal cell.

Recently, Kerr-Conte et al. reported that adult human islets became cystic structures when islets were cultured in rat tail collagen gels and Matrigel (Kerr-Conte et al., 1996). In contrast to my conclusion, they suggested that these cystic structures originated from pre-existing pancreatic exocrine cells rather than islet cells. Their islet preparations consisted of 51% nonendocrine cells, which included 30% ductal cells. Unlike Kerr-Conte et al., the human islets that were used in my experiments were well separated from attached exocrine cells and were CK-19 negative after density gradient purification. Kerr-Conte et al. reported that endocrine cells in the wall of the ductal cysts were not observed until after 5 days of culture and continued to increase thereafter, whereas I found that islet cells in the ductal cysts decreased with time. Furthermore, Kerr-Conte et al. tried to demonstrate neogenesis of islets

from ductal epithelium by using these islets as their starting materials, a situation which is completely inappropriate. In fact, a primary duct epithelial culture should have been used instead. Methods for adult pancreatic duct epithelial isolation and culture from human and other species have been well documented (Githens, 1988; Githens, 1994).

The mechanisms involved in the acquisition and maintenance of cell differentiation remain largely unknown. Stability of the cellular phenotype in adult organisms is probably related to the extracellular matrix (McDonald, 1989; Gumbiner, 1992; Hay, 1993; Biechmeier & Biechmeier, 1993), cell-cell interactions (Pitts et al., 1988; Chailakhyan, 1990; Cross & Dexter, 1991) as well as cytoplasmic and nuclear components that interact to control gene expression (DiBerarkino et al., 1984; Blau & Baltimore, 1991; Watt, 1991; MacDougald & Lane, 1995). A perturbation or loss of stabilizing factors may induce cells to change their commitment (Eguchi & Kodama, 1993). The mechanism of islet cell transdifferentiation is probably multifactorial. The factors involved may include collagenase digestion of extracellular matrix during islet isolation, growth factor stimulation, and the interaction between cultured islets and an extracellular matrix (see 4.5). It is noteworthy that isolated islets from the Syrian golden hamster rarely underwent phenotypic changes when the islets were cultured in the same environment as human islets (data not shown). Further investigation focusing on the biological differences between human and hamster islets, as well as improvement in the isolation techniques, will provide a better understanding of the mechanisms underlying the phenotypic transformation of cultured human islets.

Conclusions

1. A method for isolation and culture of intralobular ducts from the hamster pancreas has been developed. The ductal epithelial cysts slowly arise from partially digested fragments of acinar tissue and consist of flattened, cuboidal or columnar epithelial cells. This method gives a high yield of ductal cysts. Autoradiography indicated the duct cysts could have originated from either progenitor cells of ducts or those of acini.

2. The acinar cell phenotype can be maintained for over 1 month when cells are cultured in collagen. Most acinar cells retain a normal morphological phenotype and immunocytochemistry for amylase demonstrates positive staining at the apical cytoplasm of acinar cells. Uptake of ^{3}H-thymidine of cultured acinar cells increases with time.

3. The cultured acini undergo phenotypic changes when EGF and cholera toxin are added to the medium. The acinar cells gradually disappear when the cultures continue and are replaced by duct-like structures. These duct-like structures are lined by a single layer of cubical or flattened epithelium with a few acinar cells. It is also found that the duct-like cells have a limited capacity to redifferentiate into acinar cells.

4. Cultured human islets cells alter their usual phenotype and attain morphological characteristics of duct cells, which is consistent with the transdifferentiation of an islet cell to a ductal cell.

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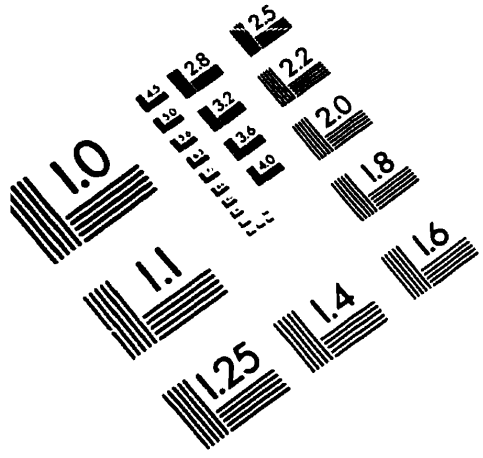
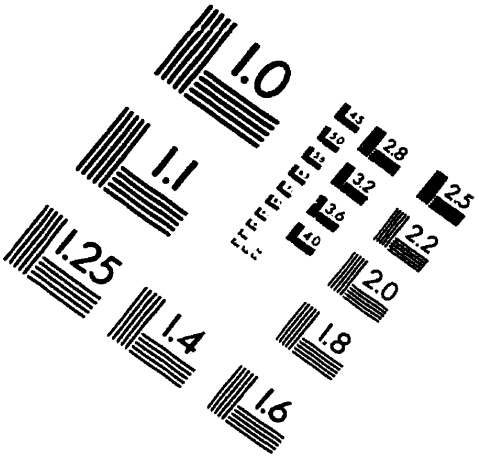
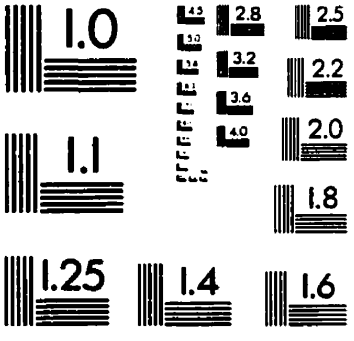
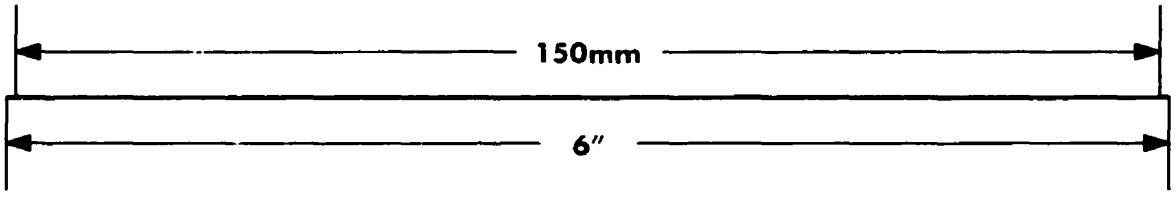
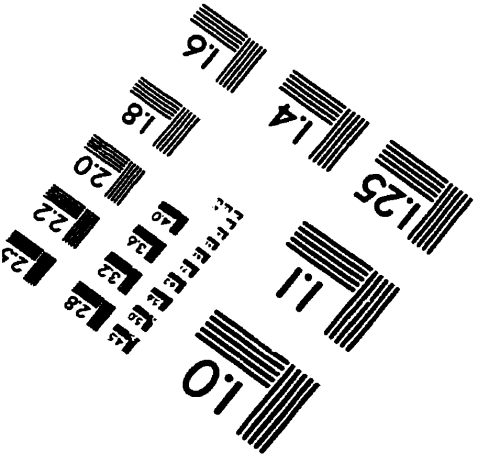
IMAGE EVALUATION TEST TARGET (QA-3)

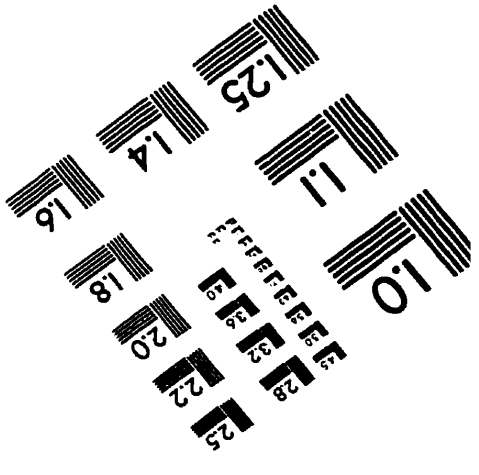












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