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Factors Controlling Pancreatic Islet Neogenesis

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We have established a model in which cellophane wrapping induces reiteration of the normal ontogeny of β-cell differentiation from ductal tissue. The secretion of insulin is physiologic and coordinated to the needs of the animal. Streptozotocin-induced diabetes in hamsters can be "cured" at least half the time. There appears to be activation of growth factor(s) within the pancreas, acting in an autocrine, paracrine, or juxtacrine manner to induce ductal cell proliferation and differentiation into functioning β cells. Given the results of our studies to date, it does not seem premature to envisage new approaches to the treatment of diabetes mellitus. Identification of the factor(s) regulating islet-cell proliferation and differentiation in our model may permit islets to be grown in culture. This concept could be extended to induce endocrine cell differentiation *in vitro* as well. Furthermore, islet-cell growth factors could be used to provide "trophic support" to islet transplants as a means of maintaining graft viability. There may also be greater scope for gene therapy when the growth factor(s) have been isolated, purified, sequenced, and cloned.

INTRODUCTION

Developmental Origin of Pancreatic Islets During Embryogenesis

The pancreas develops as an epithelial evagination of foregut endoderm into the surrounding splanchnic mesoderm. This epithelial-mesenchymal interaction is soon followed by acinar- and islet-cell differentiation [1,2]. During this early stage, a ductal stem cell may differentiate into cells that possess either an endocrine or an exocrine phenotype [3–6]. Endocrine cells develop by a budding process from the embryonic duct-like cells [7]; this process leads to the formation of primitive islets in the mesenchyme adjacent to the ducts. *In vitro* studies with fetal [8] and neonatal [9] pancreata have confirmed that the new islet tissue is derived from ductal epithelium. New islet formation may also continue in the postnatal period where foci of endocrine cells may appear scattered among the acinar tissue [10,11]. Further differentiation into cells secreting glucagon (α), insulin (β), somatostatin (δ), and pancreatic polypeptide (PP) occurs, resulting in development of an islet composed of a mixture of cell types and a sophisticated intra-islet portal vascular system. The different islet-cell types appear sequentially during development. It has been pro-

Abbreviations: APUD: amine precursor uptake and decarboxylation bp: base pair EGF: epidermal growth factor FGF: fibroblast growth factor glucokinase: ATP:D-hexose 6-phosphotransferase IGF: insulin-like growth factor MF: mesenchymal factor PCR: polymerase chain reaction PDGF: platelet-derived growth factor PP: pancreatic polypeptide RT: reverse transcription STZ: streptozotocin TGFα: transforming growth factor α TH: tyrosine hydroxylase ^{3}H-TdR: ^{3}H-thymidine

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posed that islet cells derive from the neuroectoderm because of the expression of several neuroectodermal antigens (e.g., PGP 9.5 [12], neuron-specific enolase [13], synaptophysin [14], A2B5 [15], phenylethanolamine N-methyl-transferase, and aromatic amino acid decarboxylase [16]). The endocrine cells of the islets are capable of *a*mine *p*recursor *u*ptake and *d*ecarboxylation and have, therefore, been given the acronym APUD cells [17]. The morphologic similarity of APUD cells suggested a common embryologic origin, which was believed to be the neural crest, but was later revised to include the neuroectoderm, or, in the case of some of the endocrine cells, the dorsal placoderm. This theory was attractive as it linked cells commonly involved in clinical syndromes of excess, such as multiple endocrine neoplasia, but unfortunately failed to withstand rigorous scrutiny.

Studies by LeDouarin and Teillet [18], Pictet et al. [19], and Andrew [20], based upon elegant studies of chicken and quail neuroectoderm chimeras, have cast doubt on this hypothesis, and most workers agree that these cells should be classified according to their secretory products, (i.e., gastrin, somatostatin, glucagon, PP, and so on). It is now thought, however, that β cells do not have APUD characteristics and are likely to be derived from gut, even though they express neuronal antigens such as the catecholamine biosynthetic enzyme tyrosine hydroxylase (TH) [21]. The generally held belief that the neuronal characteristics of these cells indicated an ectodermal origin during mammalian embryogenesis has therefore largely been dispelled.

During development *in vivo,* the phenotype of the mature islet cells appears sequentially. The β cells arise from progenitor cells localized in the pancreatic duct, and these precursors transiently express TH while migrating away from the duct to populate a new islet. This process suggests that the pancreatic duct is the source of endocrine stem cells throughout embryogenesis, without the need to postulate a neuroendocrine origin. This notion is supported by the finding that the embryonic pancreatic duct *in vitro* is able to regenerate a new pancreas containing exocrine and endocrine cells expressing only peptides (mature cells), and cells containing both TH and a hormone (immature cells) [21,22].

Teitelman et al. have shown that pancreatic cells of endocrine origin can indeed express several neuronal antigens in addition to the peptide hormones [21]. They further showed that, in the mouse embryo, a primitive undifferentiated cell or cells led to sequential appearance of at least four different cell types containing either a hormone (e.g., glucagon), a catecholamine enzyme (TH), or combinations of these [23]. Under appropriate conditions, these cells can be shown to differentiate into either neurites or adult endocrine cells. During regeneration, expression of neural antigens by developing cells was found to constitute an early phase to be replaced by the adult hormone-secreting counterpart. Rosenberg, Duguid, and Vinik [24] have utilized a model for islet neogenesis and have shown that pancreatic ductal cells are able to differentiate upon stimulation into adult islets capable of secreting insulin in a fully regulated manner.

The mature, fully developed islets of Langerhans are communities of cells comprising four distinct cell types. The cells are not randomly distributed within the islets nor throughout the pancreas [6,25,26]. Individual islets present a typical topographical distribution of different cell types with a central bulk of insulin-containing β cells, a peripheral rim of α and PP cells, and intermediate placed δ cells. Moreover, islets located in the body and tail of the pancreas contain relatively abundant glucagon

cells and few PP cells. The reverse occurs in islets located in the paraduodenal portion of the pancreas: these islets are rich in PP and poor in glucagon cells.

The topographical distribution of different cell types within the islets is thought to reflect a functional relationship between cells modulating each other's secretions. Endocrine differentiation proceeds, not only with development of an islet composed of a mixture of cell types, but also with neovascularization of the new islet [27]. The vascular compartment and the nature of directional flow in the portal system [28] could be a major determinant of islet-cell interactions and function. The order of islet cellular perfusion and interaction is from the β-cell core outward to the mantle, which is further subordered with the majority of δ cells downstream, or distal, to the majority of α cells. Therefore, understanding the maturation of the islet vasculature during the differentiation of new islets assumes considerable importance.

The final stage in the formation of new islets, after endocrine cell differentiation and proliferation, neovascularization, and evolution of normal topography, is the maturation of the glucose-sensing mechanism. The release of insulin from the developing pancreas in response to glucose is known to be either poor or absent, whereas, in the infant and adult, the response is considerably improved [29]. As islets evolve, they acquire the ability to recognize glucose. Central to glucose recognition is the ability of the mature β cell to take up glucose, a process modulated by glucose transporter 2, and the ability to phosphorylate glucose that is catalyzed by the tissue-specific enzyme, glucokinase. Glucokinase (ATP:D-hexose 6-phosphotransferase) catalyzes the initial step in the utilization of glucose and appears to be a key component of the glucose-sensing mechanism of the pancreatic islet.

Adjustments of islet-cell number and mass are a means by which an organism may meet changes in the demand for islet hormone production; however, the rate of mitosis is quite low in pancreatic β cells. Consequently, almost the entire complement of β cells in an adult is established during the neonatal period. This relative lack of mitotic capacity highlights the near "terminal" nature of β-cell differentiation that persists in the adult despite the presence of ductular epithelium, the potential precursor from which the islets are derived embryologically. The factors controlling islet-cell neogenesis from ductular epithelium in the adult gland remain unknown, and knowledge of their activity may explain the inability of the diabetic to regenerate an adequately functioning islet-cell mass.

There are practical reasons, therefore, to elucidate *(1)* whether islet cells in the adult pancreas retain the capacity to undergo proliferation, *(2)* whether a pool of precursor cells persists in postnatal life, *(3)* factors that can induce these cells to undergo endocrine cell differentiation, and *(4)* whether the function of newly regenerated islet cells is regulated in a normal manner.

The studies described in this report are part of our long-term plan of investigation of the concept that restoration of a functional islet-cell mass can be achieved by the induction of pancreatic endocrine cell differentiation from primitive ductal cells. Our primary objective has been to characterize the factors that control the differentiation and growth of pancreatic islet cells in order to elucidate the potential for eliciting a regenerative response in diabetes. The hypotheses underlying these investigations are enumerated below:

1. The ability of the pancreas to regenerate a functioning islet-cell mass is preserved in the postnatal period.

2. Regeneration is mediated by a growth factor or factors intrinsic to the gland and is reactivated by cellophane wrapping of the pancreas.
3. This factor, which we have termed "ilotropin," acts directly or indirectly on a stem cell within, or associated with, the ductular epithelium to induce endocrine cell differentiation.
4. Neoislet formation in this model reiterates the normal ontogeny of islet-cell development:

DEVELOPMENT OF A MODEL TO STUDY ISLET-CELL PROLIFERATION AND DIFFERENTIATION

Choice of Animal

We have elected to use the Syrian golden hamster as an experimental animal to study pancreatic regeneration, since islet regeneration can be induced in this animal without the development of neoplasia. Over the past two decades, the use of hamsters has increased due to this rodent's response to stimuli that can alter the growth and development of the pancreas, and because of the low incidence of spontaneous pancreatic tumors that would obfuscate the ability to study the normal process [30].

Choice of Experimental Model

The ability of adult pancreatic cells to undergo proliferation and differentiation toward an endocrine cell phenotype has been investigated with a variety of different models. Alloxan administration is followed by an increased number of small islets seen during the regenerative phase [31]. These islets comprise almost exclusively insulin-containing cells budding from small ductules [32,33]. In neonatal rats, the administration of the β-cell toxin streptozotocin (STZ) is followed by a similar endocrine cell renewal [34]. There is controversy, however, regarding whether this phenomenon occurs in older animals [35–37]. A related compound, the carcinogen N-nitroso-bis(2-oxopropyl)amine, induces new islet formation during carcinogenesis, but tumor eventually replaces the islets [38]. Therefore, this model is not appropriate for study of normal islet-cell differentiation.

Small islets that are closely associated with ductules are reported to develop when the rabbit pancreatic duct is ligated [39,40]. This finding has been questioned, though, because, in rabbits, duct ligation causes pre-existing islets to be broken up into small clusters. That result may give the appearance, but not the reality, of new islet formation in this situation [41]. The primary stimulus for β-cell replication in postnatal islets is persistently elevated blood glucose levels [42,43]. How glucose engenders a replicative response is, however, unclear.

A satisfactory method to induce exocrine and endocrine pancreatic growth is to resect 90 percent of the gland. The increase in β-cell mass, however, occurs as a result of the replication of existing β cells [44], although more recent data indicate some differentiation of new islets from duct cells occurs [45]. The large proportion of islets derived from previously existing islets makes the pancreatectomy model different, in this essential element, from our model of islet neogenesis in the hamster.

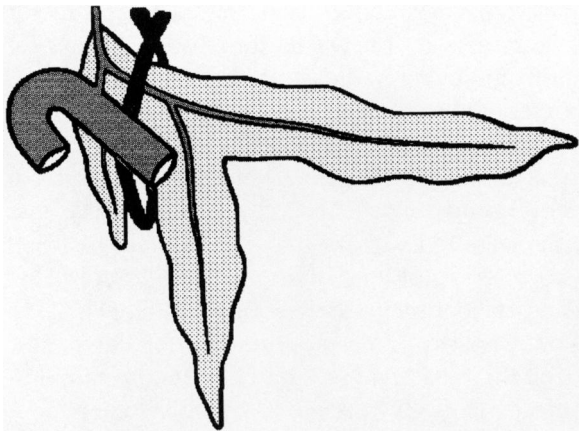

FIG. 1. The cellophane wrap model for the induction of cell proliferation and the differentiation in the pancreas. A 2 mm-wide strip of cellophane tape is placed, under direct vision with the aid of a dissecting microscope, in an avascular plane circumferentially around the head of the pancreas.

A further modification of the pancreatectomy model involves the administration of the poly(ADP-ribose) synthetase inhibitor, nicotinamide, to 90 percent pancreatectomized rats. In this model, there is exocrine and endocrine cell regeneration, and enhanced expression of *reg* protein; the gene encoding this protein has been termed the *reg* gene [46]. Human *reg* mRNA has been detected predominantly in the pancreas and, at lower levels, in gastric mucosa and the kidney [47]. *Reg* protein has also been found to be expressed ectopically in colon and rectal tumors. Expression of a homologous gene, termed *rig,* has been identified in insulinoma tissue [48]. The significance of these genes and their products remains to be determined; however, a role in regeneration is unlikely, based upon the identity of *reg* protein as pancreatic stone protein.

Endocrine development in association with duct epithelial cell proliferation is observed in transgenic mice whose β cells express interferon γ [49]. In these mice, which become diabetic following immune destruction of the β cells, islet regrowth ectopically into the duct lumen may be sufficient to outstrip the immune destruction. Little information is available on the growth factors that may be involved in this proliferative response to immunologic destruction.

Most recently, it was reported that, in the insulinoma-bearing NEDH rat, there is a profound reduction in the non-tumor islet-cell mass, and that, following resection of the tumor, there is a rapid induction of β-cell proliferation [50]. In this model, the restoration of islet mass in the native pancreas occurs as a result of the mitosis of pre-existing β cells.

In 1982, we developed a method for producing partial obstruction of the hamster pancreatic duct and noted that this procedure led to new islet formation [51]. The method consisted of wrapping a piece of cellophane tape around the head of the pancreas without duct ligation per se (Fig. 1).

The advantage of our model for the induction of islet formation is that proliferative changes can be studied in the absence of diffuse pancreatitis, autoimmune destruction, or tissue atrophy, and do not require the addition of chemical agents. As we will discuss in greater detail later in this report, the endocrine regeneration induced by cellophane wrapping appears to be primarily a reiteration of the normal ontogeny of the islet from a ductular cell precursor, and not a result of mitosis of existing β cells. The trophic effects observed in our model are mediated by a

paracrine/autocrine mechanism, and we have extracted from the pancreas a factor that appears to be responsible for the initiation of new islet formation. It is also of importance that our model uses adult animals to study factors that elicit growth potential of cells even when they have reached maturity.

The earliest response to partial obstruction of the pancreas by cellophane wrapping is seen on day 4–5 after surgery, and consists of ductular dilatation and stasis of secretions (Fig. 2). This change corresponds with a decrease in both basal and secretin-stimulated pancreatic exocrine secretion, with the lowest levels being reached after four to five days [51–53]. At two weeks, proliferation in the ductular epithelial cell population occurs, as shown by autoradiographic labeling studies (Fig. 3) [54,55]. By day 21, cells have begun to grow out from the ductular epithelium, leading to new islet formation. We have characterized three patterns of differentiation by immunohistochemistry: *(1)* islets in which glucagon, insulin, and somatostatin are expressed; *(2)* foci of new islet formation in which only one islet hormone is expressed, usually either insulin or glucagon; and *(3)* individual cells in the ductular epithelium staining for glucagon or insulin. By eight weeks, a second wave of proliferation occurs, primarily in the islet cells (Fig. 4) [54,55]. Computer-assisted morphometric analysis demonstrates that the induction of new islet formation produces a two-and-one-half-fold increase in the islet-cell mass (mean ± SD, expressed as the number of islets/mm^2) from 1.1 ± 0.9 to 2.4 ± 0.8 that is accounted for by the appearance of a new population of small islets (Table 1) [56]. These data suggest to us that the new islet cells develop by a process of endocrine differentiation from a precursor cell associated with the ductular epithelium (Fig. 5).

In spite of the increased islet-cell mass, blood glucose and insulin levels remain relatively normal after cellophane wrapping of the pancreas of normal animals [51,53]. Moreover, islets that are isolated from the wrapped pancreas of normal animals [55] and perfused *in vitro* respond with a normal biphasic insulin secretory response to glucose stimulation. These data suggest that, even with an increased islet mass, feedback regulation occurs with appropriate adjustment of insulin levels for the prevailing level of glucose.

To determine whether this insulin was biologically active, we undertook to cellophane wrap the pancreata of hamsters rendered diabetic with the β-cell toxin, STZ. Before surgery, the serum glucose (389.0 ± 18.6 mg percent) and insulin (33.9 ± 3.8 μU/ml) levels (mean ± SEM) in unoperated control animals did not differ from those in the animals having the operation (373.2 ± 18.6 mg percent; 37.9 ± 3.8 μU/ml, respectively). After seven weeks, 50 percent of the wrapped animals treated with STZ had serum glucose and insulin levels that were normal, compared to only 12 percent of the unoperated control STZ-treated animals. Islets from normoglycemic operated animals were characterized by increased numbers, including many small islets, positive immunoreactive insulin staining, and minimal vacuolation of cells. Islets from hyperglycemic operated hamsters and from the unoperated control animals were fewer in number and generally larger, demonstrated little or no immunoreactive insulin staining, and exhibited marked vacuolation of cells [57,58]. From these studies, we concluded that cellophane wrapping of the pancreas induced the formation of islets with endocrine cells that are functionally capable of reversing streptozotocin-induced diabetes.

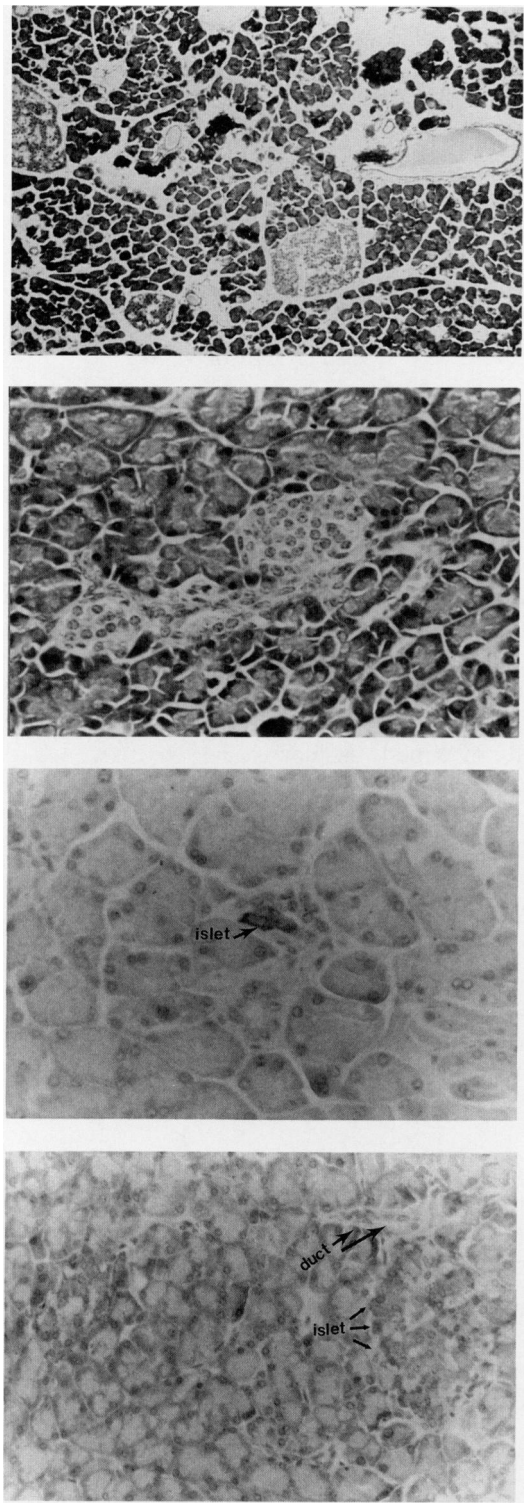

FIG. 2. A collage of hematoxylin- and eosin-stained sections (**a** and **b**), and insulin immunocytochemistry (**c** and **d**) of the cellophane-wrapped pancreas showing (**a**) the initial partial obstruction with stasis of duct secretions without an inflammatory response, (**b**) a duct with proliferation of duct cells 14 days after wrapping, culminating in (**c**) formation of a neoislet with positive insulin immunoreactivity, and (**d**) the proximity to the duct of the mature neoislet, with positive insulin immunoreactivity, supporting its ductal origin.

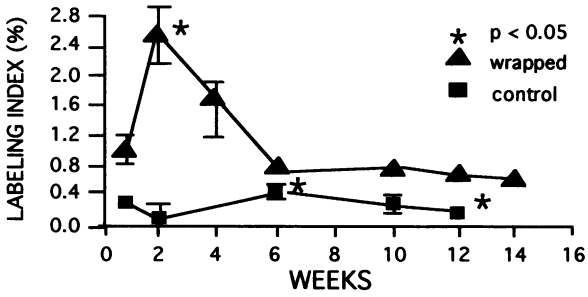

FIG. 3. The effect of cellophane wrapping on the incorporation of tritiated thymidine (^{3}H-TdR) into DNA by ductular epithelial cells (▲), expressed as an index of percentage of cells labeled, in the pancreas of the Syrian golden hamster (n = 4). (■) labeling index for sham-operated control animals (n = 4) [54,55].

THE MOLECULAR BIOLOGY OF ISLET NEOGENESIS

In our model, after cellophane wrapping, endocrine-like cells begin to form in association with the ductular epithelium as early as two weeks after the stimulus of wrapping. The initial trophic effect of cellophane wrapping is on the ductular epithelium, as shown by autoradiographic analysis following a single pulse of ^{3}H-thymidine (^{3}H-TdR). The activity of pancreatic ornithine decarboxylase, an enzyme in the DNA synthetic pathway, is increased within eight hours of trophic stimulation, followed 40 hours later by an increase in pancreatic DNA content, and a rise in ductular cell ^{3}H-TdR labeling index by seven days. If, however, the pancreata are examined six weeks after the pulse, most of the label is contained in differentiating islet cells, not ductular cells. The uptake of ^{3}H-TdR by ductular cells is maximal approximately four weeks before the peak uptake of the label by islet cells. The neoislets are identified by a lack of well-developed vascular spaces, the presence of mitotic figures, and the vesicular nature of nuclei [51]. Thus, wrapping of the pancreas induces the formation of new endocrine cells, which appear to arise from duct epithelium.

In an attempt to identify whether the primitive proto-undifferentiated cell was capable of synthesizing message (mRNA) for insulin, total RNA was isolated from hamster pancreata 0, 1, 2, 4, 8, 10, 14, and 56 days after wrapping. A 510 base pair (bp) human insulin cDNA that recognized both hamster and rat transcripts, and a 400 bp hamster insulin cDNA were used as probes in Northern blot analysis of hamster poly(A)$^+$RNA. The results were quantitated densitometrically, using β actin as control. There was an increase in insulin mRNA two and four days after

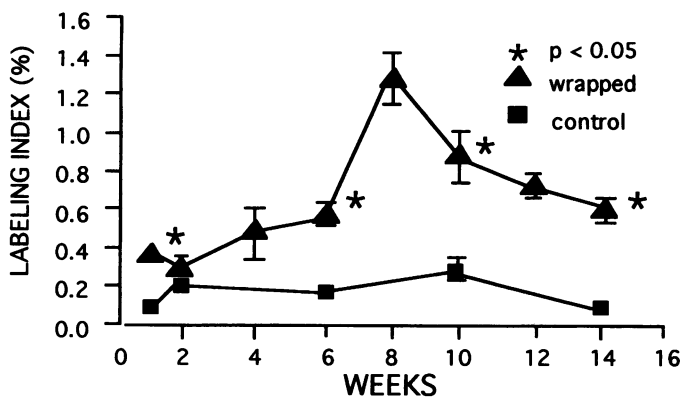

FIG. 4. The effect of cellophane wrapping on the incorporation of ^{3}H-TdR into DNA by islet cells (▲), expressed as an index of percentage of cells labeled, in the pancreas of the Syrian golden hamster (n = 4). (■) labeling index for sham-operated control animals (n = 4) [54,55].

TABLE 1
Distribution of Islet Size in Control and Wrapped Hamsters

	Islet Population (%)	
Islet Diameter (μm)	Control	Wrapped
>100	63	33
<100	32	67
<48	1	26

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wrapping, which thereafter declined to control, non-wrapped levels after eight weeks. In contrast, glucagon gene expression, as seen both by Northern blot analysis, using a 1,200 bp hamster glucagon cDNA and reverse transcription of RNA followed by polymerase chain reaction (RT-PCR), was not increased. Preliminary experiments using a human insulin cRNA probe for in situ hybridization showed an increase in abundance within two days of wrapping, with considerable heterogeneity between adult islets. After ten days, insulin-positive cells were visible within ductular epithelium (Fig. 5).

Somatostatin mRNA is normally low in abundance, but increased markedly after four days of wrapping. Budding from ductules was observed as early as 14 days, with small foci of cells with insulin-positive immunocytochemical reactivity. Within this period, there was diminished expression of insulin mRNA in pre-existing large islets, accompanied by a decrease in islet insulin content. Corresponding with the decrease in islet insulin content, fasting serum insulin levels were depressed, and the blood glucose responses were in the diabetic range on glucose tolerance tests. Normal glucose homeostasis was restored by four weeks after wrapping. Thus, it seems that indeed there may be proto-undifferentiated cells in the ducts of the pancreas that, when stimulated appropriately, are induced to grow and, very early in their differentiation, are capable of synthesizing the message for insulin; however, these cells could be already committed to a path of endocrine function. To elaborate further upon this issue, we are attempting to find antigens that are both unique to ductal cells, and found only in acinar and endocrine tissue. Such antigens would verify the transitional nature of the ductal proto-undifferentiated cell and its ability to transform into a fully functional endocrine organ. Meanwhile, whatever the origin of the cell that becomes an endocrine organ, there is a need to understand the regulatory mechanisms involved.

Since rat models of islet-cell regeneration have demonstrated significantly enhanced expression of *reg* gene, we studied *reg* gene expression in our model. Northern blot analysis, using a rat *reg* cDNA probe, showed no pancreatic expression of *reg* mRNA in either control or cellophane-wrapped pancreas, whereas a strong signal was detected in control rat pancreas. Using RT-PCR, we amplified, isolated, and sequenced a 197 bp product, which was identified as a fragment of hamster *reg*. There was a marked increase in the expression of hamster *reg* within 24 hours of cellophane wrapping, and, after four days, levels of *reg* fell, approaching control. Thereafter *reg* mRNA further decreased to levels markedly lower than control. In situ studies demonstrated that *reg* was expressed only in the exocrine pancreas, mainly in areas of

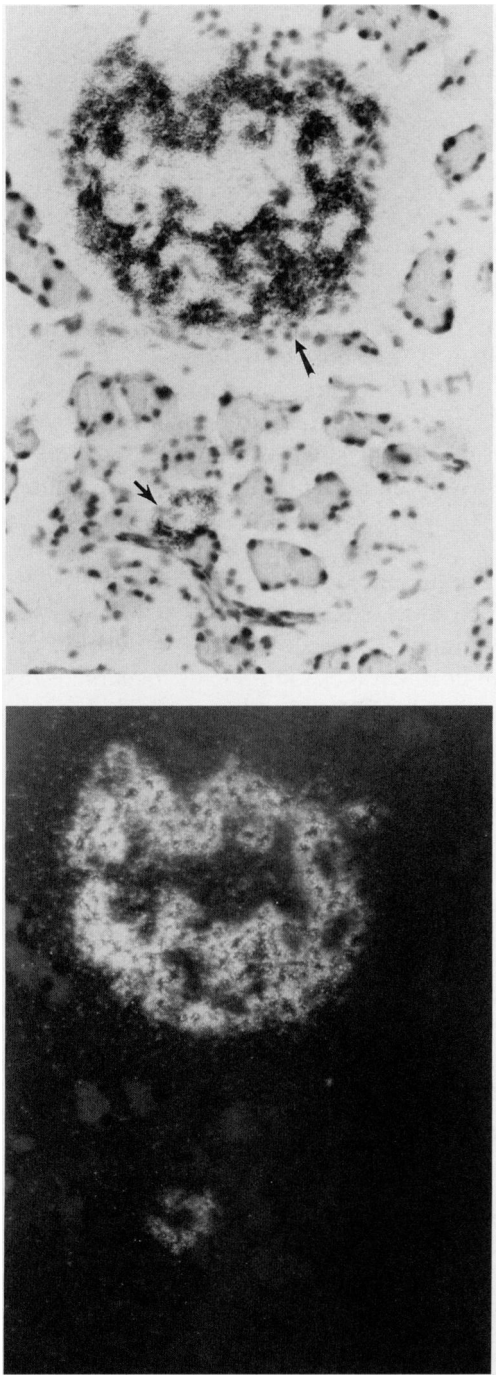

FIG. 5. Localization of insulin mRNA by in situ hybridization is demonstrated on a pancreas ten days after wrapping. The cryostat section was hybridized with ^{35}S labeled antisense single-stranded insulin RNA probe transcribed from 510 bp cDNA. These are a bright-field (**a**) and a filtered-field (**b**) photograph of a mature adult pancreatic islet, showing high abundance of insulin mRNA, and a neoislet budding off a duct (*arrows*, **a**).

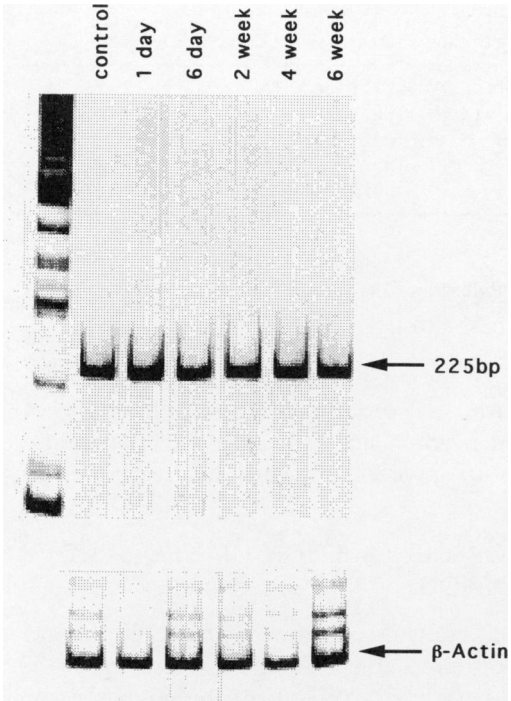

FIG. 6. Northern blot analysis of IGF-1 gene expression in control hamsters and one day to six weeks after wrapping. Note the absence of a significant change in message. The hybridization of β actin is shown to indicate equivalent recoveries at the different time periods.

inflammation and lymphocyte infiltration, but there was no expression in islet cells (data not shown). These data suggest that the increase in *reg* mRNA found in certain models of regeneration might be related to the inflammatory process in traumatized pancreata, when cells undergo extensive dedifferentiation, but not to the islet regeneration itself. This theory would explain why *reg* mRNA is found in some models of islet formation, but not others. Of the other candidate growth factors, we have only examined IGF-I using Northern blot analysis for quantitation of mRNA; IGF-I was unaffected by the wrapping process (Fig. 6).

EVIDENCE FOR AN AUTOCRINE, PARACRINE, OR JUXTACRINE REGULATION OF NEOISLET FORMATION IN THE CELLOPHANE WRAP MODEL

The mechanism by which partial obstruction in our model initiates cell growth, proliferation, and differentiation is unknown. To determine if humoral factors are involved, and in particular whether the trophic effect is the result of the local release of growth factor or factors, we studied cellophane wrapping of the hamster pancreas using a parabiotic experimental design.

Pairs of parabiotic hamsters were established with common cross-circulation demonstrated using the technetium pertechnetate method of Markowitz [59]. Partial duct obstruction was produced by wrapping one parabiont from each pair. At six weeks, the pancreatic gastric lobe weight, DNA, and protein content of the wrapped parabionts showed significant increases of 32 percent, 20 percent, and 28 percent, respectively, over the non-wrapped parabionts (Table 2) [56]. Morphometric analysis of the pancreatic splenic lobes demonstrated the presence of new islet formation in the wrapped pancreata with a 100 percent increase in the number from 0.90 ± 0.50 to

TABLE 2
The Trophic Effect of Cellophane Wrapping of the Pancreas Using a Parabiotic Model

Parabiont	Tissue Weight (mg)	Tissue Protein (μg/100 g body wt)	Tissue DNA (μg/100 g body wt)	No. Islets/mm^2
Control	130 ± 17	21.2 ± 1.9	795 ± 159	0.90 ± 0.50
Wrapped	167 ± 21[a]	25.4 ± 2.7[a]	1,052 ± 206[a]	1.80 ± 0.70[b]

[a]Significantly ($p < 0.05$) different from control
[b]Significantly ($p < 0.01$) different from control
From [56]; reproduced with permission of Plenum Publishing Corporation, New York

1.8 ± 0.7 islets/mm^2 over non-wrapped parabionts. These data suggest that the trophic effects observed in this model of islet-cell proliferation and differentiation appear not to be mediated by a humoral mechanism, and control of pancreatic endocrine growth in this model appears to involve paracrine and/or autocrine regulatory mechanisms [60,61].

EVIDENCE FOR GROWTH FACTOR REGULATION OF NEOISLET FORMATION

Identifying genes and their products that control cell growth, proliferation, and differentiation is basic to understanding both normal growth and abnormal growth. The stimulation of hepatic regeneration, for example, has been attributed to a factor that has been isolated from liver cytosol [62], and increase in the expression of immediate-early response genes has been reported [63]. Embryologically, the liver and pancreas are derived from the same area of foregut endoderm. Therefore it seems likely that the sensitive control demonstrated in the regenerating liver may have its counterpart in the pancreas, and that the mitogenic effect of partial obstruction may be mediated through a tissue-specific growth factor and/or activation of multiple gene expression. It is also essential to establish that the newly formed islet expresses the genes encoding the constellation of hormones required to maintain normal physiology. The final stage of phenotypic expression and cellular differentiation in the pancreas may be controlled by the proximity of cells to a local stimulus originating in the gland itself [64,65]. Co-transplantation of fetal foregut mesenchyme with pancreatic duct epithelium has been shown to result in the development of islet-like cell clusters [66]. Whether these cells were actually at an early stage of islet formation is unknown. The possible mechanisms of action include: *(1)* secretion of an autocrine or paracrine inducing or transforming factor; *(2)* information exchange through cell-to-cell contact; and *(3)* production of extracellular matrix that contains critical trophic factors [2,67–69].

There is good evidence for autocrine and paracrine activity within the pancreas. The presence of a specific mesenchymal factor (MF) that promotes differentiation in the developing pancreas has been hypothesized [70]. Older rat embryo or adult organ extracts do not, however, contain MF activity [11,71,72], nor has there been any indication of the nature of the factor.

Soluble peptide growth factors are a group of trophic substances that regulate both cell proliferation and differentiation. These growth factors are multi-functional and may trigger a broad range of cellular responses [73], including effects on extracellular matrix formation. Their actions can be divided into two general categories: effects on

cell proliferation and effects on cell function. They are different from the polypeptide hormones in that they act in an autocrine and/or paracrine manner [74,75]. One family of growth factors that has been associated with ductal proliferation and differentiation is the somatomedins, insulin-like growth factors I and II (IGF-I and IGF-II). IGF-I is synthesized and secreted by the β cells of fetal and neonatal rat islets in tissue culture [76,77]. Smith et al. have reported that, following 90 percent pancreatectomy in the rat, IGF-I gene expression is enhanced in the regenerating pancreas [45]. In these studies, IGF-I is localized to the capillary endothelium and to ductular epithelial cells, but not to endocrine cells. The role of IGF-I in the cellophane wrap model has yet to be determined. IGF-II has been identified in human fetal pancreas [78]. Both somatomedins have a mitogenic effect on fetal and neonatal pancreatic endocrine cells [76,77] and may act in concert with their binding proteins [79]. Platelet-derived growth factor (PDGF) also stimulates fetal islet replication, and this effect is additive to that of IGF-I [80]. IGF-I may be the primary mediator of β-cell replication in fetal and neonatal rat islets [42,80–83]. The temporal expression of these growth factors in the progression from ductal cell to neoislet in our model is unknown.

There is *in vitro* evidence that overproduction of transforming growth factor α (TGFα), might result in an autocrine increase in growth signals. Ductal epithelial cells and 95 percent of pancreatic carcinomas are immunohistochemically positive for TGFα [84]. Transgenic mice bearing a fusion gene consisting of the mouse metallothionine-1 promoter and a human TGFα cDNA exhibit pleiotropic effects in a number of tissues, including the liver and mammary glands. In the pancreata of these animals, there is a florid acinoductular metaplasia during which acinar cells degranulate, dedifferentiate, and assume the characteristics of intercalated or centroacinar ducts [85,86]. Although TGFα has diffuse effects, transgenic mice with the gene directed to the pancreas by fusion with the elastase promoter undergo metaplastic changes in the pancreas in what appears to be an autocrine-regulated manner. Ultimately the process results in progressive interstitial fibrosis. In none of these situations, however, has there been documentation of an increase in islet-cell mass. If the TGFα gene is combined with the gastrin gene in transgenic animals, then the fibrotic reaction disappears. If these animals are inbred with others carrying the transgene, then gross overexpression of gastrin and TGFα are accompanied by an increase in islet mass [87]. While this process may be an exaggeration of what occurs physiologically or pathophysiologically, nonetheless, it seems that TGFα alone, perhaps acting upon the epidermal growth factor (EGF) receptor, or in combination with gastrin, may play an important role in duct cell proliferation, organogenesis, and neoplastic transformation.

For reasons that are not entirely clear, the development of a fully functional islet organ dictates a need for correct topographic alignment of the various islet cells, as well as a vascular supply consonant with directional flow in a centrifugal direction [88], perfusing initially the β cells and thence the α cells and δ cells. Angiogenesis is a complex process that involves proteolysis of basement membrane as well as endothelial cell migration and proliferation [89]. Several peptides, including angiogenin, acidic fibroblast growth factor (FGF) [90], basic FGF [91], and TGFα [92], are capable of inducing vascular growth in various experimental situations.

These proteins have been postulated to regulate vascular growth, and the FGFs are potent endothelial mitogens with established receptors. TGFα binds to the EGF

receptor in microvascular endothelial cells. TGFα is abundant in the first week of gestation and declines to undetectable levels by day 13, which is compatible with a role in vasculogenesis [93]. With maturation of the islet portal system, endothelial cells express factor VIII and vimentin. The development of islet vascular topography will be evaluated by measuring these markers.

Thus, some growth factors may promote the development of β cells from fetal tissue, but may not promote the growth of new islets from established adult duct epithelium. Furthermore, the role of those factors in the fetus and the neonate that act in the maintenance and replacement of a functional islet-cell mass in the adult pancreas remains obscure, particularly with respect to which are primary regulators of neoislet formation and which mediate secondary growth responses.

In our model, the initiation of proliferation and differentiation associated with new islet formation appears to be mediated in an endocrine or paracrine manner. As a result of this finding, we prepared extracts of wrapped pancreas and identified one that contained trophic activity that could not be ascribed to known hormones and growth factors. An extract prepared from a non-wrapped pancreas had no such activity [94]. We therefore hypothesized that the trophic activity contained in the tissue extract of wrapped pancreas was due to potentially novel islet-cell growth factor(s). We have since prepared crude tissue extract from the cellophane-wrapped pancreas, and the extract had a positive trophic effect on pancreatic tissue when injected into hamsters (Fig. 7). This extract contains a protein in which the biologically active fraction appears to be in the 29–45 kDa range (Fig. 8) [95], because both the pooled fractions from 66 kDa to 40 kDa and 40 kDa to 20 kDa retain equal bioactivity. On gel electrophoresis, two proteins are seen (Fig. 9) [95], in that size range, that are enhanced with the wrapping process [96]. A growth-promoting effect was not observed when a tissue extract prepared from the non-wrapped pancreas was administered. Thus, using a variety of classical protein chemistry techniques, we have identified a soluble polypeptide that we have termed ilotropin. Ilotropin has been only partially characterized as protein or proteins that are trypsin-sensitive, heat-stable, acid-stable, alcohol-precipitable, with an apparent molecular weight in the range of 29–45 kDa, and which are not sialated. We have therefore begun to examine the model for candidate growth factors with these characteristics.

We have used an *in vivo* bioassay to track the trophic activity of ilotropin at successive stages of purification. Daily injection for two days of a partially purified preparation of ilotropin increased pancreatic weight and DNA content by 40 percent (Fig. 7a) and 15 percent (Fig. 7b), respectively [97]. After three weeks, ^{3}H-TdR incorporation into ductular cells significantly increased tenfold and into islet cells increased sixfold [98]. Endocrine cell differentiation was comparable to that produced by cellophane wrapping.

To implicate ilotropin directly in the trophic effects of wrapping, we treated diabetic hamsters for six weeks with twice-daily intraperitoneal injections of ilotropin. Using this regimen, diabetes was stabilized or reversed in 60 percent of the ilotropin-treated animals, compared to only 10 percent of saline-treated controls (Fig. 10) [99]. Animals treated with extracts of non-wrapped pancreas fared no better than saline-treated animals, at most undergoing remission in 12 percent of animals. The successful treatment of diabetes in this setting was achieved by the induction of a new population of insulin-producing β cells.

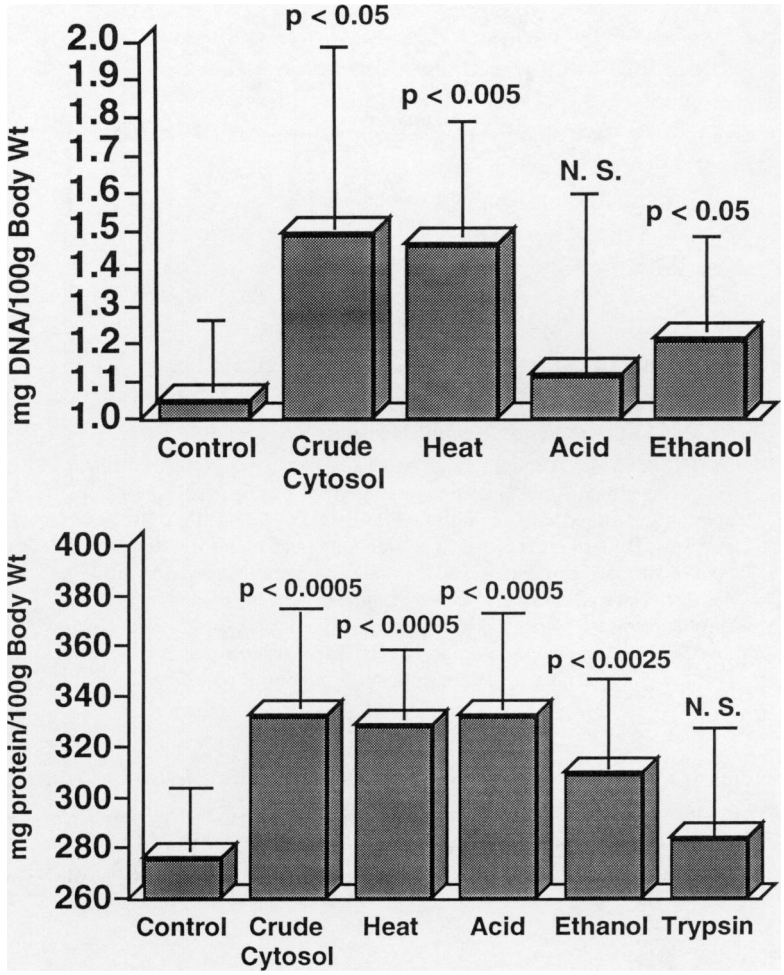

FIG. 7. **a.** The bioactivity of wrapped pancreatic cytosol extract on pancreatic DNA content was compared to control injections by injecting extract 2× daily for two days into each of seven Syrian golden hamsters. Stability of the bioactivity was tested by pre-treatment with either heat (65°C for 20 minutes), acid (10 percent v/v perchloric acid for 20 minutes), or ethanol precipitation (70 percent for two hours at 4°C). Only acid treatment appeared to destroy the ability of wrapped cytosol extract to increase pancreatic DNA content. All statistics are expressed versus control levels. **b.** The bioactivity of wrapped cytosol extract on pancreatic weight was compared to control injections by injecting extract 2× daily for two days into each of seven Syrian golden hamsters. Stability of the bioactivity was tested by pre-treatment with either heat (65°C for 20 minutes), acid (10 percent v/v perchloric acid for 20 minutes), ethanol precipitation (70 percent v/v for two hours at 4°C), or trypsinization (0.1 M for 30 minutes). Only trypsinization destroyed the ability of wrapped cytosol extract to increase pancreatic weight. All statistics are expressed versus control levels.

We examined the effects of wrapping on pancreatic islet regeneration in different species to determine whether this result might be a species-specific effect. The effects of cellophane wrapping of the pancreas could also be induced in the pancreas of the cat, the rat, and the mouse. Therefore, the trophic effects of partial duct obstruction

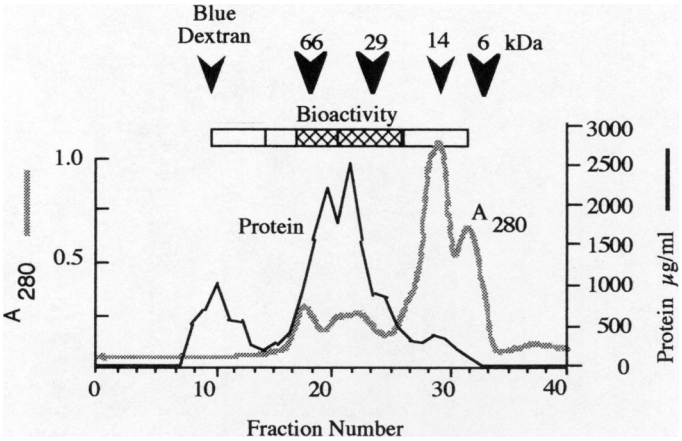

FIG. 8. Cytosol extract, which had been partially purified with heat, acid, and ethanol precipitation, was applied to a Superose-12® FPLC column and eluted with an imidazole (50 mM), KCl (200 mM), glycerol (5 percent), DTT (5 mM) buffer. The fractions were monitored for protein content, using absorbance at 280 nm and subsequently assayed for protein content. The fractions were pooled as shown by the boxes, and assayed for trophic activity, using the bioassay described previously. The cross-hatched boxes indicate those pooled fractions which contained bioactivity (From [95]; reproduced with permission of Plenum Publishing Corporation, New York).

are not species-specific, but may presumably be generalized to other large mammals, possibly even including man. Over 50 years ago, the pancreatic exocrine outflow in diabetic children was partially obstructed in a manner similar to the technique of cellophane wrapping described here. This surgical manipulation reportedly pro-

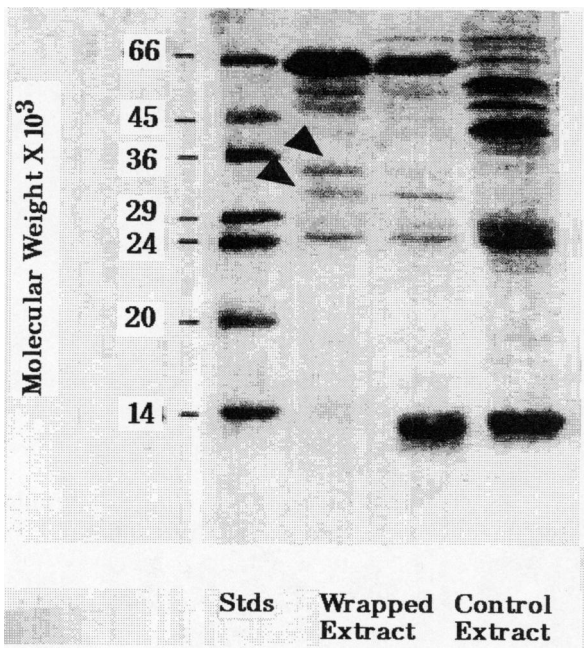

FIG. 9. SDS-PAGE gel showing control versus wrapped pancreatic cytosol extract in the molecular weight region determined for bioactivity on FPLC. Two proteins can be seen in the wrapped cytosol extract *(arrows)*, which are not represented in the control extract preparation. Whether one or both of these proteins might be the pancreatic duct/islet growth promoting factor, or related to the factor, remains to be determined (From [95]; reproduced with permission of Plenum Publishing Corporation, New York).

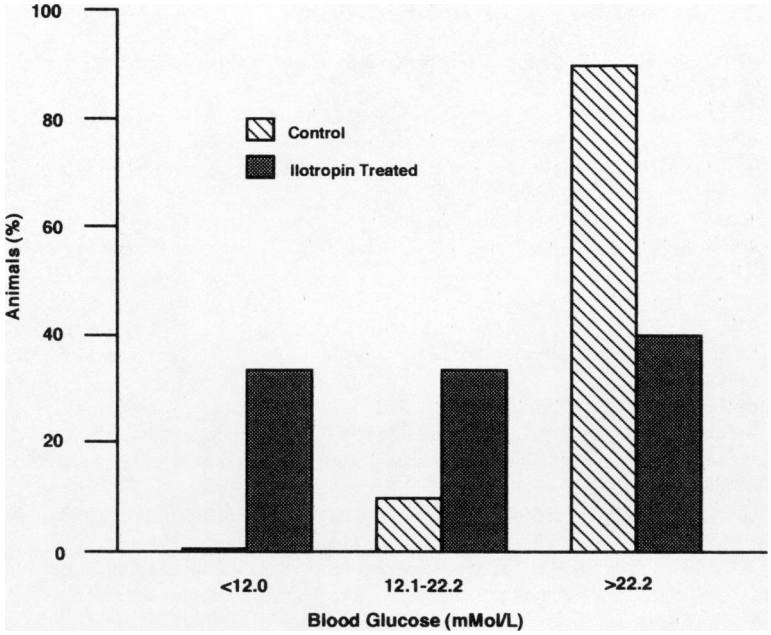

FIG. 10. This graph illustrates the incidence of spontaneous remission of diabetes in streptozotocin-treated animals and the dramatic enhancement of remission with ilotropin treatment.

duced an increase in "sugar tolerance" compatible with our observation in lower mammals [100,101], but we can only speculate upon whether the apparent improvement in glucose homeostasis was due to the regeneration of a new β-cell population.

SUMMARY

We have established a model in which cellophane wrapping induces reiteration of the normal ontogeny of β-cell differentiation from ductal tissue. The secretion of insulin is physiologic and coordinated to the needs of the animal. Streptozotocin-induced diabetes in hamsters can be "cured" at least half the time. There appears to be activation of growth factor(s) within the pancreas acting in an autocrine, paracrine, or juxtacrine manner to induce ductal cell proliferation and differentiation into functioning β cells. Given the results of our studies to date, it does not seem premature to envisage new approaches to the treatment of diabetes mellitus. Identification of the factor(s) regulating islet-cell proliferation and differentiation in our model may permit islets to be grown in culture. This concept could be extended to induce endocrine cell differentiation *in vitro* as well. Furthermore, islet-cell growth factors could be used to provide "trophic support" to islet transplants as a means of maintaining graft viability. There may also be greater scope for gene therapy, when the growth factor or factors have been isolated, purified, sequenced, and cloned.

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