Nerve growth factor induces neuron-like differentiation of an insulin-secreting pancreatic beta cell line

(pancreas/neural crest/endoderm/development/trk oncogene)

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ABSTRACT Nerve growth factor (NGF) is the best understood of a class of trophic proteins that are important for the survival of neurons and the elaboration of their characteristic processes. Here we demonstrate that RINm5F, a rat insulinoma cell line representing an early stage in pancreatic beta cell differentiation, expresses both the Trk and p75 NGF receptors and responds to NGF by extending neurite-like (neurofilament-containing) processes. NGF treatment of RINm5F cells also induces the expression of genes normally responsive to NGF in neurons, including the NGF-1A gene. Inasmuch as pancreatic beta cells arise from the embryonic endoderm, these results suggest that NGF may play a wider role during development than previously thought—a role not restricted to cells of neuroectodermal origin—and that endocrine and neuronal cells share a developmental pathway. The specific effect of NGF on an early pancreatic beta cell line also suggests that this neurotrophic factor might form the basis of a therapeutic treatment for some types of diabetes by inducing the proliferative differentiation of islet cells.

The defining characteristic of neurons is that they respond to various extracellular signals by initiating a self-propagating change in electrical potential across the cell membrane that ultimately leads to the release of specific chemicals—neurotransmitters—that transmit the signal to the target cell. The insulin-secreting beta cells of the pancreas resemble neurons in a number of ways, and it was once suggested that both cell types might arise from a common precursor during development (1). For example, beta cells secrete insulin in response to ligands in a process similar to neurotransmitter release from neurons (2). Glucose and the neurotransmitter acetylcholine depolarize the plasma membrane of the beta cell, which allows the influx of Ca^{2+} ions and initiates the exocytosis of insulin-containing vesicles. Beta cells also resemble neurons in expressing some neurotransmitter biosynthetic enzymes, such as tyrosine hydroxylase, dopa decarboxylase, and glutamate decarboxylase (an autoimmune target in type 1 diabetes mellitus), as well as neurofilament protein and the neural cell adhesion molecule N-CAM (3–10).

However, despite the fact that beta cells express a large number of neuronal antigens and properties, currently they are thought to arise from the endodermal rather than the neuroectodermal lineage during development (1, 5, 9, 11, 12). This conclusion is most strongly supported by transplantation and ablation experiments in which normal, endogenous pancreatic development proceeded in the absence of the neural crest or in the presence of a transplanted neural crest from a different species (5, 11, 12).

Neurons require certain “growth” factors in order to establish and maintain their distinctive processes, and even to survive. We have investigated the neuronal character of pancreatic beta cells by asking whether molecules known to be important for the differentiation and survival of neurons and other neural crest-derived cells can also affect insulin-secreting cells. Specifically, we have tested two neuronal differentiation/survival factors with clear effects on developing neurons, nerve growth factor (NGF) and laminin. NGF is a protein that is produced by target tissues during nerve innervation and is essential for the survival of sympathetic, peripheral sensory, and central cholinergic neurons and for the development of their distinctive processes (13–16). Laminin is an extracellular matrix molecule that plays an essential role in the attachment, migration, regeneration, and axon outgrowth of developing neurons (17, 18). This very large (1000 kDa) multifunctional protein contains a domain which, like NGF, has a neuronal differentiation function *in vitro* and is particularly localized along the routes of migrating neurons and axons during development.

We have studied the effect of NGF and laminin on two insulin-secreting beta cell lines, one representing an early stage in pancreatic development (RINm5F) and the other representing a middle stage (β TC3). The RINm5F cell line extends neurite-like processes when exposed to either NGF or laminin, whereas the more mature β TC3 cell line responds only to laminin. This differential response is paralleled by the presence of both the p75 and Trk NGF receptors only on the first cell line. These results demonstrate that pancreatic beta cells can undergo a neurotypic response to neuronal differentiation factors and suggest that neurons and pancreatic endocrine cells share an overlapping developmental program.

METHODS

Cell Culture. Two pancreatic beta cell lines were studied with respect to their response to neuronal growth factors. RINm5F is a rat insulinoma cell line that constitutively secretes insulin and, to a lesser degree, glucagon and somatostatin, just as do undifferentiated fetal beta cells (19–21). β TC3 is an insulinoma cell line derived from a transgenic mouse into which a simian virus 40 T-antigen gene linked to the insulin promoter was introduced (22). β TC3 cells represent a later, more mature state of beta cell differentiation than

Abbreviations: NGF, nerve growth factor; BSA, bovine serum albumin.

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RINm5F in that they have developed a much greater ability to respond to glucose by increasing their insulin secretion, though not with the same rapid kinetics of response as adult beta cells (21). The first RINm5F cells used were at passage 27 from the initial line; their ability to respond to NGF by growing neurites has held constant until at least passage 37.

PC12 is a rat pheochromocytoma cell line derived from a neural crest-derived tissue—the adrenal medulla—and provides a standard example of a cell line that is induced to undergo neuronal differentiation by NGF (23). Rat hepatoma cells (FAO) served as a nonneural, control cell line.

The cell lines were cultured in RPMI 1640 supplemented with 5% (RINm5F cells) or 10% (β TC3, PC12, and hepatoma cells) fetal bovine serum. Three days before the experiments, the cells were transferred to the same medium, but without serum and containing insulin (5 μ g/ml), transferrin (5 μ g/ml), sodium selenite (30 nM), putrescine (100 μ M), and progesterone (20 nM), which enable the cells to grow for at least 2 weeks without the addition of serum or growth factors. The cells were harvested with 10 mM EDTA and replated at low density. NGF (100 ng/ml; Boehringer Mannheim), laminin (10 μ g/ml; GIBCO/BRL), anti-NGF antibody (500 ng/ml; Boehringer Mannheim), or bovine serum albumin (BSA, 2 mg/ml; Sigma) was added. Factors were renewed every 48 hr. At day 4, the cells were photographed.

Immunocytochemistry. For neurofilament immunocytochemistry, RINm5F cells were grown, as described above, on poly(L-lysine)-coated glass coverslips and treated with either NGF or laminin. Coverslips were washed in Dulbecco's phosphate-buffered saline (DPBS; GIBCO/BRL), and the cells were then permeabilized in cold ethanol for 1.5 min and washed again. They were incubated for 1 hr with a mouse monoclonal antibody directed against the 160-kDa component of neurofilaments (Boehringer Mannheim), diluted 1:10 in DPBS/10% horse serum with BSA at 1 mg/ml. After the unbound antibody was washed away with DPBS, a secondary antibody, fluorescein-conjugated rabbit anti-mouse antibody (Boehringer Mannheim) was diluted 1:500 as above and added for 1 hr in the dark, followed by washing in DPBS. Neuron-like PC12 cells, with and without NGF, were treated in parallel and served as a positive control for neurofilament detection; hepatoma cells served as a negative control. The low-affinity NGF receptor was similarly assayed with a mouse monoclonal antibody (Boehringer Mannheim), except that the permeabilization step was omitted so as to allow only cell surface labeling.

Northern Blot Hybridization. After treatment with NGF, cells were harvested in guanidinium isothiocyanate, and total cytoplasmic RNA was isolated by centrifugation through a CsCl cushion. After electrophoresis in formaldehyde/agarose gels, the RNA was analyzed for message corresponding to NGF receptor by Northern blotting. Hybridizations were carried out with p75 and Trk NGF receptor gene probes, labeled by random hexamer priming. To assure that equal amounts of RNA had been loaded, all gels were visualized by staining with ethidium bromide.

To assess increases in NGF-1A mRNA in the RINm5F pancreatic cells after exposure to NGF, the cells were first cultured for 24 hr in the absence of serum as above and then supplemented with NGF (100 ng/ml). RNA was harvested after 30 and 60 min and prepared as described above, and the resulting Northern blot was hybridized to a probe from the NGF-1A gene.

RESULTS

Response to NGF. To investigate the effects of neuronal differentiation factors on pancreatic beta cells, we studied two rat insulinoma cell lines. RINm5F cells resemble incompletely differentiated fetal beta cells in that they contain and

secrete minimal amounts of insulin and respond only weakly to glucose by increasing their insulin secretion. They also secrete small amounts of glucagon and somatostatin, which are normally produced only by the alpha and delta cells of the adult pancreas (19, 21). The second insulinoma, β TC3, consists of cells that are more differentiated than RINm5F cells: like adult beta cells, they respond in a graded manner to glucose by secreting insulin, and they lack glucagon and somatostatin (24). On the basis of these characteristics, RINm5F and β TC3 appear to represent the early and middle to late stages, respectively, of pancreatic beta cell differentiation.

The pancreatic cells were first grown in serum-containing medium and then transferred for 3 days to defined medium without growth factors (see *Methods*). In the absence of added neuronal differentiation factors, the cells remained round. However, when NGF (100 ng/ml) was added to the medium, about 60% of the RINm5F cells extended multiple processes within 48 hr (Fig. 1). A similar response was obtained with NGF at 50 ng/ml (data not shown). Many of these processes were several cell diameters in length, and all of them terminated in growth-cone-like structures. The addition of monoclonal anti-NGF antibodies to the culture prevented the appearance of NGF-induced processes. In contrast to its effect on RINm5F cells, NGF did not induce outgrowth of processes in the more differentiated β TC3 beta cell line.

FIG. 1. Relative ability of NGF and laminin to induce neuron-like processes in RINm5F and β TC3 pancreatic cells. NGF (100 ng/ml), laminin (10 μ g/ml), NGF together with anti-NGF antibody (α NGF, 500 ng/ml), or fetal bovine serum (10%) was added as indicated. (Upper) Representative RINm5F cells in the absence (Left) or presence (Right) of NGF. (Lower) Quantitative data on the number of cells containing processes after different treatments. The cells remained round in the presence of anti-NGF antibody alone, or in the presence of control proteins, BSA and basic fibroblast growth factor (data not shown).

Characterization of Processes. To establish that the processes induced on the pancreatic cells by NGF were similar to those found on authentic neurons, the cells were analyzed by immunocytochemistry using antibodies directed against the 160-kDa subunit of neurofilaments, the characteristic intermediate-filament protein found in neurons. The RINm5F cells contained neurofilament proteins in the cytoplasm prior to differentiation and in the processes after treatment with NGF, just as did the control PC12 cells (Fig. 2).

Detection of NGF Receptor on Responding Cells. Since NGF induces neuronal differentiation in the RINm5F but not the β TC3 cells, it would be expected that the former (but not the latter) should express a functional receptor for NGF. Two types of NGF receptors, with high and low affinity for the NGF protein, have been described (25–30). The low-affinity NGF receptor (p75^{NGFR}) is a transmembrane glycoprotein that binds NGF with a $K_d \approx 1$ nM. It is present on all cells responsive to NGF but lacks a readily apparent cytoplasmic signaling domain (12, 26). In addition, the product of the protooncogene *trk* (p140^{proto-trk}) is a tyrosine kinase enzyme that can act independently as a functional receptor for NGF when introduced into cells by transfection (29, 30). It has been suggested that Trk must combine with the low-affinity receptor to form a high-affinity ($K_d \approx 0.02$ nM) receptor with full biological activity (27, 29). The physiological importance of the Trk protein in the NGF response is further indicated by its presence on sympathetic neurons, sensory neurons, and PC12 cells, all of which respond to NGF and are derived from the neural crest (31).

We tested for the presence of both forms of NGF receptor on the beta cell lines. By immunocytochemistry, the RINm5F cells, which undergo neuron-like differentiation upon treatment with NGF, were found to express the p75 NGF receptor in a punctate pattern similar to that seen with PC12 cells (Fig. 3). The control hepatoma cells were unlabeled. The presence of the low-affinity NGF receptor was confirmed by Northern blot analysis, which showed that the RINm5F cells contained the 3.8-kb mRNA for p75 (Fig. 3D). mRNA for the “high-affinity” NGF receptor, Trk, was not found in the RINm5F cells by Northern blot analysis using a human *trk* probe (data

not shown) but was detected at low levels by using the recently identified rat *trk* sequence (33) as probe (R.S., A. Tazi, M.P., C. Kanaka, and P. Czernichow, unpublished work; see Fig. 3E). The more differentiated β TC3 cells, which are unresponsive to NGF, correspondingly did not express mRNA for the p75 NGF receptor (data not shown), but have recently been shown to express *trk* mRNA at low levels (R.S., A. Tazi, M.P., C. Kanaka and P. Czernichow, unpublished work).

mRNA Changes Induced in RINm5F Cells by NGF. In addition to causing morphological changes in neuronal cells, NGF also induces rapid changes in gene expression (34–37). We tested for such nuclear evidence of the NGF–receptor interaction by assaying the induction of the NGF-1A gene. NGF-1A (also called *zif/268*, *egr1*, and *Krox-24*; refs. 37–40) is an “early response” gene that is transcriptionally activated within minutes after the addition of NGF to PC12 cells or other neuronal cells; its product is a DNA-binding protein believed to be involved in activating genes required for the subsequent neuronal differentiation of the cells. When RINm5F cells were treated with NGF, they exhibited an ≈ 10 -fold increase in the level of the 3.1-kb NGF-1A mRNA (Fig. 4). The neuron-like PC12 cells also showed an induction of NGF-1A mRNA, while the control hepatoma cells were unresponsive to NGF. For both the responding RINm5F and PC12 cells, the level of NGF-1A mRNA reached a peak at about 30 min and then began to decline by 60 min, a time course that is characteristic of early response genes.

Neuronal Differentiation in Response to Laminin. Finally, we tested a neurite-outgrowth protein of the extracellular matrix for its ability to promote neuronal differentiation in the pancreatic beta cell lines. Specifically, when laminin (10 μ g/ml) was added to the cultures, cells from both the RINm5F and β TC3 lines extended processes essentially identical to those induced by NGF in the RINm5F cells (see Fig. 1).

DISCUSSION

NGF-Induced Differentiation of Pancreatic Beta Cells. Our data demonstrate the influence of neural differentiation factors on pancreatic cells. Specifically, the response of two insulinomas, one (RINm5F) representative of an early stage and one (β TC3) of a middle-late stage of pancreatic beta cell differentiation, to the neurotrophic factors NGF and laminin was studied. NGF caused RINm5F insulinoma cells to elaborate neuronal-like processes and to undergo changes in gene expression. These results indicate that the growth factor interacted with a functional receptor protein on the cell surface. Indeed, expression of mRNA for the low- and high-affinity NGF receptors, p75 and Trk, was detected in the RINm5F cells. The relatively high level of NGF required to induce process outgrowth from RINm5F cells was consistent with their low level of Trk expression. While NGF induced specific morphological and biochemical responses in RINm5F cells, it did not show a differentiation effect on the β TC3 cells. Correspondingly, β TC3 cells did not express the p75 NGF receptor mRNA. However, both cell lines responded to laminin by extending processes.

Potential Role for NGF in Pancreatic Development. One area of relevance of these data is the ontogeny of the endocrine pancreas. A possible neuroectodermal origin for the pancreatic islet cells has been an attractive hypothesis since it was first suggested by Pearse (1, 11, 12). However, transplantation and ablation experiments argue strongly against this model (5, 11, 12, 41). If pancreatic beta cells are not derived from the neuroectoderm, then the evidence presented here that NGF can induce neuronal differentiation of beta-like RINm5F cells suggests that the role of NGF in development may extend to cells of endodermal as well as neuroectoder-

FIG. 2. NGF-induced processes of the RINm5F cells contain neurofilaments. (A) Prior to the addition of NGF, RINm5F cells were round and showed a perinuclear staining pattern when labeled with an antibody to the 160-kDa subunit of neurofilaments. (B) After addition of NGF, the induced processes also were labeled with anti-neurofilament antibody. (C and D) Negative controls showing RINm5F cells with the first (anti-neurofilament) antibody omitted (C) and neurofilament-labeled hepatoma cells (D). (E and F) Neuron-like PC12 cells served as a positive control before (E) and after (F) NGF addition.

FIG. 3. Presence of the p75 NGF receptor on RINm5F cells, and Northern blot analysis for p75 and *trk* mRNA. Cells were labeled with a mouse monoclonal antibody against the low-affinity (p75) NGF receptor. (A) RINm5F cells were labeled both before and after NGF addition. (B) Neuron-like PC12 cells. (C) Negative labeling of control hepatoma cells. (D) Presence of the low-affinity NGF receptor mRNA in RINm5F cells (lane 1) and control PC12 cells (lane 2), detected with a p75^{NGFR} probe. (E) Presence of "high-affinity" NGF receptor mRNA in RINm5F cells (lane 1) and in PC12 cells (lane 2), as detected by hybridization with a probe to the rat *trk* protooncogene (32). The filters in D and E were rehybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA to establish the relative amounts of RNA in each lane. (In E, it was necessary to load more RINm5F RNA than PC12 RNA in order to detect the low expression of *trk* in the pancreatic beta cell line.) Positions of 28S and 18S rRNA are indicated.

mal origin. Specifically, the results suggest a role for NGF (and also the extracellular matrix protein laminin, which has a functionally analogous neurite-outgrowth domain) in the development of the islet cells of the endocrine pancreas, which, like neurons, are ligand-activated, depolarizing, secretory cells.

The finding that pancreatic cells respond to NGF also suggests a role for the factor in the regeneration of the endocrine pancreas. An *in vivo* experiment is consistent with this hypothesis. The DNA synthesis that occurs in the pancreata of young rats after administration of a β -adrenergic receptor agonist, such as isoproterenol, requires either intact salivary glands (which secrete NGF and epidermal growth factor into the circulation) or the addition of exogenous NGF or epidermal growth factor (42). It is not known whether endocrine islet cells are involved in this response or whether NGF might act indirectly by stimulating the sympathetic innervation of the pancreas to induce regeneration. Our results suggest that NGF may exert its effect directly on the pancreatic cells themselves.

Differentiation Effect of Laminin. As a differentiation and survival factor, NGF functions on a restricted class of neurons, primarily sensory and sympathetic neurons of the peripheral nervous system and the few large cholinergic neurons of the central nervous system. Other neurotrophic factors each nourish a subset of the remaining majority of neurons in the brain (14). In contrast, laminin, a large extracellular matrix molecule that contains a domain with

FIG. 4. NGF mediates increases in mRNA of the early response gene NGF-1A in RINm5F cells. (A) NGF induced increases in NGF-1A mRNA in RINm5F pancreatic cells and neuron-like PC12 cells (Left and Center). No induction of NGF-1A expression by NGF was observed in control hepatoma (HEPA) cells (Right). When cells were grown in medium containing fetal bovine calf serum (FCS) (with numerous growth factors), the NGF-1A gene was induced in all cell types. Time points of 30 and 60 min are shown for each treatment. (B) To confirm that equal amounts of RNA were loaded in each lane, the filter in A was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase probe.

neurite outgrowth-inducing activity, affects the growth and differentiation of many classes of nerve cells (17). This paper demonstrates that laminin has a similarly general differentiation effect on pancreatic cells, promoting neurite outgrowth from two independently derived insulinoma cell lines. Indeed, the capacity of pancreatic beta cells to display processes extends to a fraction of the cells in the adult pancreas. If islets are dissociated and plated on culture dishes whose adhesion properties have been designed to mimic an extracellular matrix, about 15% of beta cells extend processes (9).

In sum, these results suggest that while undergoing their differentiation, the precursor cells of the endocrine pancreas slowly lose some of their neuronal properties, starting with their responsiveness to NGF, but retain their ability to respond to the neurite-outgrowth domain of laminin. This model of islet development is reminiscent of the ontogeny of the adrenal medulla, whose endocrine cells (and their derived tumor cell line PC12) can also be induced to display neuronal properties in response to NGF (23). The major difference is that the adrenal medulla is derived from the neural crest, while the endocrine pancreas is believed to be derived from the endoderm. Thus it appears that a neural-type differentiation program involving ligand activation, depolarization, secretion, and process formation can be elicited from a subset of endodermal cells, a putative "neuroendoderm."

Possible Function for Neurite-Like Processes in Islet Differentiation. In the nervous system, cellular processes perform two major functions: (i) to receive and transmit signals between neurons and their target cells and (ii) to establish physical connections early in development as a prelude to neuronal or glial cell migration (17, 44, 45). Either or both of these functions might be served by NGF-induced processes in developing pancreatic beta cells. For example, beta cells are known to be responsive to neurotransmitters: insulin secretion is increased in response to acetylcholine, and catecholamines either increase or decrease insulin release depending on whether β - or α -adrenergic receptors are activated (46). Moreover, in addition to their external innervation, islet cells also communicate neurochemically with each other. For example, delta cells release somatostatin, which

inhibits the release of insulin from the beta cells (47). It seems reasonable that islet-cell processes could function to increase the sensitivity or target-cell selectivity of such neurohormonal stimulation.

In addition to neurotransmitter communication, a second role for cellular processes is in directing the migration and placement of cells during development (17, 44, 45). For example, after a neuronal process establishes a connection with a target tissue, the nucleus of the nerve cell appears to migrate inside the process, bringing the cell to its final location (17, 48). It is possible that processes serve a somewhat similar function in the developing pancreas by helping—together with neural cell adhesion molecules such as N-CAM, known to be present on beta cells (8)—to bring together like cells to form islets in which, eventually, a beta-cell center is surrounded by a halo of alpha and delta cells. In fact, studies with chimeric mice have shown that pancreatic islets are indeed polyclonal, rather than each being descended from a single, multipotential progenitor cell (32). Processes would allow a developing islet cell to effectively reach out beyond its immediate vicinity to sample many more other cells than it is initially in contact with and determine whether they contain the same or different cell surface molecules. Then, once an appropriate contact between two precursor-islet-cell processes was established, the cells could profitably migrate toward each other and form an aggregate, a nascent islet. Such multiple exploratory processes potentially constitute a more rapid and efficient sorting system than one based solely on random cell migration and adhesion, in which the search is limited to immediately adjacent cells. Of course, the final stages of islet development could rely simply on cell sorting, with the required structure of the islet being determined by the relative strengths of the adhesion molecules on the various islet cells.

In sum, this paper describes the ability of NGF (and laminin) to induce pancreatic beta cells, represented by two insulinoma cell lines, to elaborate neuronal-like processes and to undergo specific changes in gene expression. These results indicate that neuronal growth factors may play a wider role in development than previously believed, specifically on endodermal cells. Indeed, the similarity between pancreatic beta cells and neurons—not only in their basic secretory functions, but also in their response to differentiation factors—implies that these two cell types, thought to arise from different lineages during development, are, in fact, examples of the same fundamental program of differentiation. This relatedness also suggests that NGF may be useful in the treatment of degenerative pancreatic diseases. Evidence for the regenerative capacity of the adult pancreas comes, for instance, from the finding that solid insulinomas introduced into NEDH rats result in a severe reduction in the number of beta cells, which are then apparently regenerated following excision of the tumor (43).

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