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Ontogeny of Hepatocyte Growth Factor and c-met/hgf Receptor in Rat Pancreas

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Hepatocyte growth factor has several activities in epithelial cells, including mitogenesis, dissociation of epithelial sheets, stimulation of motility, and promotion of matrix invasion. HGF is the ligand of a dimeric transmembrane tyrosine kinase receptor encoded by the met proto-oncogene. The objective of this study was to characterize by Northern blot the HGF and c-met mRNA expression in rat pancreas during development. Our findings show that simultaneous expression of both messengers occurs during the early development of the rat pancreas. Pancreatic HGF and c-met mRNA levels were higher between late fetal and 11 post-natal days and then decreased to a very low level at 26 days. This low level was maintained until the adult age. We conclude that HGF gene expression and c-met pancreatic gene expression are age dependent with the highest expression before weaning. These new data demonstrate that HGF and its receptor may be implicated in normal pancreas development and maturation.

Hepatocyte growth factor (HGF) was first purified from rat platelets as a potent mitogen for mature hepatocytes (1). While HGF was considered to have a narrow-cell specificity, it is now accepted that it is a pleiotropic factor produced by mesenchymal cells which acts on various epithelial cells (2,3). The observation that HGF stimulated the rapid tyrosine phosphorylation of a 145 kDa protein provided an early clue concerning the identity of its receptor (4). By using antibodies specific for different tyrosine kinases, it was determined that the 145 kDa phosphoprotein was the B subunit of the c-met proto-oncogene product (5). Further studies firmly established the c-met protein as the HGF receptor (5).

HGF can stimulate DNA synthesis, cell migration and morphogenesis in a variety of epithelial cell types in culture (6), and was postulated as a factor that helps to maintain mesenchymal-epithelial homeostasis (7). Although HGF's role *in vivo* remains obscure, recent evidence, however, suggests that it may be involved in cellular functions such as wound repair, organ growth and regeneration (8).

In rabbit, staining with an anti-HGF antibody was observed in acinar cells of the pancreas, in the ductal cells of the salivary glands and in the Brunners glands of the duodenum (9). The presence of HGF was also noticed in rat fetal pancreas at days 17 and 19 of gestation by immunohistochemistry (7) and in both man and rat pancreas islet A-cells (10). HGF is a potent mitogen for primary cultures of human exocrine pancreatic cells (11), and a potent stimulus for the formation of islet-like cell clusters in primary cultures of human fetal pancreas (12). Moreover, c-met mRNA seems to be overexpressed in ductal carcinomas of the pancreas while it is barely detectable in normal acinar cells. This finding strongly suggests a pivotal role for this growth factor and/or its receptor in growth and development of the pancreas.

Organogenesis during developmental processus involves morphogenesis and cell proliferation and locomotion. Pancreatic organogenesis has been a classic example of epithelio-mesen-

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chymal interaction (13), and mesenchymal factors seem to be essential for the formation of acinar structures (14). As HGF is a mesenchymal cell-derived regulator for epithelial cell growth, motility and morphogenesis, a potential role in organogenesis and maturation of parenchymal cells remains to be determined.

This study was performed to analyze changes in HGF mRNA and its receptor, the c-met proto-oncogene product, in the rat pancreas during late fetal and postnatal development in order to determine potential roles for HGF in growth, maturation and maintenance of gland homeostasis. Pancreatic messengers RNA expressions were compared with those in the liver since both HGF and c-met mRNA transcripts have been best documented in this tissue previously (15).

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were fed Purina rat chow ad libitum and were kept in a room with controled temperature and light (20 °C; 12 h light, 12 h dark daily). All studies were conducted in accordance with the principles and procedures outlined in the Guidelines for Care and Use of Experimental animals. Sprague-Dawley 19 day-old rat fetuses (F19), newborn (NB) and 5, 11, 26 and 70 day-old rats were used (P5-P70). Fetal pancreata were obtained from timed pregnant rats 19 days after the appearance of vaginal plugs, and pancreas were pooled from multiple fetus (both male and female). Rats were sacrificed by decapitation, and their pancreata and livers were rapidly removed and submerged in liquid nitrogen before processing.

Pancreatic RNA isolation. Total RNA was isolated by a modification of the procedure of Chirgwin *et al.* (16). Frozen tissues were rapidly homogenized at room temperature in a minimum of 10 vol of 4 M guanidine thiocyanate, 0.1 M Tris HCl (pH 7.5), 10 mM 2-mercaptoethanol and sarcosil 0.5 %. The homogenate was adjusted to 80 mM acetic acid and 0.1 M potassium acetate (pH 5.0). RNA was precipitated by the addition of 0.75 vol of pure ethanol and placed for 2 h at −20 °C. A pellet was then obtained at 10,000 × g for 10 min, and was resuspended in 7.5 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol. The resuspended pellet was adjusted to 0.1 M potassium acetate (pH 5.0) and precipitated for 2 h at −20 °C by adding 0.5 vol of pure ethanol. After centrifugation (10,000 × g for 10 min), the resulting precipitate was again collected, redissolved and precipitated as before. The final RNA pellet was dissolved in 25 mM EDTA (pH 7.0), 0.1 % sodium dodecyl sulphate (SDS) for 3 min at 65 °C and extracted with 2 vol of water-saturated chloroform:1-butanol (4:1 v/v). After centrifugation at 8000 × g for 5 min, RNA in the aqueous phase was precipitated by adding 0.1 vol of 2 M sodium acetate (pH 7.0) and 2.5 vol of cold pure ethanol at −20 °C overnight. The RNA was pelleted at 10,000 × g for 15 min, rinsed with 75 % ethanol and dissolved in DEPC-treated water.

Preparation of the probes. The HGF complementary RNA (cRNA) probe was transcribed from the 1400-base pair EcoRI rat HGF cDNA fragment, inserted into the plasmid vector Bluescript KS II+. The templates were linearized with BamHI, and T3 RNA Polymerase was used for *in vitro* (antisense) transcription. The c-met cRNA probe was transcribed from the 710-base pair Cla I - Sal I human c-met cDNA fragment, inserted into the plasmid vector Bluescript SK II+. The templates were linearized with Sal I, and T3 RNA Polymerase was used for *in vitro* (antisense) transcription. After linearization, antisense cRNA probes were labelled with [^{32}P]UTP using Promega Transcription Riboprobe System (Promega, Madison, WI). The rat amylase complementary DNA (cDNA) probe was a 1,100-base pair insert from clone pcXP100 (generously provided by J-Ch.Dagorn). The cDNA probes were labelled with [^{32}P]dCTP by random priming using a commercial kit (Megaprime DNA Labelling System, Amersham Canada Limited, Oakville, Ont, Canada).

Northern blot analysis. Twenty μg of total RNA (quantitated by measurement of absorbance at 260 nm) were size-fractionated on a 1 % agarose gel containing 2.2 M formaldehyde and transferred to nylon membranes (Nytran Plus, Schleicher and Schuell, Keene, NH) as described (17). The membranes were prehybridized at 65 °C for 4-6 h in 5× SSC, 50 mM Hepes (pH 6.8), 1% SDS, 5× Denhardth, 2 mM EDTA, 50 % formamide and sonicated salmon sperm DNA (200 μg/ml). Hybridization was performed at 65 °C during 16-20 h in the above solution containing [^{32}P]UTP RNA probe (1×10^6 cpm/ml). After the hybridization, filters were washed with 0.1× SSC/0.1% SDS twice, 30 min at 75°C and exposed to Kodak X-Omat AR film (Eastman Kodak Company, Rochester, NY), with an intensifying screen (E.I. Dupont) at −80 °C for variable periods of time. A 18 S ribosomal [^{32}P]dCTP-labelled cDNA probe was used as a control probe to evaluate RNA loading and transfer. The size of the RNA transcripts were estimated according to the position of the 18S and 28S rRNAs or RNA ladder. Autoradiograms were quantified using laser densitometer (BIORAD Imagin densitometer Model GS-670, Biorad Laboratories Ltd., Mississauga, Ont., Canada). The relative density of the bands was expressed in arbitrary absorbance units. To correct for differences in loading of total RNA on Northern Blots, a ratio of the relative density of each band with the relative density of the 18S ribosomal RNA band was calculated before comparisons were made.

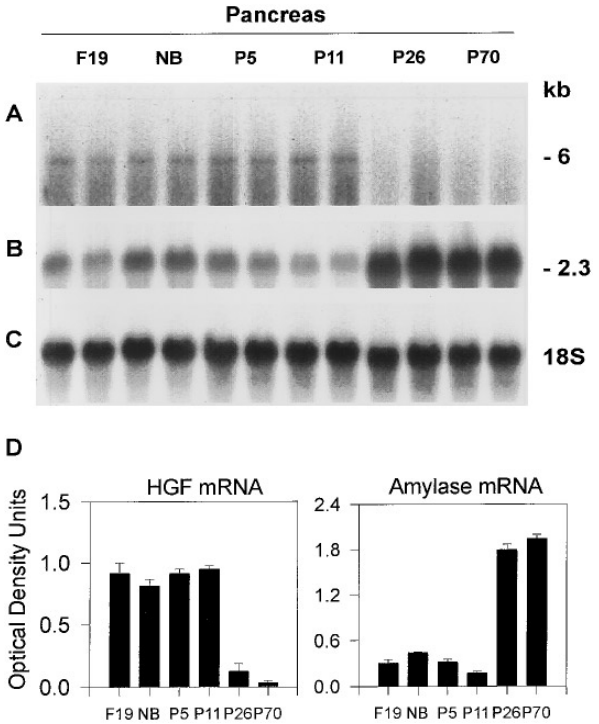

FIG. 1. HGF and amylase mRNA expression in developing rat pancreas. Two separate samples of twenty micrograms of total RNA from pancreata of 19-day-old fetus (F19), newborn (NB) and postnatal age 5, 11, 26 and 70 days (P5 - P70) were hybridized for HGF mRNA using a [^{32}P]-labeled cRNA probe (A), and amylase cDNA labeled probe (B). A 18S ribosomal cDNA probe was used to evaluate relative amounts of mRNA transferred to the membrane (C). The size of the RNA transcripts revealed by hybridization are indicated in kilobases (kb). Quantitative analysis of autoradiograms (D) of total RNA (20 μg) from rat pancreata at different ages was prepared and blotted onto nylon membranes as described in Materials and Methods. Blots were hybridized with HGF cRNA and Amylase cDNA probes and then scanned on a densitometer. HGF and Amylase mRNAs abundance at each time point were normalized to the abundance of 18S rRNA. Results represent the mean values ± SE of four different samples.

RESULTS

HGF and Amylase mRNA Expression in Developing Rat Pancreas and Liver

The results of the HGF mRNA expression in rat pancreas during ontogeny are shown in Figure 1. The Northern blot reveals one mRNA transcript with an estimated size of 6.0 kb. Pancreatic HGF mRNA expression was examined during late fetal development (19 days) up to adulthood (70 days) (Fig.1A); its expression was elevated in late fetal life, at birth and during the first 11 days post partum; it dropped abruptly thereafter and became undetectable in 26 days and adult pancreata. Amylase mRNA expression measured on the same filter exhibits a single hybridizing transcript of 2.3 kb and shows an opposite pattern (Fig.1B). The highest levels of amylase mRNA were observed after weaning, in the 26 (P26) and 70 (P70) days old rats. A relatively high level was also observed in the fetus (F19) and in the newborn (NB) with the lowest level present at 11 days after birth (P11) (Fig.1B). A presentation of the 18 S cDNA probe hybridization is also included (Fig.1C) and served for the estimation of RNA loading on the gels. The abundance of HGF and amylase mRNA were calculated by densitometric scanning and normalized to the abundance of the 18S ribosomal RNA (Fig. 1D). These quantitative analysis reveal that in fetal pancreata (F19) up to 11 days postpartum (P11),

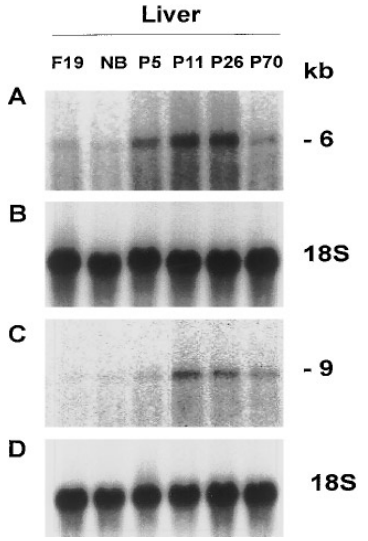

FIG. 2. HGF and c-met mRNA expression in developing rat liver. Twenty micrograms of total RNA from liver of 19-day-old fetus (F19), newborn (NB) and postnatal age 5, 11, 26 and 70 days (P5-70) were hybridized for HGF (A), and c-met (C) mRNA using a [^{32}P]-labeled cRNA. A 18S ribosomal cDNA probe was used to evaluate relative amounts of mRNA transferred to the membrane (B and D). The sizes of the RNA transcripts revealed by hybridization are indicated in kilobases (kb).

pancreatic HGF mRNA abundance was significantly greater that in the adult pancreas (Fig.1D). Conversely, amylase mRNAs were significantly higher in 26 and adult pancreata (P26, P70) (Fig.1D).

In this study, samples of the liver were taken because HGF and c-met mRNA expression have been clearly demonstrated (15); therefore, this organ can serve as a positive control. In the liver (Fig.2A), HGF mRNA also exhibits one transcript of 6.0 kb as previously described (15). Hepatic HGF mRNA levels are detectable in fetus and at birth and then increased from day 5 after birth to reach a maximum on day 26. Later on, HGF expression decreased to a low level observed at day 70.

C-met mRNA Expression in Developing Rat Pancreas and Liver

As shown in figure 3A, Northern blot hybridization of pancreatic RNA reveals one evident c-met transcript with an estimated size of 9.0 kb. C-met mRNA was strongly expressed in 19 day fetus; the receptor mRNA expression is already decreased at birth to reach almost undetectable levels at day 70. A presentation of the 18 S cDNA probe hybridization is also included (Fig.3B) and served for the estimation of RNA loading on the gels. The abundance of c-met mRNA calculated by densitometric scanning and normalized to the abundance of 18S ribosomal RNA is shown in figure 3C. The largest amount of c-met receptor was observed in tissue collected from 19-day-old embryos (a 8.3-fold increase relative to the amount measured in tissue from P26). The amount of c-met mRNA in pancreas from adult (P70) was barely detectable and was at the limit of the method sensitivity. Contrary to the pancreas, the c-met mRNA expression is hardly detectable in the liver (Fig.2C) before P5, and its expression follows closely the developmental pattern of liver HGF mRNA expression (Fig.2A) with the highest expression before and around weaning.

DISCUSSION

The hepatocyte growth factor (HGF) is an important paracrine mediator of interactions between the epithelial and stromal compartments of various tissues during development and

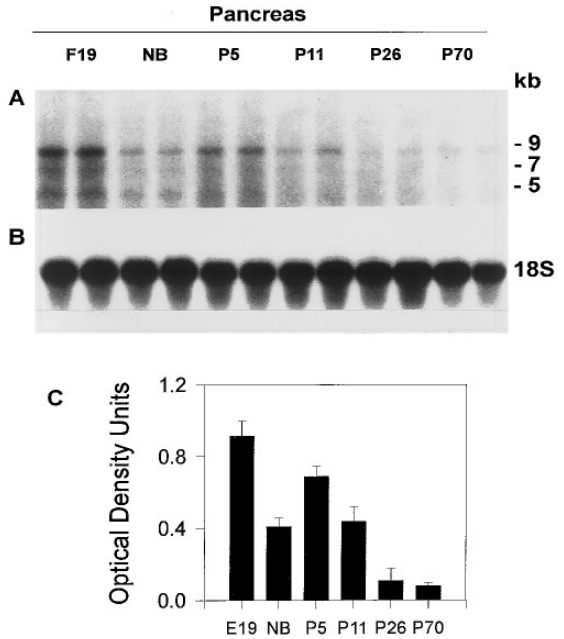

FIG. 3. C-met mRNA expression in developing rat pancreas. Twenty micrograms of total RNA from pancreata of 19-day-old fetus (F19), newborn (NB) and postnatal age 5, 11, 26 and 70 days (P5 - P70) were hybridized for c-met mRNA using a [^{32}P]-labeled cRNA probe (A). A 18S ribosomal cDNA probe was used to evaluate relative amounts of mRNA transferred to the membrane (B). The size of the RNA transcripts revealed by hybridization are indicated in kilobases (kb). Quantitative analysis of autoradiograms (C) of total RNA (20 μg) from rat pancreata at different ages was prepared in duplicate and blotted onto nylon membranes as described in Materials and Methods. Blots were hybridized with c-met cRNA probe and then scanned on a densitometer. C-met mRNA abundance at each time point was normalized to the abundance of 18S rRNA. Results represent the mean values ± SE of four different samples.

in the maintenance of homeostasis in adult tissues (18). HGF is thought to elicit its various biological activities by binding to a membrane-spanning tyrosine kinase receptor encoded by the c-met proto-oncogene (5). Transduction of the HGF signal results in either increased division/differentiation of the cells or in cell dissociation and increased motility with loss of adhesion and functional communication. The growth regulating effect of HGF on various cell types, other than hepatocytes, is now well documented (19). HGF is believed to be the most potent epithelial morphogen inducing formation of branching tubules and gland-like structures in epithelial cells derived from kidney or mammary tissues in vitro (20, 21); furthermore, recent findings show that simultaneous expression of HGF and c-met occurs during the early development of the mouse kidney (22) as well as in lung and central nervous system (18, 23).

Recently Vilá (11) reported HGF as a potent mitogen for normal human pancreatic cell *in vitro*, and suggested that HGF and c-met may play a role in growth regulation of human pancreas. Immunolocalization has indeed identified HGF in the exocrine pancreas (9), but immunopositivity does not distinguish between sequestration or de novo synthesis. Our present results suggest that beside the known mitogenic effect of HGF on human pancreatic cell *in vitro*, HGF may also be implicated in normal rat pancreatic growth and that both ligand and receptor are expressed simultaneously in the pancreatic gland. On the other hand, the expression in the liver presents a different picture with a slight expression during late fetal life and increasing expression after birth as previously shown (24).

In this study, we demonstrated that pancreatic HGF and c-met genes were activated during late gestation and the first days after birth, but downregulated at 26 and 70 days. These data

support Gittes' hypothesis (14) that mesenchymal cells may be essential only for the formation of acinar structures, and that the concentration of the necessary ''mesenchymal factor(s)'' may determine the proportion of acinar differentiation. Furthermore, the potential involvement of tyrosine kinase receptors and their specific ligands in this type of interactions proposes a molecular basis for such exchange of signals in pancreatic development.

After birth, pancreatic development is characterized by the rapid proliferation of duct, islet, and acinar cells, as indicated by the presence of numerous mitotic figures in those cells, and by increases in total pancreatic DNA (25, 26). The rate of proliferation decreases steadily from prenatal day 13 to birth as indicated by evaluations of both total DNA synthesis and nuclear labelling indices of epithelial cells (27, 28). During this late fetal period, we clearly found detectable levels of expression of HGF and c-met, and their expression follows the rate of pancreatic cell proliferation; indeed, a fairly constant expression is seen during the first three postnatal weeks to decline after weaning, up to the adult age (27-30).

In conclusion, HGF and c-met pancreatic genes expression are age dependent with the highest expression before weaning. Both messengers are simultaneously expressed and their period of maximal expression matches the period of rapid proliferation of the pancreatic gland. These data suggest that HGF may be implicated in the normal development of the pancreatic gland.

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