Pancreatic pattern of expression of thyrotropin-releasing hormone during rat embryonic development

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Abstract

In rodents, the first insulin-producing cells appear in the pancreas at mid-gestation around embryonic day 11 (E11). However, on the basis of various features, such as morphology or hormonal coexpression, it is apparent that these initial insulin-expressing cells are different from those that develop after E15. In the present study, the pancreatic expression of both thyrotropin-releasing hormone (TRH) mRNA and insulin was studied during embryonic and fetal life. We report here that in the rat, while insulin mRNA is detected in the pancreas as early as E12, TRH mRNA cannot be detected before E16. At that stage and later on during fetal and early postnatal life, TRH mRNA

is detected in insulin-producing cells, no signal being detected in other endocrine cell types or in exocrine tissue. It was also noted, by means of triple staining performed at E17, that the expression of TRH mRNA was restricted to insulin-expressing cells negative for glucagon, whereas the few insulin-expressing cells present at that stage, which coexpress insulin and glucagon, did not express TRH mRNA. Taken together, these data indicate that TRH is a marker of insulin-expressing cells, which develop after E15.

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Introduction

In rodents, during embryonic and fetal life, pancreatic development follows a specific pattern that can be divided into three main steps. During the first step, named primary transition and starting around embryonic day 10 (E10), the first insulin-positive cells appear. Their number remains stable during the next 3–4 days, a period called the protodifferentiation state. Finally, the number of insulin-positive cells increases very rapidly from E15, during a period called the secondary transition (Pictet & Rutter 1972, Herrera *et al.* 1991). Various features indicate that the insulin-expressing cells found before E15 are different from the ones found after E15 (Pictet & Rutter 1972, Pang *et al.* 1994, Miralles *et al.* 1999).

Thyrotropin-releasing hormone (TRH) is a hypothalamic hormone that has been shown to be expressed in the pancreas (Morley *et al.* 1977). In this organ, its expression is tightly regulated spatially. Indeed, TRH is specifically found in beta cells in the islets of Langerhans (Martino *et al.* 1978, Leduque *et al.* 1987, Scharfmann *et al.* 1988), where it colocalizes with insulin in the same granules. Its expression is also tightly regulated temporally. Indeed, a large number of studies have demonstrated that the levels of TRH are high in pancreatic beta cells during the

neonatal period (Martino *et al.* 1980, Aratan-Spire *et al.* 1984) and decrease rapidly, when postnatal development progresses (Martino *et al.* 1980, Aratan-Spire *et al.* 1984, Ouafik *et al.* 1987, Scharfmann *et al.* 1988). To the best of our knowledge, the prenatal pattern of pancreatic TRH expression remains poorly studied.

The objective of the present study was thus to study the pattern of pancreatic expression of TRH during embryonic and fetal life in insulin-expressing cells. More specifically, our goal was to determine whether insulin-expressing cells developed during the primary and the secondary transition express TRH. Our results demonstrate that while insulin mRNA can be detected in the pancreas as early as E12, TRH expression is not detected before E16. At E16 and later on, TRH mRNA is specifically detected in insulin-expressing cells. These data suggest that TRH mRNA is a marker of mature beta cells that develop during the secondary transition.

Materials and Methods

Animals and tissue preparation

Non-pregnant and pregnant Wistar rats were purchased from the Janvier breeding center (Le Genet, France). The

morning of the discovery of the vaginal plug was designated as E0.5. The pregnant rats were killed by cervical dislocation at different stages of gestation. The embryonic pancreases were obtained after microdissection under a Leitz microscope. The tissues were either frozen for RNA extraction or fixed for *in situ* hybridization or immunohistochemistry.

Cell culture

INS-1 cells derive from a rat insulinoma (Asfari *et al.* 1992) and were grown in RPMI 1640 supplemented with penicillin (100 µg/ml), streptomycin (100 µg/ml), 10% fetal calf serum, 1 mM sodium pyruvate, 10 mM Hepes and 50 µM 2-mercaptoethanol. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

In situ hybridization

The pancreases were either fixed overnight at 4 °C in 4% paraformaldehyde in PBS, briefly rinsed with PBS, cryo-protected overnight at 4 °C in 30% sucrose and frozen or fixed in formalin and embedded in paraffin. Sections were cut and collected on Superfrost⁺ slides (CML, Nemours, France). Prehybridization was done at 70 °C in hybridization buffer (50% formamide, 5 × SSC, 5 × Denhardt's solution, 250 µg/ml yeast RNA, 500 µg/ml herring-sperm DNA). RNA probes were labeled with DIG-UTP by *in vitro* transcription using the DIG-RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Hybridization was initiated by the addition of fresh hybridization buffer containing probe (1 µg/ml) and then continued overnight at 70 °C. Thereafter, the slides were washed with decreasing concentrations of SSC. Non-specific sites were blocked with 2% blocking reagent (Boehringer Mannheim) in Tris (25 mM pH 7.5), NaCl (140 mM), KCl (2.7 mM) and Tween 20 (0.1%) for 30 min at room temperature. Then slides were incubated overnight at 4 °C with alkaline phosphatase-conjugated polyclonal sheep anti-DIG antibody (diluted 1:1000; Boehringer Mannheim). The reaction product was visualized by an enzyme-catalyzed color reaction using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate medium (Boehringer Mannheim). Sections were incubated until the colored reaction product developed at the sites of hybridization. The slides were washed in H₂O, mounted and visualized using a DMRD light microscope (Leica, Heerbrugg, Switzerland). Each experiment was performed at least three times.

The following riboprobes were used: rat proinsulin (Aratan-Spire *et al.* 1990) and rat proTRH (Segerson *et al.* 1987).

Immunohistochemistry

The paraffin sections, first used for *in situ* hybridization as described above, were next used for immunohisto-

chemistry. For that purpose, the sections were first permeabilized using 0.1% Triton X100 in PBS, incubated for 30 min in Tris-buffered saline (TBS) containing 3% BSA. Subsequently, the sections were incubated overnight at 4 °C with the primary antibodies. After washing in TBST (TBS containing 0.1% Tween 20), the sections were incubated with the appropriate fluorescent secondary antibodies. Finally, the sections were extensively washed in TBST and mounted with a fluorescence-protecting medium. The sections were examined and photographed with a DMRD microscope (Leica).

The antisera employed in this study were guinea-pig anti-porcine insulin (1:200; Dako S.A., Copenhagen, Denmark) and mouse anti-porcine glucagon (1:2000; Sigma Chemical Co., St Louis, MO, USA). The fluorescent secondary antibodies were fluorescein anti-guinea-pig antibodies (1:500; Dako S.A.) and Texas-red anti-mouse antibodies (1:200; Jackson Immunoresearch).

RNA preparation and comparative PCR

Total RNAs were extracted from pancreases and reverse-transcribed as described previously (Atouf *et al.* 1997). For comparative PCR, series of twofold dilutions of the cDNAs were prepared and used as templates in the PCR reactions.

The oligonucleotides used for amplification were as follows: insulin (forward), 5'-CCTAAGTGACCAGCTACA-3'; insulin (reverse), 5'-GTAGTTCTGCAGTTGTA-3'; TRH (forward), 5'-ATTCTTGTGGAAAGACCTCCAGC-3'; TRH (reverse), 5'-GACATCTGAGACCAGGAATCCA-3'; TRH receptor 1 (TRHR1; forward), 5'-AGATGTTTCAACAGCACCGTTTC-3'; TRHR1 (reverse), 5'-TCTGTGCTAAAGCGGTCTGACTC-3'; TRHR2 (forward), 5'-GGTTCTTCCTGGTGGATCTCAAT-3'; TRHR2 (reverse), 5'-GAGCAGTACCAGTGTGCGGTAAG-3'; cyclophilin (forward), 5'-CAGGTCCTGGCATCTTGTC-3'; and cyclophilin (reverse), 5'-TTGCTGGTCTTGCCATTCCT-3'.

Typically, 35 cycles of amplification were performed. Amplification parameters included a 30 s denaturation step at 96 °C, a 30 s annealing step at 57 °C and a 30 s extension step at 73 °C. The products of amplification were separated on a 1.5% agarose gel and photographed. Each experiment was performed at least three times.

Results

RT-PCR analysis of the expression of proinsulin and proTRH mRNA in the pancreas in development

As shown in Fig. 1, RNA coding for insulin could be amplified from rat embryonic pancreases as early as E12.5. Some increase in the proinsulin mRNA level was found at

Figure 1 RT-PCR analysis of proinsulin and proTRH mRNA expression in the developing pancreas. (A) Ethidium bromide staining of 1.5% agarose gels containing proinsulin and proTRH PCR products of reverse-transcribed total RNA from a series of twofold dilutions of cDNAs prepared from rat pancreases at different stages of development. (B) Cyclophilin amplification in non-saturating conditions was used as the control.

E14 when compared with E12. The insulin mRNA level next remained stable between E14 and E16, whereas a sharp increase was seen at E18 and birth.

ProTRH mRNAs were amplified neither at E12.5 nor at E14. A low level of expression was detected at E16, followed by a sharp increase at E18 and birth (Fig. 1).

In situ hybridization for proinsulin and proTRH mRNA in the pancreas in development

In situ hybridization was next performed at different stages of pancreatic development, using proinsulin and proTRH riboprobes. As expected, as shown in Fig. 2, proinsulin mRNA was detected in the pancreas from adult and 2-day-old rats, whereas proTRH mRNA was not detected in the adult pancreas, but was detected in the pancreas of 2-day-old rats. At that stage, proTRH staining was found in the islets of Langerhans. No staining was found when sense probes were used.

The pancreatic patterns of expression of proinsulin and proTRH mRNA were next followed at different prenatal stages on tissue sections. Proinsulin mRNAs could be detected at E20, E18, E16 and E14. On the other hand, while proTRH mRNAs were detected at E20, E18, E16, no expression of proTRH mRNA was detected at

E14 (Fig. 2). Such data correlate with those found by RT-PCR analysis and described above.

Definition of the cell types expressing TRH in the developing pancreas

Pancreatic sections from 4-day-old rats and embryos at E17 were first hybridized with a proTRH riboprobe, followed by double immunohistochemistry using antibodies specific for insulin and glucagon. As shown in Fig. 3 (left-hand panels), in the pancreas of 4-day-old rats, all of the insulin-positive cells expressed TRH, whereas no cells coexpressing TRH-mRNA and glucagon were detected.

At E17, the vast majority of the insulin-expressing cells coexpressed TRH, whereas TRH mRNA was never detected in glucagon-expressing cells (Fig. 3, right-hand panels). At that specific stage, some cells positive for both insulin and glucagon were detected. Such double-positive cells stained negative for TRH (Fig. 3, right-hand panels).

Expression of TRHRs in the developing pancreas

To define whether TRH, which is produced in the pancreas during embryonic life, could act in an autocrine/

Figure 2 Expression and localization of proinsulin and proTRH mRNA in the rat pancreas at different stages of development. Frozen pancreatic sections from postnatal (adult and 2-day-old) and prenatal (E20, E18, E16 and E14) rats were hybridized with proinsulin or proTRH antisense DIG-labeled riboprobe. Arrows: islets of Langerhans. Original magnification: $\times 100$.

Figure 3 Coexpression of proTRH mRNA and insulin in the developing pancreas. Paraffin-embedded pancreatic sections from postnatal (4-day-old) and prenatal (E17) rats were first hybridized with a proTRH riboprobe (blue staining). The sections were next stained for insulin (green fluorescence) and for glucagon (red fluorescence). Arrows show cells coexpressing insulin and glucagon but negative for Pro-TRH mRNA. Magnification: $\times 100$.

Figure 4 RT-PCR analysis of TRHR1 and TRHR2 mRNA expression in the developing pancreas. The pictures show ethidium bromide staining of 1.5% agarose gels containing TRHR1 and TRHR2 products of reverse-transcribed total RNA from cDNAs prepared from rat pancreases at different stages of development, INS-1 cells and brain.

paracrine fashion, we searched for TRHR expression in the pancreas in development. As shown in Fig. 4, mRNA coding for TRHR1 could be amplified from pancreases at E12, E14, E16 and birth. No major differences were detected at the different stages tested. TRHR1 could also be amplified from the insulin-producing cell line INS-1. On the other hand, while TRHR2 could be amplified from the brain, no amplification of TRHR2 could be detected in the pancreas at the different stages tested or in INS-1 cells (Fig. 4).

Discussion

In the present study, we demonstrate that TRH is expressed in the rat pancreas during late-embryonic life and early postnatal life. During this period, TRH mRNA is found exclusively in insulin-expressing cells. Moreover, during the same period, TRHR mRNA is expressed in the developing pancreas. On the other hand, before E16, the insulin-positive cells present in the pancreas do not express TRH mRNA.

It has been known for many years that TRH is expressed in the rat pancreas (Morley *et al.* 1977). Its

pattern of expression is tightly controlled in a temporal and cell-type-specific manner. Indeed, in the pancreas, TRH is found at high levels specifically in insulin-expressing cells at around the time of birth, the TRH levels decreasing very rapidly during the first days of postnatal life (Martino *et al.* 1980, Leduque *et al.* 1987, Scharfmann *et al.* 1988). Such data were mainly obtained at the protein level. Our data, obtained at the RNA level, do confirm this specific pattern of expression. However, to the best of our knowledge, very few data have been available concerning the expression of TRH in the pancreas during prenatal life. In fact, there was one report indicating that TRH mRNA was detected in the pancreas of rat at E19 (Dutour *et al.* 1987). However, in that study, no time points prior to E19 were studied. Moreover, the study (performed by Northern blot analysis) did not permit definition of the pancreatic cell types expressing TRH mRNA at those prenatal stages of development. The present study clearly indicates that before birth TRH mRNA is specifically found in beta cells.

Our data, obtained by comparative RT-PCR, confirm previous data indicating that, both in the rat and the mouse, insulin levels increase between E12 and E14 (a period called the primary transition). These levels remain

quite stable between E14 and E16 and increase very rapidly after E16, during a period called the secondary transition (Pictet & Rutter 1972, Herrera *et al.* 1991). The present data indicate that, while the insulin-expressing cells that develop during the secondary transition express TRH, the ones that develop during the primary transition stain negative for TRH. In fact, previous data from the literature have indicated that, on the basis of different criteria, insulin-expressing cells present in the pancreas during early embryonic life are different from the ones present later during prenatal life. Prior to E15, insulin-expressing cells contain neither granules resembling those found in mature beta cells (Pictet *et al.* 1972) nor Rab3A and SNAP-25 (Miralles *et al.* 1999), two molecules important for the control of insulin secretion (Sadoul *et al.* 1995, Regazzi *et al.* 1996). Moreover, prior to E15, insulin-expressing cells stain negative for the glucose transporter Glut2 (Pang *et al.* 1994). Finally, such embryonic insulin-expressing cells coexpress glucagon (Alpert *et al.* 1988, Pang *et al.* 1994, Jackerott *et al.* 1996, Miralles *et al.* 1998, 1999). Our results, described in the present study, indicate that such cells, which coexpress insulin and glucagon, stain negative for TRH, whereas the insulin-expressing cells present later, after E16, express TRH. Thus TRH does represent a new marker for beta cells, which developed during the secondary transition.

In endocrine organs, such as the pituitary, two populations of thyrotrope cells develop. While the first population appears at E12 in the rostral tip of the developing gland, the second one develops 3 days later in the caudomedial region of the gland. In this organ, it has been proposed that these two cell populations arise independently (Lin *et al.* 1994). In the pancreas, the relationship between the insulin-expressing cells that develop during the primary and secondary transitions remains unknown. It had been proposed, on the basis of indirect evidence, that the insulin-expressing cells that develop during the primary transition could represent precursor cells for the insulin-expressing cells, which develop later on (Alpert *et al.* 1988). However, the fact that a large number of markers such as, for example, Glut2 (Pang *et al.* 1994), Pdx-1 (Ahlgren *et al.* 1996) or proTRH (the present study) are absent from insulin-containing cells which develop early during development could indicate that these two cell populations develop independently.

Recently, it was shown that mice with a targeted disruption of the TRH gene exhibited hyperglycemia and impaired insulin secretion in response to glucose (Yamada *et al.* 1997). One hypothesis for this was that TRH produced in the pancreas could act in a paracrine/autocrine fashion to regulate pancreatic development. In that case, TRHR would have to be expressed in the developing pancreas. To the best of our knowledge, no information has been available concerning the expression of TRHR in the developing pancreas. In rodents, two different TRHRs have been cloned. TRHR1 has been

found to be expressed in the anterior pituitary (Straub *et al.* 1990, de la Pena *et al.* 1992) and in the hypothalamus, testes and thymus (Montagne *et al.* 1999). More recently, a cDNA encoding a novel subtype of TRHR has been cloned. This type of receptor was found to be expressed specifically in the brain (Cao *et al.* 1998, Itadani *et al.* 1998). We demonstrate in the present study that mRNA coding for TRHR1, but not TRHR2, is detected in the embryonic developing pancreas. Taken together, these data strongly suggest that TRH is a marker of insulin-expressing cells that develop after E15. It could play a role, in an autocrine/paracrine fashion, in the development of the pancreas.

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