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Localization of transthyretin-mRNA and of immunoreactive transthyretin in the human fetus

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Summary. Five human fetuses at mid-term (16–20 weeks) and one with a gestational age of 8 weeks were investigated. The cellular localization of transthyretin (TTR)-mRNA in different organs was demonstrated by in situ hybridization with a ^{35}S-labelled, single-stranded RNA probe. Immunoreactive TTR (TTR-IR) was localized with a monoclonal antibody to TTR. In all fetuses, choroid plexus epithelial cells demonstrated intense labelling for TTR-mRNA as well as strong TTR-IR. Hepatocytes, on the other hand, showed weak in situ labelling and weak or, in some cases, non-demonstrable TTR-IR. In all mid-term fetuses, but not in the 8 week fetus, TTR-mRNA and TTR were also expressed in pancreatic endocrine A-cells. The degree of in situ labelling in these A-cells was moderate, whereas that of TTR-IR was strong. Despite negative findings for TTR-mRNA in the gut and the kidney, endocrine cells of the gut and epithelial cells of the renal proximal convoluted tubules showed TTR-IR in some of the fetuses. The investigation provides evidence for TTR synthesis in the human fetal choroid plexus, liver and endocrine pancreas. However, further studies are required to demonstrate TTR synthesis in the gut and the kidney.

Key words: Transthyretin – Human – Fetus – In situ hybridization – Immunocytochemistry

Introduction

Transthyretin (TTR, prealbumin) has an important role in the transport of two substances, thyroxine and retinol (Robbins and Bartalena 1986; Goodman 1976), both of which are essential for normal fetal growth and development (Dussault and Ruel 1987; Wilson et al. 1953). The 55 kDa protein consists of four identical subunits and the amino acid sequence as well as the three-dimensional structure are known (Kanda et al. 1974; Blake et al. 1978). The structure of the TTR gene and its location on the human chromosome 18 have also been determined (Wallace et al. 1985). Mutant TTR is the major component of the amyloid deposits in various forms of familial amyloidotic polyneuropathy (FAP) and prenatal diagnostic tests for these diseases are currently being evaluated (Cornwell et al. 1987). In adults, the liver is believed to constitute the main site for TTR production (Felding and Fex 1982). The choroid plexus is a further site for TTR synthesis, explaining the relatively high concentration of TTR in the cerebrospinal fluid (Dickson et al. 1986; Herbert et al. 1986; Jacobsson 1989a). Recently, evidence for TTR synthesis also in the adult human endocrine pancreas and in endocrine tumours of the pancreas and gut was presented (Jacobsson 1989a; Jacobsson et al. 1989b).

In previous studies, immunological methods were used to determine the presence and distribution of TTR in human fetal tissues (Gray et al. 1985, Jacobsen et al. 1982). On this basis it is not possible to state definitely whether the protein is produced or absorbed by the TTR-containing cells or even whether the protein is maternally or fetally derived. For these reasons, and because of the important role TTR may play in normal fetal development, the cellular distribution of TTR-mRNA correlated with that of TTR immunoreactivity (IR) in the human fetus has been investigated.

Materials and methods

The investigation of five legally aborted human fetuses with gestational ages ranging from 16 to 20 weeks was approved by the local ethical comittee. Fetal ages were calculated both

by considering the menstrual age and the crown-heel lengths. Specimens from the choroid plexus, liver, pancreas, oesophagus, stomach, duodenum, jejunum, ileum, colon, kidney, adrenal, spleen, thyroid, lung, thymus and heart were snap-frozen in liquid nitrogen and stored at −70° C for up to 2 weeks before being sectioned. The interval between the completion of abortion and freezing of tissues varied between 2 and 4 h. The frozen sections (4 μm) were stored at −20° C for up to 6 months before being immersed in 4% phosphate-buffered formaldehyde for 5 min for in situ hybridization or in Carnoy's fixative for 10 min for immuno-histochemical stainings (see below).

Pancreatic specimens from the five fetuses at mid-term were also fixed immediately after removal of the tissues in 4% phosphate-buffered formaldehyde for 24 h prior to paraffin-embedding.

One fetus with a gestational age of 8 weeks, obtained after surgery for an extrauterine pregnancy, was also included in the study. The fetus was formaldehyde-fixed and paraffin-embedded in toto as described above for pancreatic specimens.

All fetuses were free from disease and the tissues showed no pathological changes in haematoxylin/eosin-stained control sections.

For the in situ hybridization procedure a 500 base pair cDNA insert (specifying an almost full-length human TTR-mRNA), cloned into SP 65 plasmids at the ECO R1 site (Soprano et al. 1985), was used. The linearized template DNA was transcribed to produce sense or antisense ^{35}S-labelled RNA sequences with a specific activity of approximately 10^9 cpm/μg (Jacobsson 1989a).

The formaldehyde-fixed frozen sections were pre-treated, hybridized and post-treated as previously described for deparaffinized sections (Jacobsson 1989a) with the exception of the pre-treatment steps no. 3–5 (see below), which were omitted.

The pre-treatment steps for deparaffinized sections included: (1) Acetylation in acetic anhydride/triethanol amine, pH 8.0 (Hayashi et al. 1978) (2) Immersion in 0.1 M Tris-HCl, pH 7.0/0.1 M glycine for 30 min (3) Treatment with 0.2 M HCl for 20 min (4) Immersion in 2 × SSC for 30 min at 50° C (5) Incubation with proteinase K (Boehringer, Mannheim, Germany) 1 μg/ml for 30 min at 37° C (6) Dehydration in ethanol and air-drying. Brief washings in 2 × SSC were performed between each of the different steps described above. The hybridization mixture contained ^{35}S-labelled RNA probe (10^8 cpm/ml), 50% deionized formamide, 0.6 M NaCl, 50 mM Na phosphate buffer, pH 7, 0.05% herring sperm DNA (Type IV, Sigma), 0.05% yeast total RNA (type III, Sigma), 0.005% yeast tRNA (Sigma), 0.005% polyadenylic acid (Sigma), 1 mM EDTA, 0.02% Ficoll (mw 400000, Sigma), 0.02% polyvinyl pyrrolidine (mw 40000, Sigma), 0.02% bovine albumin (Sigma) and 10–100 mM dithiothreitol. The mixture, heated at 90° C for 10 min, was applied to the sections (10 μl/18 × 18 mm cover slip) and the hybridization was carried out at 50° C for 3 h as recommended by Cox et al. (1984).

The post-treatment steps included: (1) Rinsing in 50% formamide/2 × SSC at 52° C for 30 min. (2) Treatment with RNase A (100 μg/ml, Sigma) and RNAse T (1 μg/ml, Sigma) in 2 × SSC for 30 min at 37° C. (3) Rinsing in 50% formamide/2 × SSC at 52° C for 5 min. (4) Dehydration in ethanol and air-drying. The sections, coated with G5 emulsion (Ilford, Mobberley, England), were developed after 5 days in G150 and fixed in G334 (Agfa-Gevaert). After light counterstaining in hematoxylin, the sections were dehydrated and mounted.

Consecutive deparaffinized sections (4 μm) from the formaldehyde-fixed pancreatic specimens were prepared in order to compare the distribution of TTR-mRNA as revealed by the in situ hybridization procedure with that of TTR-IR as described below.

An indirect immunofluorescence (IF) or immunoperoxidase (IP) method was used on the Carnoy-fixed frozen sections or the deparaffinized formaldehyde-fixed sections, respectively (Jacobsson et al. 1989c). Briefly, the sections were pre-treated with 1% hydrogen peroxide (IP-method only) followed by 5% albumin prior to the application of a monoclonal antibody to TTR (MAb, clone 25) (Collins et al. 1986) with overnight incubation at room temperature. A fluorescein- or peroxidase-conjugated rabbit antibody to mouse IgG (DACO, Santa Barbara, USA) was applied for 30 min and diaminobenzidine was employed as the chromogen in the IP-method. Substitutions of the MAb with buffer and non-immune mouse serum were included as controls. Sections from the choroid plexus, liver and pancreas of one mid-term fetus were included in each run of immunohistochemical staining or in situ hybridization to provide an internal standard in the semiquantitative grading of the results from different experiments.

Results

In situ hybridization, to determine the localization of TTR-mRNA, was used on formaldehyde-fixed frozen sections (Table 1) and, in some cases (see below), on sections from formaldehyde-fixed and paraffin-embedded specimens. The sensitivity was found to be higher when the in situ method was used on frozen sections as compared to that obtained on deparaffinized sections. The specificity of the method was shown by obtaining autoradiographic labelling only with the anti-sense probe (complementary to human TTR-mRNA) and not with the control sense probe.

In the deparaffinized whole-body sections from the fetus at 8 weeks of gestation, a strong specific labelling of epithelial cells of the primitive choroid plexus was demonstrated (Fig. 1), whereas weak labelling was observed in hepatocytes. Only background level of grains was seen in other parts of

Table 1. The degree of in situ labelling for TTR-mRNA and of TTR immunoreactivity (IR) in different organs from the five human fetuses at mid-term (16–20 weeks)[a]

	Choroid plexus epithelial cells	liver hepatocytes	pancreas A-cells	gut endocrine cells	kidney tubular cells
TTR-mRNA	++++	+	++	−	−
TTR-IR	++++	+/−	++++	+++/−	++/−

[a] ++++ very strong, +++ strong, ++ moderate, + weak, − negative

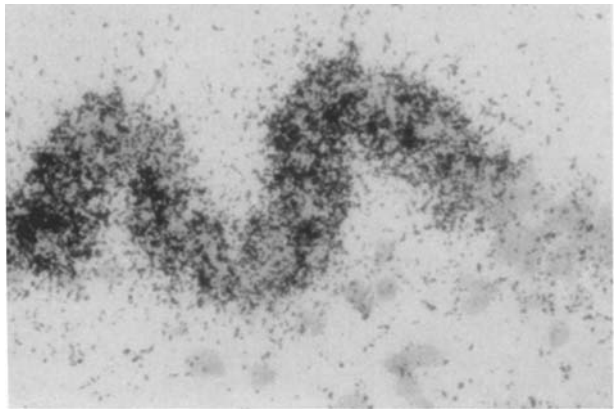

Fig. 1. In situ demonstration of TTR-mRNA in the primitive choroid plexus of the human fetal brain (lateral ventricle) at 8 weeks of gestation. A ^{35}S-labelled, single-stranded anti-sense RNA probe was hybridized to a section (4 μm) from a formaldehyde-fixed and paraffin-embedded specimen. Note the intense labelling of the epithelial cells (300 ×)

the body including the pancreas, gut, kidney and heart. The sections did not, however, include the yolk sac.

All fetuses at mid-term (Table 1) also demonstrated an intense specific labelling for TTR-mRNA in the epithelial cells of the choroid plexus and a weak specific labelling in almost all hepatocytes. Only background grains were observed in the brain adjacent to the choroid plexus and in the hematopoietic cells of the liver. In addition to the findings in the 8 week fetus, all mid-term fetuses exhibited TTR-mRNA in pancreatic endocrine cells (mainly A-cells, see below) (Fig. 2a) and the intensity of labelling per A-cell was found to be intermediate between that of choroid plexus epithelial cells and hepatocytes (Table I). No specific signal could be detected in the different parts of the gut nor in the kidney, adrenal, spleen, thyroid, lung, thymus and heart. In 2 of the 5 mid-term fetuses, a few cells of tissue macrophage appearance in the spleen, thymus and small bowel were labelled both by the sense and the anti-sense probe, hence the labelling was considered non-specific.

Indirect immunofluorescence or immunoperoxidase techniques, to determine the localization of the TTR protein, were applied to frozen or deparaffinized sections, respectively. Both techniques were found to be equally sensitive in localizing TTR.

The 8 week fetus demonstrated strong TTR-IR in the epithelial cells of the developing choroid plexus, whereas hepatocytes were only weakly TTR immunoreactive. TTR-IR of moderate degree was also seen in tubular epithelial cells of the developing kidney, despite the fact that this organ was

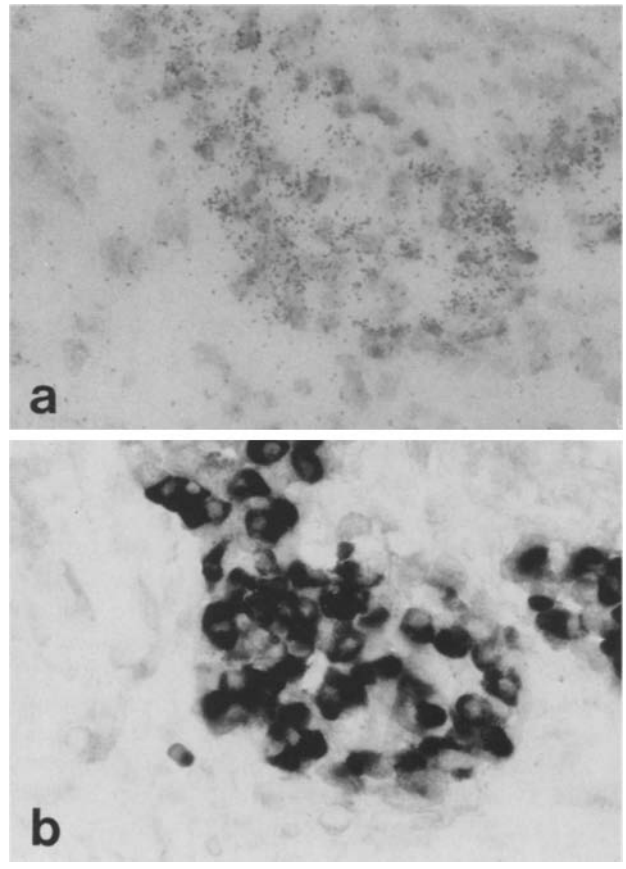

Fig. 2. In situ demonstration of TTR-mRNA and of TTR immunoreactivity (TTR-IR) in the human fetal pancreas at mid-term. Consecutive sections (4 μm) from a formaldehyde-fixed and paraffin-embedded specimen showing that both the specific labelling of TTR-mRNA **a** and the TTR-IR **b** are confined to islet(A)-cells. **a** In situ hybridization with a ^{35}S-labelled, single-stranded anti-sense RNA probe. **b** Immunostaining with a monoclonal antibody to TTR followed by a peroxidase-conjugated antibody to mouse IgG and diaminobenzidine as the chromogen (300 ×)

negative for TTR-mRNA as judged by the in situ method. No TTR immunoreactive cells were found in the pancreas, gut or heart.

All mid-term fetuses (Table 1) demonstrated strong TTR-IR in the epithelial cells of the choroid plexus, whereas adjacent parts of the brain were TTR-negative. Hepatocytes were TTR immunoreactive in only 3 of the 5 cases and then to a slight degree. A strong TTR-IR, comparable to that of choroid plexus epithelial cells, was seen in pancreatic islet cells (Fig. 2b) as well as in a few single cells and small cell clusters dispersed in the exocrine parenchyma. These TTR positive cells of the pancreas have previously been shown to represent glucagon-containing A-cells (Gray et al. 1985). Comparison of paired serial deparaffinized sections showed coexistence of TTR-mRNA and its

corresponding protein in these A-cells (Fig. 2). Despite there being no evidence for TTR-mRNA in the gut and kidney, some mid-term fetuses demonstrated a few TTR immunoreactive cells of endocrine type in the gastric (1 of the 5 fetuses), duodenal (2/5), jejunal (3/5) and the ileal (1/5) mucosa as well as TTR-IR in epithelial cells of the renal proximal convoluted tubules (2/5). No TTR positive cells were observed in sections from the oesophagus, colon, adrenal, spleen, thyroid, lung, thymus or heart. TTR-IR was, however, seen in serum inside blood vessels and, in some specimens, also in interstitial fluid.

Discussion

The present data clearly indicate TTR synthesis in the primitive choroid plexus and liver as early as the 8th week of gestation. The data also indicate TTR synthesis in the choroid plexus and liver as well as in the pancreas of the human fetus at mid-term. Although not included in the present study, the yolk sac is a further site for TTR synthesis in the early stages of fetal development (Gitlin and Gitlin 1975; Soprano et al. 1986).

The choroid plexus epithelial cells demonstrated intense labelling for TTR-mRNA as well as strong TTR-IR. This was in contrast to hepatocytes, which showed weak labelling for TTR-mRNA and weak or non-demonstrable TTR-IR. This relationship between the two cell types (TTR-mRNA and TTR in choroid plexus epithelial cells $\gg$ TTR-mRNA and TTR in hepatocytes) remained the same throughout the human gestational weeks 8–20 and a similar relationship between the corresponding cell types has previously been observed in adult humans (Jacobsson 1989a). The result obtained for the human choroid plexus and liver are in agreement with findings in the rat (Thomas et al. 1988; Fung et al. 1988) and mouse (Wakasugi et al. 1986; Murakami et al. 1987) – the animal models mainly used in TTR studies.

The present data also clearly indicate that TTR synthesis occurs in the human fetal endocrine pancreas although it begins at a later stage in fetal development than in the choroid plexus and liver.

Gray et al. (1985) have previously shown TTR-IR in the secretory granules of the pancreatic A-cells, thus suggesting TTR synthesis. The present demonstration of TTR-mRNA in these cells provides proof of such synthesis. In a similar manner the A-cells of adult human pancreas have also been shown to synthesize TTR (Jacobsson 1989a). It is notable that the presence of TTR-mRNA in the endocrine pancreas has not previously been reported in the rat (Soprano et al. 1985) nor in the mouse (Murakami et al. 1987).

The presence of TTR-IR in endocrine cells of the gut as well as in renal tubular epithelial cells are in agreement with results obtained in previous human studies (Jörnvall et al. 1981; Gray et al. 1985). The failure to detect TTR-mRNA in these cells could be due to the in situ method not being sufficiently sensitive to detect very small amounts of TTR-mRNA per cell. This hypothesis is supported by the findings in the mouse, which showed the presence of TTR-mRNA by blotting analysis of renal tissue homogenates (Wakasugi et al. 1986), whereas no specific signal for TTR-mRNA was obtained in kidney sections by in situ hybridization (Murakami et al. 1987). Gray et al. (1985), on the other hand, have shown that the TTR-IR in renal tubular epithelial cells is located in large, polymorphic vacuoles in the cytoplasm at the EM level and these vacuoles are thought to contain protein reabsorbed from the tubular lumen. Further studies are thus required to demonstrate TTR synthesis in the human gut and kidney.

During the preparation of this manuscript, evidence for TTR synthesis in the adult rat and bovine eye was reported (Martone et al. 1988). Although the eyes of the mid-term fetuses were not included in the present study, neither TTR-mRNA nor TTR could be detected in the eye of the 8 week fetus.

The TTR synthesized by the liver has an important role in the transport and distribution of thyroxine and retinol. The TTR derived from the choroid plexus seems to be mainly involved in the transfer of thyroxine from the bloodstream to the cerebrospinal fluid and in the distribution of thyroxine to the brain (Dickson et al. 1987). The TTR derived from the yolk sac may be involved in the transfer of thyroxine and retinol from the maternal blood supply to the fetus in the early stages of development (Soprano et al. 1986). The functional role for the TTR synthesized by the endocrine pancreas is presently unknown. A relationship to gastro-intestinal prohormones has been suggested (Jörnvall et al. 1981) and a thymic hormone-like activity intrinsic to the TTR molecule has recently been demonstrated (Burton et al. 1987). TTR may also have a role in the packaging and sorting of regulatory peptides and/or of biogenic amines or function as a carrier for such substances. Studies along these lines are in progress.

In summary, evidence for TTR synthesis in the human fetal choroid plexus, liver and endocrine pancreas has been presented. However, further stu-

dies are needed to show that TTR synthesis occurs in the human gut and kidney.

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