# Ontogeny of Cells Containing Insulin, Glucagon, Pancreatic Polypeptide Hormone and Somatostatin in the Bovine Pancreas

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#### Abstract

Antibodies to insulin, glucagon, pancreatic polypeptide hormone (PP) and somatostatin were used in the immunofluorescence histochemical procedure to study the ontogeny of pancreatic endocrine cells containing the four hormones in the bovine fetus of approximately 100 days gestation to term. Pancreatic sections from the bovine neonate and adult were also examined for the cellular distribution of the four hormones. Immunoreactive cells staining for insulin, glucagon, PP and somatostatin were present in the pancreas of all fetuses studied. Each endocrine cell type displayed a characteristic distribution within the developing pancreas and in the neonate and adult. The presence of the four islet hormones relatively early in bovine fetal life suggests that they may be important in intra- and extra-islet metabolism in the fetus.

### Introduction

It is now recognized that insulin, glucagon, pancreatic polypeptide hormone (PP) and somatostatin have widespread distribution in mammals, with the pancreas being the major site of production of the first three hormones. Glucagon, PP and somatostatin have also been demonstrated in cells of extra-pancreatic tissue such as those of the gastrointestinal tract (Hokfelt *et al.* 1975; Larsson *et al.* 1976; Leduque *et al.* 1982) and more recently insulin-like material has been reported to be present in the brain (Baskin *et al.* 1983; Birch *et al.* 1984). A number of biologically active peptides previously found in the hypothalamus and other regions of the brain have now been shown to exist in the pancreatic islet. These include thyrotrophin releasing factor (TRF),  $\beta$ -endorphin and vasoactive intestinal peptide (Said 1980; Watkins *et al.* 1980; Koivusalo *et al.* 1981). The physiological significance of these recently discovered pancreatic peptides is, however, still unclear.

Ontogenic studies of pancreatic insulin, glucagon, PP and somatostatin have been carried out for the human and rat (Orci *et al.* 1969; Assan and Boillot 1973; Dubois *et al.* 1975; Alumets *et al.* 1977; Sundler *et al.* 1977; Paulin and Dubois 1978; Stefan *et al.* 1983). More recently, Alumets *et al.* (1983) have reported on the ontogeny of several pancreatic and gut peptides in the fetal pig by immunocytochemistry. In ruminant species similar ontogenic studies have been lacking. In the fetal sheep, concentrations of PP in pancreatic tissue and serum have been measured during pregnancy by radioimmunoassay (Shulkes and Hardy 1982) and we have recently reported on the cellular localization of PP and somatostatin in the adult and fetal pancreas of late gestation (Reddy and Elliott 1984; Reddy *et al.* 1984).

In the bovine fetus, neither the cellular localization of the four major pancreatic hormones nor their concentrations in serum and pancreas at different stages of pregnancy have been reported previously. In the present investigation we report on the ontogeny of insulin, glucagon, PP and somatostatin cells in the bovine fetal pancreas from approximately 100 days of gestation until term as assessed by the immunofluorescence histochemical procedure. The distribution of the endocrine cells in the pancreas of the neonate and adult animal is also examined and compared with that in the fetus.

### **Materials and Methods**

Almost the entire pancreas was removed from 16 fetal calves of various gestational ages immediately after the pregnant cows were killed at the Auckland City Council Abattoirs. Estimates of gestation were obtained by measurement of crown to rump lengths of the fetuses (Swett *et al.* 1948) and ranged from 17-20 cm at 90-120 days to 90-96 cm at term:

Crown-rump length (cm):	17, 20	31, 33	44, 46, 47	56, 58, 59	76, 78	90, 90, 95, 96
Gestational age (days):	90-120	121-150	151-180	181-210	211-240	270-term

Portions of pancreas from the splenic region were also obtained from four neonatal calves (3-7 days old) and four adult cattle from the same abattoirs. The glands were excised into approximately 0.5 by 0.5 by 0.5 cm pieces and fixed in Bouin's fluid for 24 h at room temperature. Tissues were then dehydrated through increasing concentrations of ethanol, cleared in xylol and embedded in paraffin (56°C). Sections (5  $\mu$ m) were cut and prepared for histological examination.

As controls, pieces of pancreas from normal Wistar rats and diabetic BB Wistar rats were also similarly processed for histology.

Guinea pig anti-insulin serum was prepared by Dr J. R. Crossley (Auckland, New Zealand) according to the procedure of Robinson and Wright (1961). The antigen was bovine insulin from Sigma, St Louis, U.S.A. Rabbit anti-bovine pancreatic polypeptide (anti-BPP) serum and highly purified BPP antigen were kindly supplied by Dr R. E. Chance (Lilly Research Laboratories, Indianapolis, U.S.A.). Antibodies to porcine glucagon (Eli Lilly & Co., Indianapolis, U.S.A.) coupled to bovine serum albumin were raised in rabbits according to the procedure of Holst and Aasted (1974) and were supplied by Dr J. E. Livesey (Christchurch, New Zealand). This antiserum showed only minimal cross-reaction with glicentin (J. E. Livesey and C. Redekopp, personal communication).

Somatostatin was obtained from Bachem (California, U.S.A.) and rabbit anti-somatostatin serum was prepared by Dr J. R. Oliver (Adelaide, South Australia) and characterized as reported previously (Buckerfield *et al.* 1981). The indirect immunofluorescence procedure was carried out as described by Nairn (1969). Bovine and normal rat pancreatic sections were incubated with anti-insulin, anti-glucagon, anti-BPP or anti-somatostatin sera at a dilution of 1 : 40 at  $37^{\circ}$ C for 1 h with phosphate-buffered saline (PBS) as the diluent. After the sections were washed in excess PBS they were incubated with either sheep anti-rabbit  $\gamma$ -globulin or sheep anti-guinea pig  $\gamma$ -globulin labelled with fluorescein isothiocyanate (Wellcome Laboratories, U.K.) at a dilution of 1 : 10 in PBS for 30 min at  $37^{\circ}$ C. After further washing, sections were mounted in glycerol-saline and the immunofluorescence observed with a Nikon fluorescence microscope. Following immunofluorescence examination sections were stained with haematoxylin and eosin (H and E).

Pancreatic sections from diabetic BB rats were stained for insulin by the immunofluorescence procedure described above.

The specificity of the immunoreaction was tested by replacing the primary antisera with either normal rabbit or guinea pig serum, PBS, or application of the antisera to tissue sections after absorption with the corresponding antigens (40–50  $\mu$ g per millilitre of undiluted antiserum) in the immunofluorescence procedure.

# Results

Immunofluorescent cells staining for insulin, glucagon, PP and somatostatin were observed in all bovine pancreatic tissues studied. In the fetal tissue, insulin

**Figs 1-5.** Representative photomicrographs to show the localization by immunofluorescence of insulin (1A-5A), glucagon (1B-5B), PP (1C-5C) and somatostatin (1D-5D) immunoreactive cells in the fetal pancreas of 90–120 days gestation (Fig. 1), 181–210 days gestation (Fig. 2), 270 days gestation to term (Fig. 3), in the neonatal pancreas 3–7 days old (Fig. 4) and in the adult pancreas (Fig. 5). The scale shown in Fig. 5D = 75  $\mu$ m and is applicable to all photomicrographs.



immunoreactive cells were arranged in clusters of 3-4 to 30-40 cells within the developing islets (Figs 1A, 2A, 3A). The distribution of insulin cells was essentially similar in fetuses ranging from 90 to 240 days of gestation. In fetuses close to term, the neonate, and in the adult animal, insulin immunoreactive cells comprised the major islet population (Figs 3A, 4A, 5A). Glucagon immunostained cells were located in the periphery of the fetal islets (Figs 1B, 2B, 3B). However, not all peripheral cells in the fetal islet showed immunoreaction to anti-glucagon serum. In the neonatal and adult pancreas glucagon cells were observed both in the islet periphery and internally (Figs 4B, 5B). These cells were less numerous than in the fetus. In the fetal and neonatal pancreas PP-stained cells were observed usually either singly or in groups of 2–3 cells within a few islets only and were less prominent than those staining for insulin, glucagon or somatostatin (Figs 1C, 2C, 3C, 4C). In the adult PP cells showed bright staining and usually occupied a dense region of the islet (Fig. 5C). Somatostatin immunoreactive cells in the fetus and the neonate were located predominantly in the islet periphery whereas in the adult these cells were observed both in the periphery and within the islet in a ribbon-like pattern (Figs 1D, 2D, 3D, 4D, 5D).

In the normal rat pancreas insulin-immunostained cells occupied the central region of the islet, whereas glucagon, PP and somatostatin were located in cells of the islet periphery (results not shown). These observations were similar to those reported previously by others (Erlandsen *et al.* 1976; Orci *et al.* 1976). No immunofluorescent staining was observed when pancreatic sections obtained from a diabetic BB rat were stained for insulin by the immunofluorescence procedure (Patel *et al.* 1983).

Immunoreactive cells were absent from pancreatic sections of the bovine and normal Wistar rat when the four primary antisera were replaced by normal rabbit or guinea pig serum, PBS, or antigen-absorbed antisera in the immunohistochemical procedure.

# Discussion

In the past, ontogenic studies on pancreatic insulin, glucagon, PP and somatostatin have been confined to the rat (Yoshinari and Daikoku 1982), human (Leduque et al. 1982; Stefan et al. 1983), and pig (Alumets et al. 1983). The present study on the fetal pancreas from the bovine has demonstrated that endocrine cells containing insulin, glucagon, PP and somatostatin appear at least as early as 100 days of gestation. The distribution of insulin-immunoreactive cells observed was similar to that reported for the pig, rat and human of comparable gestational ages (Yoshinari and Daikoku 1982; Alumets et al. 1983; Stefan et al. 1983). In the less mature pancreas of other mammals examined, for example the pig, insulin cells have been reported to be distributed either singly or in small clumps within the parenchyma of the developing pancreas (Alumets et al. 1983). Whether this is also true in the bovine pancreas of less than 100 days of gestation is being investigated. The distribution of glucagon immunoreactive cells in the fetal pancreas of greater than 100 days observed in this study was almost entirely in the islet periphery and is similar to that in other species examined, for example, the human (Leduque et al. 1982). In the neonate and adult, glucagon immunoreactive cells were also observed within the islet. Cells staining for PP were far less numerous in the bovine fetus and neonate than in the fetal sheep close to term and the human of greater than 21 weeks of gestation (Stefan et al. 1983; Reddy and Elliott 1984). Our findings suggest that in the bovine fetus and neonate the pancreatic content of PP may be lower than insulin, glucagon and somatostatin. The distribution of somatostatin immunoreactive cells in the islet periphery observed in the fetus and neonate is similar to that reported in the adult rat (Orci 1982). In the pancreas of the adult, however, somatostatin cells were observed in the periphery and within the islet, similar to that reported in the sheep (Reddy *et al.* 1984).

It is now recognized that insulin, glucagon, PP and somatostatin are stored in distinct cell types within the pancreatic islet of mammals. Using immunohistochemical procedures  $\beta$ -endorphin immunoreaction has been observed in the D cell of the rat, guinea pig, and human pancreas (Watkins *et al.* 1980). More recently, Tung and Cockburn (1984) have detected  $\beta$ -endorphin-like immunoreactivity in pancreatic extracts of the bovine fetus by radioimmunoassay. In the fetal rat, glucagon cells have been also shown to contain peptide YY (Ali-Rachedi *et al.* 1984). The cellular distribution of the latter peptides in the bovine pancreas requires examination.

In the human and pig, the presence of insulin, glucagon, PP and somatostatin immunoreactive cells have been demonstrated early in fetal life (Alumets *et al.* 1983; Stefan *et al.* 1983). In the fetal pig, for example, pancreatic cells staining for the four hormones were observed in the fourth week of pregnancy (Alumets *et al.* 1983). In the rat, however, insulin, glucagon and somatostatin cells appear on day 11, 12, and 15 respectively, but the PP cells do not appear until just after birth (Sundler *et al.* 1977; Yoshinari and Daikoku 1982). In the fetal pancreas of the human glucagon-immunoreactive cells are preceded by cells containing glicentin which finally disappear in later stages and are replaced by the adult-type glucagon cells (Stefan *et al.* 1983). Such ontogenic changes have yet to be examined in the bovine fetal pancreas.

The presence of insulin, glucagon, PP and somatostatin relatively early in fetal life implies metabolic roles for the hormones. Studies in the experimental rat have shown the inhibitory effect of exogenously administered somatostatin on the release of pancreatic insulin and glucagon (Tannenbaum *et al.* 1982) and it is possible that somatostatin from bovine islet cells could fulfil this role in a 'paracrine' manner. Others have shown trophic effects of insulin and PP on acinar tissue (Greenberg *et al.* 1977; Henderson *et al.* 1981). These reported intra- and extra-islet functions of pancreatic hormones in the adult may also be important in the bovine fetus.

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