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Netrin-1 expression in fetal and regenerating rat pancreas and its effect on the migration of human pancreatic duct and porcine islet precursor cells

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Abstract

Aims/hypothesis. We investigated the expression and function of netrin-1, a diffusible laminin-like protein known to regulate neuronal-cell migration in the pancreas. We questioned whether this factor regulates migration of pancreatic epithelial cells and whether this could be involved in islet neogenesis.
Methods. We studied fetal and adult rat pancreas wherein duct ligation induced islet neogenesis. Netrin-1 expression was analysed by RT-PCR, western blot and immunohistochemistry. In vitro cell migration was measured with a human pancreatic duct cell line (CAPAN-2) and with fetal porcine islet cells. We also studied the expression of two netrin-receptors, neogenin and deleted in colorectal cancer.
Results. We found a transient expression of netrin-1 mRNA and protein in fetal pancreas from E15 to E18, and in adult pancreas after duct ligation. In normal adult pancreas there was very little netrin-1 expression. Netrin-1 expression was observed both in endocrine and exocrine cells. At the immunohistochemical level, it was expressed by islet cells during tissue regeneration. We could show that netrin-1 increases the migration of fetal islet cells and of a ductal cell line, mainly via a chemokinetic effect. From the two well-established netrin receptors, DCC and neogenin, we only found neogenin to be expressed in the pancreas. Neogenin expression coincided with the period of netrin-1 up-regulation.
Conclusion/interpretation. Netrin-1 is involved in pancreatic morphogenesis and tissue remodelling and plays a role in the regulation of duct-cell and fetal-islet cell migration. This can be of importance in islet regeneration, where migration of islet precursors takes place. [Diabetologia (2003) 46:926–933]

Keywords Netrin, pancreas morphogenesis, cell migration, islet neogenesis, stem cells.

When injury is induced in the pancreas, for instance by obstructing exocrine ducts, tissue remodelling occurs and this is characterized by acinoductal metaplasia of the exocrine tissue, increase in mesenchyme, and hyperplasia of the endocrine cells as a result of islet neogenesis [1, 2]. One of the mechanisms that are involved in islet morphogenesis, both in the fetus and during regeneration, is the migration of cells from exocrine to endocrine tissue. The migratory capacity of pancreatic epithelial cells and its molecular regulation has not been well documented. Nevertheless, molecules have been identified which are involved in regulating the adhesiveness of cells in the fetal pancreas [3] and in the degradation of the extracellular matrix [4, 5], which are functions necessary for cell migration.

We have focussed on the expression and possible role of netrin-1 in pancreas regeneration using the model of pancreatic duct ligation (PDL) [1, 2]. Netrins

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Abbreviations: CNS, Central nervous system; DCC, deleted in colorectal cancer; FPIC, fetal porcine islet cells; PDL, pancreatic duct ligation.

are laminin-like diffusible chemotactic proteins which have been shown to act as guidance cues for developing axons and migrating neuroblasts [6, 7]. In addition to having a guidance function, netrins also promote outgrowth of neurites [6, 8]. A possible role in neuronal response to injury has also been proposed [9]. Netrins continue to be expresssed in the adult vetrebrate CNS [10]. In the nervous system the glial cells [8] and the neurons are the source of netrin. Outside the nervous system netrin-1 has been localized in human osteoblasts, osteosarcoma cell lines and osteoclasts [11]. Northern blot analysis also showed expression of netrin-1 at various embryonic stages and in adults in several mesodermally- and endodermally-derived tissues, including heart, lung and ovary [12]. Netrin-1 is also involved in the formation of the semicircular cannals in the mouse inner ear where it is expressed in the otic epithelium [13]. Given that there exist cell biological and developmental similarities between neural cells and pancreatic islet cells [14], it seemed interesting to study the effect of neural guidance cues on pancreatic cells.

Material and methods

Animals and animal procedures. Adult male Wistar rats (Janvier, Le Genest-St-Isle, France) of 250 to 300 g body weight (12 weeks of age) were housed according to the guidelines of the Belgian Regulations for Animal Care. To induce pancreatic regeneration or tissue remodelling, rats were subjected to ligation of the exocrine ducts draining the splenic half of the pancreas, as described previously [1]. For the collection of fetal tissue, timed pregnant rats were killed at gestational times of 15, 18, 20 and 21 days (gestation =21–22 days). The experiments were approved by the Ethics Committee of the Free University of Brussels.

Culture of CAPAN-2. CAPAN-2, a human adenocarcinoma cell line [17] was obtained from American Type Culture Collection (Rockville, Md., USA). Cells were cultured in McCoy's 5A medium with glutamax-1 (Invitrogen, San Diego, Calif., USA), 1000 U/ml penicillin, 1000 µg/ml streptomycin (BioWhittaker, Verviers, Belgium) and 10% of fetal bovine serum (Invitrogen). When 80 to 90% confluence was reached cells were detached by trypsin/EDTA (0.05 % trypsin, 0.53 mmol/l EDTA) digestion and subcultured in a 1:2 ratio.

Isolation and culture of rat cells. Pancreas from adult rats was dissociated with collagenase and islets were isolated by collagenase digestion [18]. Exocrine cells were isolated and cultured as described [19].

Fetal porcine islet cells. Fetal porcine islet cells (FPIC) were obtained from Beta-Cell N.V. (Brussels, Belgium). The cells were prepared according to the method described in European patent application EP 1 146 117. In brief, Belgian Landrace pregnant sows of 108 to 114 days of gestation were used for the collection of fetal pancreases (University of Ghent Veterinary School, Merelbeke, Belgium). Pancreatic tissue was minced with scissors and dissociated for 30 min with collagenase-P (0.3 mg/ml; Roche, Basel, Switzerland) at room temperature. The suspension was filtered over a 100 µm mesh screen and the filtrate was pumped into a Beckman J6B elutriator centrifuge equipped with a JE10x rotor (Palo Alto, Calif., USA) at 1500 rpm and at a flow rate of 25 ml/min. By increasing the flow rate to 190 ml/min, a fraction was collected with a particle diameter of less than 15 µm. The elutriated fraction was then centrifuged for 20 min at 2500 rpm on a discontinuous two-layer Percoll gradient of 1.040 and 1.075 g/ml to remove contaminating exocrine cells. An endocrine-cell enriched preparation was collected at the interphase between both layers. This fraction contained between 30 and 50 million cells per pancreas with a viability of approximately 90%. The cells are composed of minimally 70% endocrine cells (30–50% insulin-positive, 15–45% glucagon-positive and 5–10% somatostatin-positive cells), 10 to 25% non-granulated cells and less than 5% acinar cells. They are considered as immature islet cells with a five-fold lower insulin content compared to mature beta cells, and with immunoreactivity for the ductal marker cytokeratin-7 [20]. Cells were cultured for 3 days in Ham F10 (Invitrogen) containing Albumax I BSA (Beta-Cell N.V.), 50 µmol/l IBMX (BioWhittaker), 5 mmol/l nicotinamide (BioWhittaker), 2 mmol/l L-glutamine (BioWhittaker), 1 µmol/l hydro-cortisone (Beta-Cell N.V.), 100 U/ml penicillin and 100 µg/ml streptomycin (BioWhittaker) before migration assay was carried out.

RT-PCR. Pancreatic total RNA was extracted with TRIzol (Invitrogen, Merelbeke, Belgium). cDNA was prepared by reverse transcription on 500 ng total RNA. PCR (n=3) was done with Platinum Taq DNA Polymerase (Invitrogen). The primer pairs for rat netrin-1 were: forward 5′-GCAAAGCCTGTGATTG-CCA-3′ and reverse 5′-CGCCTTCAGAATGTGGATCTG-3′. Those for rat neogenin were: forward 5′-CAATGCGACT-GAAGGAGACGA-3′ and reverse 5′-ATCTCCAAACTGCCT-CCTGCT-3′. Those for human neogenin were: forward 5′-AG-CCAACCAGAACCTTCCTCA-3′ and reverse 5′-CCTGAAG-CAACACATGGCAAC-3′ The primers for rat DCC were: forward 5′-ACATCCGACGTTCGGCTTT-3′ and reverse 5′-TG-ATTTTCCCATTGGC-TTCC-3′. The primers for rat B-actin were: forward 5′-ACTATCGGCAATGAGCGGTTC-3′ and reverse 5′-AGAGCCACCAATCCACACAGA-3′. The thermal cycle profile for neogenin, DCC and B-actin was as follows: denaturation at 94°C for 90 s, 10 cycli (denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min) followed by 20 cycli (denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min) and final extension at 72°C for 10 min. The thermal cycle profile for netrin-1 was as follows: denaturation at 94°C for 90 s, 33 cycli (denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min) and final extension at 72°C for 10 min. A 10 µl sample was loaded onto 1.2% agarose gel.

Immunohistochemical staining. For immunohistochemical demonstration of netrin-1 and neogenin, tissue was fixed in buffered 4% formaldehyde and embedded in paraffin. Sections of 4 µm thickness were treated with microwave (2×5 min in citrate bufer, pH 6.0) and immunostained with streptavidin-biotin method as described [15]. Rabbit anti-netrin-1 antibody (1/100) (Ab-1, Oncogene Research Products, San Diego, Calif., USA) and a biotinylated anti-rabbit antibody (1/300) (Amersham, Roosendaal, The Netherlands) were used respectively as first and secondary antibody for the staining of netrin-1. For the staining of neogenin we used a goat anti-neogenin antibody (1/100) (C20, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and a biotinylated anti-goat antibody (1/200) (Vector Laboratories, Burlingame, Calif., USA). The controls consisted in pre-absorbing the antiserum with excess of blocking peptide which resulted in complete absence of staining.

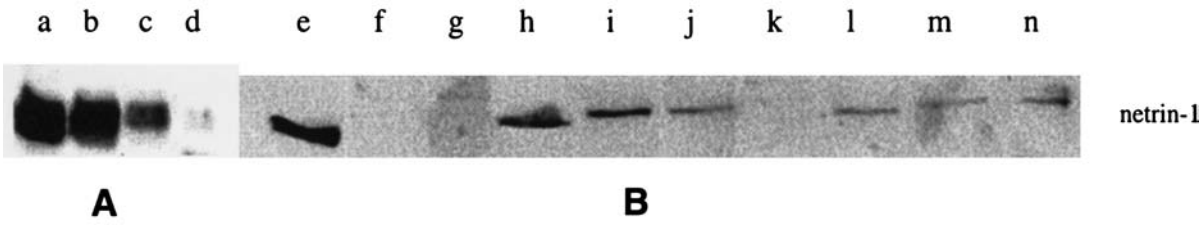

Fig. 1A, B. Western blot of netrin-1 expression in rat in embryonic (**A**) and duct-ligated pancreas (**B**) (different percentages of acrylamide were used in the gel for electrophoresis between **A** and **B**). **A** brain of an embryo of 15 days (a), pancreas of an embryo of 20 days (b) and 21 days (c) and pancreas of a neonatal rat (d). **B** brain of an embryo of 15 days (e), part of the normal pancreas near the spleen (f), part of the normal pancreas near the duodenum (g), ligated part of the pancreas on day 7 after PDL (h), on day 5 after PDL (i) and day 3 after PDL (j), non-ligated part of the pancreas on day 7 after PDL (k), day 5 after PDL (l) and day 3 after PDL (m), and CAPAN-2 cell line (n)

Western blot. Protein extraction from pancreas tissue and immunoblotting (n=3) were done as described [16]. A sample of 50 µg was loaded on the gel. Anti-netrin-1 antibody (1/100) (Ab-1, Oncogene Research Products) and horseradish peroxidase-coupled anti-rabbit antibody (1/1000) (Amersham) were used respectively as first and secondary antibody. Fetal rat brain (E 15) was used as a positive control for netrin-1.

Migration assay. In vitro cell migration was measured by a micropore migration assay. For this assay, cells suspended in 200 µl medium are added to a transwell chambre (diameter of 6.5 mm) inserted in a well of a 24-well plate. The bottom of the insert is composed of a polycarbonate membrane with 8 µm pore size (Costar, Corning, N.Y., USA). Control medium or medium with 10 µg/ml netrin-1 (R&D Systems, Abingdon, UK) was added to the wells. Cells (1×10^6 CAPAN-2 or 5×10^5 FPIC) were incubated overnight at 37°C in 5% CO_2 atmosphere. After removing the transwell, cell migration was quantitated by counting the number of cells that had migrated from the insert into the well in ten microscopic fields at a magnification of 320× under phase contrast [21].

Statistical analysis. The results are given as means ± SD. Statistical significance was assessed with Student's t test for paired data (p value <0.05).

Results

Expression of netrin-1 in rat pancreas. Netrin-1 expression was analysed by western blot and RT-PCR and these analyses were repeated at least three times independently and were found to give very consistent results. Because it is known that netrin-1 is expressed in fetal brain, we used fetal-rat brain at E15 as a positive control. In western blots, netrin-1 antiserum detected a protein band of approximately M_r 78 in fetal brain. In fetal pancreas, the same netrin-1 band was present at E20, very faintly at E21 and it was absent in neonatal pancreas (Fig. 1). In adult pancreas, netrin-1 protein remained undetectable (Fig. 1). After duct

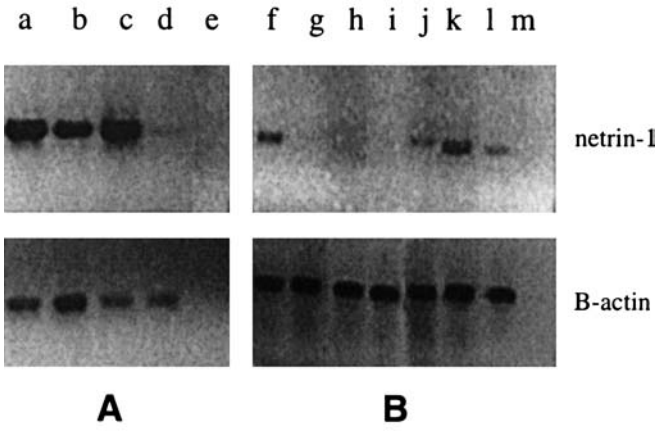

Fig. 2A, B. RT-PCR of netrin-1 expression in rat in embryonic (**A**) and duct-ligated pancreas (**B**). **A** brain of an embryo of 15 days (a), pancreas of an embryo of 15 days (b), 18 days (c), 20 days (d) and water (e). **B** islets of a normal pancreas (f), non-ligated part of the pancreas on day 3 after PDL (g), on day 5 after PDL (h) and day 7 after PDL (i), ligated part of the panceas on day 3 after PDL (j), day 5 after PDL (k) and day 7 after PDL (l) and water (m)

ligation, netrin-1 protein re-appeared and was observed on day 3, 5 and 7 post-ligation in the ligated part of the pancreas. However, there was also netrin-1 protein in the head part after 3 and 5 days although the bands were less dense than in the tail part (Fig. 1). The pancreatic tail represents the ligated part where tissue remodelling occurs, whereas the head represents the unligated part of the tissue. However, histological inspection showed that there occurs some tissue remodelling also in part of the head, namely close to the ligation site. Therefore, we harvested parts from both ends of the pancreas more distantly from the ligation site and excluded the middle part. This more stringent separation of head and tail parts was followed by RT-PCR analysis of netrin-1 expression. RT-PCR confirmed that netrin-1 was expresssed in fetal brain (E15) and fetal pancreas (E15-E20), with a very low expression at the end of gestation (E20) (Fig. 2). In adult pancreas, netrin-1 mRNA was practically undetectable. After duct ligation, its expression was very low in the head part of the pancreas whereas it was strongly upregulated in the tail (=ligated) part at 3, 5 and 7 days post-ligation with the strongest expression at 5 days (Fig. 2).

To find out which tissues express netrin-1, we also carried out an RT-PCR analysis on islets and on exocrine epithelial cells that were isolated from adult rat

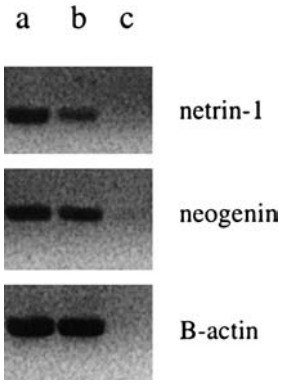

Fig. 3. RT-PCR of netrin-1 and neogenin expression in rat: monolayer of exocrine epithelial pancreatic cells (a), brain of an embryo of 15 days (b) and water (c)

pancreas. Netrin-1 mRNA was present in both types of tissue, indicating that the different types of pancreatic epithelial cells can synthesize netrin-1 (Fig. 2, Fig. 3). Netrin-1 protein was also detected by western blot in the human pancreatic duct cell line CAPAN-2 (Fig. 1).

Immunohistochemistry showed netrin-1 immunoreactivity in all islets of the ligated pancreas, whereas unligated tissue and control pancreas tissue were completely negative (Fig. 4). The immunoreactive islet cells were mainly insulin-containing cells. There was a certain heterogeneity in staining intensity with most intense netrin-1 staining being preferentially seen in irregularly shaped islets, or parts of islets, which likely represent newly generated ones. In fetal pancreas, we observed weak immunoreactivity for netrin-1 which was mainly seen in the exocrine cells.

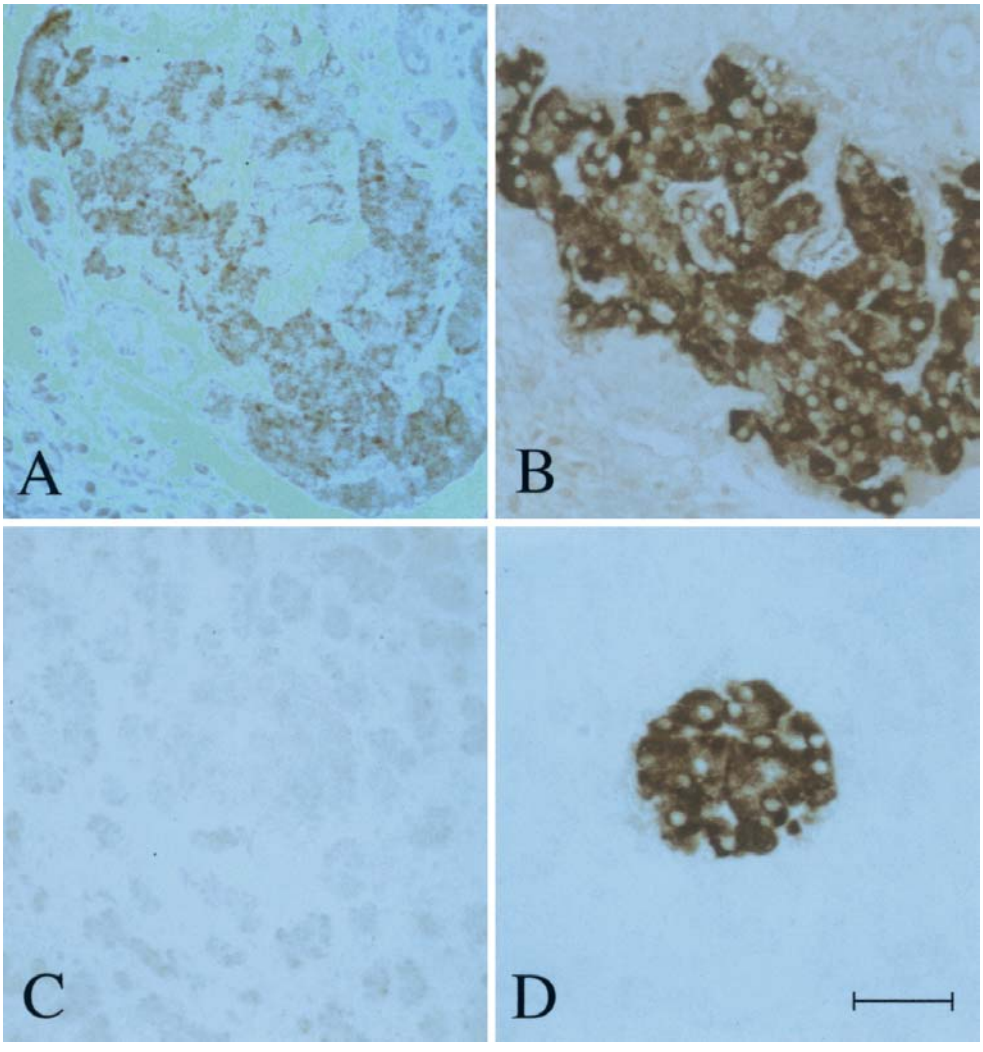

Fig. 4A–D. Immunohistochemical staining of netrin-1 (**A**, **C**) and insulin (**B**, **D**). A and B show the same islet in serial sections from the duct-ligated part, 7 days after duct ligation. **C** and **D** show the same islet in serial sections from the unligated part of the same pancreas. Note that netrin-1 immunoreactivity is detected only in the islet from the ligated part (**A**, **B**), whereas the islet in the unligated part is negative (**C**, **D**). Bar=20 μm

Table 1. Migration assay of FPIC and CAPAN-2: mean of migrated cells/field ± SD and significance with *n* the number of experiments

	Control (cells/field)	Netrin-1 (cells/field)	significance
FPIC (*n*=7)	4.3±1.9	10.4±4.1	0.001
CAPAN-2 (*n*=11)	14.8±8.1	20.9±11.03	0.015

Effect of netrin-1 on epithelial cell migration. Based on observations concerning the function of netrin-1 in regulating the migration of neural cells in vertebrates [6] and of the *C. elegans* netrin homologue UNC-6 in the migration of axons and mesodermal cells [22], we tested whether netrin-1 can also affect the in vitro migration of pancreatic epithelial cells. A micropore migration assay was used with fetal porcine pancreatic cells, and CAPAN-2, a human duct cell line. At a 10 µg/ml concentration netrin-1 stimulated the migration of both fetal cells and the tumor-derived duct cells (Table 1). Addition of netrin-1 to the upper compartment of the Transwell system led to a comparable increase in cell migration, suggesting that the major effect of netrin-1 on cell migration was chemokinetic. Thus, netrin-1 stimulates the migration of fetal pancreatic islets cells and of an adult pancreatic epithelial cell line.

Expression of neogenin and DCC in rat pancreas and CAPAN-2. We wondered whether the established netrin-receptors DCC and neogenin are expressed in the pancreas. By RT-PCR, we found that there was a strong expression of neogenin in fetal brain (E15) and fetal pancreas at E15-E18 but thereafter decreased to the same level that was observed in the adult pancreas (Fig. 5). In adult pancreas, low levels of neogenin expression were found in head and tail regions of the normal pancreas, and there was a marked up-regulation in the ligated part during tissue remodelling (3–7 days post-ligation) (Fig. 5). The other netrin-receptor, DCC, was present in fetal brain but was undetectable in adult pancreas with or without ligation (Fig. 6) and in fetal pancreas. In the CAPAN-2 pancreatic duct cell line, neogenin mRNA was also expressed whereas DCC was not. Also, we found neogenin expression in primary exocrine epithelial cell cultures (Fig. 3) and in isolated islets.

Immunohistochemical staining revealed immunoreactivity for neogenin in the plasma membrane of islet cells and a weak cytoplasmic staining of duct cells from ligated pancreas (Fig. 7). We couldn't detect heterogeneity of staining in the islets. In unligated or control pancreas, immunostaining was absent in ducts but was still weakly present in islets.

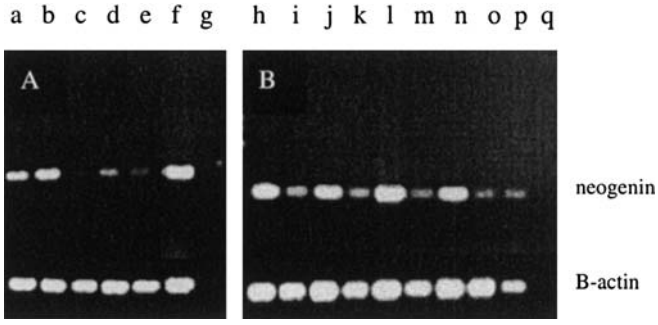

Fig. 5A, B. RT-PCR of neogenin expression in rat from embryonic (**A**) and duct-ligated (**B**) pancreas. **A** pancreas of an embryo of 15 days (a), 18 days (b), 20 days (c), 21 days (d), and birth (e), brain of an embryo of 15 days (f) and water (g). **B** Brain of an embryo of 15 days (h), non-ligated part of the pancreas on day 3 after PDL (i), day 5 after PDL (k), day 7 after PDL (m), ligated part of the pancreas on day 3 after PDL (j), day 5 after PDL (l), day 7 after PDL (n), part of the normal pancreas near the duodenum (o), part of the normal pancreas near the spleen (p), and water (q)

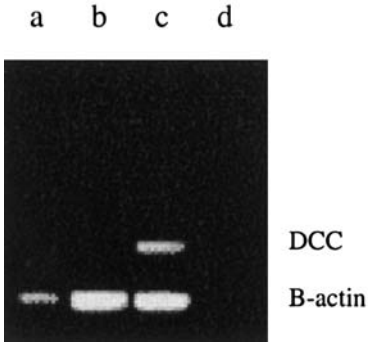

Fig. 6. RT-PCR result of DCC expression in the rat: non-ligated part of the pancreas on day 7 after PDL (a) ligated part of the pancreas on day 7 after PDL (b), brain of an embryo of 15 days (c) and water (d)

Discussion

We focussed on the expression of netrin-1 at the mRNA and protein level within the rat pancreas. We noted expression of netrin-1 in fetal pancreas during late gestation, which is the period of islet morphogenesis. In neonates and adults, netrin-1 protein was no longer detected but it re-appeared when islet neogenesis and tissue remodelling are induced by the procedure of duct ligation. This procedure is known to induce neogenesis of islets during the first week post-ligation [1, 2]. These observations suggest that netrin-1 plays a role in pancreatic morphogenesis, both prenatally and in the regenerating adult rat pancreas. We found netrin-1 mRNA to be expressed by islet cells, exocrine cells with ductal characteristics [2, 19] as well as by a human pancreas duct-cell line. Immunohistochemical staining revealed netrin-1 protein in islets from regenerating pancreas, whereas normal islets

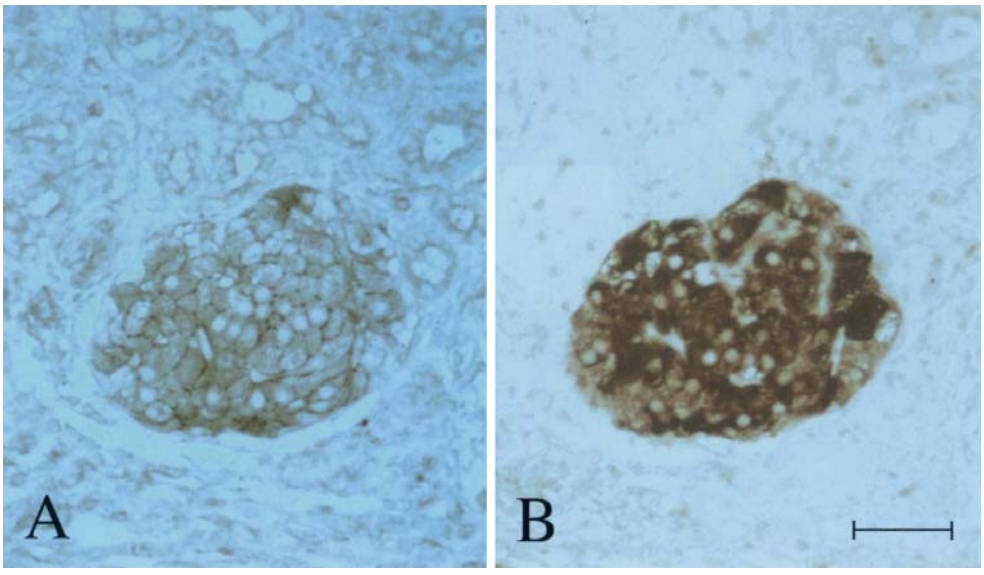

Fig. 7A, B. Immunohistochemical staining of neogenin (**A**) and insulin (**B**) of the duct-ligated part, 7 days after duct ligation. Note immunoreactivity in the plasma membrane of islet cells. Bar=20 μm

were negative. These observations suggest that the highest level of netrin-expression is present in regenerating islets. RT-PCR, immunohistochemistry and western blot showed no expression of netrin-1 in the normal pancreas but netrin-1 mRNA was detected by RT-PCR in isolated islet cells. It might be that netrin-1 expression is induced by the tissue dissociation and isolation procedure of islets. Another explanation could be that islets have a very low netrin-1 protein expression, which remains below the detection limit of immunohistochemistry. Since the pancreas consists mainly of exocrine tissue, a possible netrin-1 expression in islets could remain undetected in whole-pancreas extracts.

Netrin is a laminin-like substance and can have several different functions such as in cellular adhesion, differentiation or survival [23, 24], but so far only a role in cellular migration has been well documented. It is tempting to speculate that netrin-1 from islets might stimulate the migration of precursor cells which are known to be located in the ductal complexes induced by the ligation [1, 2]. Netrin has been shown to regulate the migration of neural cells and their axons [6]. We show that netrin-1 also positively influences the in vitro migration of pancreatic epithelial cells, including a duct-cell line and fetal-islet cells. The observed chemokinetic function of netrin-1 could thus be involved in the migration of islet precursor cells from ducts to islets during the process of "budding" islet morphogenesis.

Netrins are heparin-binding proteins and could therefore become bound to the extracellular matrix via glycosaminoglycans limiting the range of diffusion [6, 12]. Interaction with extracellular matrix proteins such as laminin-1 [25] can also influence the effect of netrin. This interaction could be involved in haptotactic direction of cell migration along an extracellular matrix gradient towards the islets. The fetal-islet cells that we used for the migration assay are immature cells which can differ in migratory capacity from mature islet cells. Islet cells from normal adult islets failed to migrate in this in vitro assay.

There is very little published information on the function of netrin-1 in epithelial tissue. As far as we know its function on epithelial cells is only described in the formation of the semicircular canals in the mouse inner ear. There, netrin-1 is required for the local disruption of the basement membrane and to induce a local proliferation of the adjacent mesenchymal cells to push the epithelial walls together [13].

Netrin could have different receptors on the cell surface. We considered the first family of established netrin receptors, namely those which were shown to direct neuronal migration in response to netrin. This family consists of DCC and neogenin [26]. We found expression of neogenin in the pancreas and in the CAPAN-2 cell line, but not of DCC. The latter is in accordance with a previous study showing neogenin but no DCC expression in the CAPAN-2 cell line [27]. Neogenin expression was transiently increased concomitantly with netrin expression in the fetal and regenerating pancreas, suggesting that neogenin could serve as a netrin receptor in these conditions. However, direct evidence for this is lacking at the moment. Studies on the expression of neogenin and DCC done by in situ hybridisation in the mouse embryo already indicated that neogenin is the predominant member of this subfamily in other tissues than the neuronal tissue, while DCC and neogenin could play complementary roles in the generation of the ful-

ly functional central nervous system [28]. However, other families of netrin receptors will have to be considered, such as the vertebrate unc-5 homologues [29] which were shown to convert the netrin-induced chemoattraction via DCC into repulsion when both receptors are simultaneously expressed [30, 31]. Recently the adenosine A2b receptor has also been described as a netrin co-receptor of DCC necessary for the cAMP-induced outgrowth of axons by netrin-1 [32].

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