Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors

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Accepted 24 March; published on WWW 23 May 2000

SUMMARY
Pancreatic acini and islets are believed to differentiate from common ductal precursors through a process requiring various growth factors. Epidermal growth factor receptor (EGF-R) is expressed throughout the developing pancreas. We have analyzed here the pancreatic phenotype of EGF-R deficient (−/−) mice, which generally die from epithelial immaturity within the first postnatal week. The pancreata appeared macroscopically normal. The most striking feature of the EGF-R (−/−) islets was that instead of forming circular clusters, the islet cells were mainly located in streak-like structures directly associated with pancreatic ducts. Based on BrdU-labelling, proliferation of the neonatal EGF-R (−/−) beta-cells was significantly reduced (2.6±0.4 versus 5.8±0.9%, P<0.01) and the difference persisted even at 7-11 days of age. Analysis of embryonic pancreata revealed impaired branching morphogenesis and delayed islet cell differentiation in the EGF-R (−/−) mice. Islet development was analyzed further in organ cultures of E12.5 pancreata. The proportion of insulin-positive cells was significantly lower in the EGF-R (−/−) explants (27±6 versus 48±8%, P<0.01), indicating delayed differentiation of the beta cells. Branching of the epithelium into ducts was also impaired. Matrix metalloproteinase (MMP-2 and MMP-9) activity was reduced 20% in EGF-R (−/−) late-gestation pancreata, as measured by gelatinase assays. Furthermore, the levels of secreted plasminogen activator inhibitor-1 (PAI-1) were markedly higher, while no apparent differences were seen in the levels of active uPA and tPA between EGF-R (−/−) and wild-type pancreata. Our findings suggest that the perturbation of EGF-R-mediated signalling can lead to a generalized proliferation defect of the pancreatic epithelia associated with a delay in beta cell development and disturbed migration of the developing islet cells as they differentiate from their precursors. Upregulated PAI-1 production and decreased gelatinolytic activity correlated to this migration defect. An intact EGF-R pathway appears to be a prerequisite for normal pancreatic development.

Key words: EGF receptor, Gene targeting, Pancreas, Matrix-metalloproteinases, Mouse

INTRODUCTION
The pancreas develops from foregut endoderm through the fusion of dorsal and ventral pancreatic buds. It undergoes branching morphogenesis, in which endoderm-mesoderm interactions are needed for differentiation (Go et al., 1986). Gene targeting experiments have shown the necessity of transcription factors for this process, e.g. Pdx-1 (Ahlgren et al., 1996; Jonsson et al., 1994), Pax-4 and -6 (Sosa-Pineda et al., 1997; St-Onge et al., 1997), NeuroD/Beta2 (Naya et al., 1997) and Hb9 (Harrison et al., 1999; Li et al., 1999), but the extracellular regulatory mechanisms are not well understood. The default setting for embryonic pancreatic epithelium appears to be formation of islets, while mesenchyme-derived signals, such as fibroblast growth factors (FGFs), are needed for the development of acinar structures (Gittes et al., 1996; Miralles et al., 1998b, 1999).

Genes crucial for pancreatic cytodifferentiation have been characterized by various methods, the most sensitive of which is reverse-transcription polymerase chain reaction (RT-PCR). According to these studies transcription of the pancreatic endocrine genes has already begun in the mouse foregut before the 26-somite stage (E9.5) when pancreas formation starts (Gittes and Rutter, 1992). Somatostatin mRNA is the first to appear. It is expressed at the 10-somite stage (E8.5) throughout the foregut. Insulin and glucagon mRNAs can be detected ‘premorphogenetically’ already at the 20-somite stage (E9.0) in the wall of foregut restricted to the area eventually forming the pancreatic diverticulum (Gittes and Rutter, 1992). Pancreatic polypeptide is detectable from the 30-somite stage (E9.0) in the wall of foregut restricted to the area eventually forming the pancreatic diverticulum (Gittes and Rutter, 1992). Pancreatic polypeptide is detectable from the 30-somite stage (E10). Expression of exocrine enzyme coding genes such as carboxypeptidase A and amylase begins approximately 1 day after the pancreatic diverticulum has formed at E10.5-12. The pancreatic epithelium begins to branch and forms the early acini at the 48-somite stage (E11.5).

Epidermal growth factor receptor (EGF-R) is a prototype
tyrosine kinase receptor (Schlessinger and Ullrich, 1992). It is expressed throughout the developing pancreas, and its ligands, particularly transforming growth factor-alpha (TGF-α), are also abundantly expressed (Miettinen and Heikinheimo, 1992). Interestingly, TGF-α colocalizes to β-cells with insulin, but at least during human development it does not have any effect on insulin mRNA expression (Miettinen, 1993; Miettinen and Heikinheimo, 1992). Transgenic mice overexpressing TGF-α develop metaplastic pancreatic ducts and islet neogenesis, suggesting that it might be involved in duct cell proliferation and differentiation (Sandgren et al., 1990; Wang et al., 1997). Furthermore, studies with γ-interferon transgenic mice have suggested a role for EGF-R-mediated signalling in the differentiation of acini into duct-like structures (Arnush et al., 1996).

EGF-R has been implicated in the development of organs requiring epithelial-mesenchymal interactions, e.g. the lung, kidney and tooth (Warburton et al., 1992). Indeed, inactivation of EGF-R affects epithelium and, in particular, organs undergoing branching morphogenesis, resulting in death during the first postnatal week of life (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Without functional EGF-R, lungs and mammary gland exhibit impaired branching (Miettinen et al., 1997; Wiesen et al., 1999), development of astrocytes is abnormal and apoptosis of forebrain structures is increased (Kornblum et al., 1998; Sibilia et al., 1998), and craniofacial abnormalities ensue (Miettinen et al., 1999). Since the pancreas also develops through branching morphogenesis and the EGF family has been implicated in this process, we used EGF-R (−/−) animals as a model to study the role of EGF-R signalling in pancreatic development.

MATERIALS AND METHODS

Generation of EGF-R (−/−) mice and genotyping

EGF-R was disrupted as described previously (Miettinen et al., 1995). Embryos and newborn pups used in this study derived from intercrosses between EGF-R (+/+) mice. Plug-date was considered as E0.5. E12.5-15.5 embryos were genotyped by PCR and Southern blot analysis (Miettinen et al., 1995), while older EGF-R (−/−) embryos could be recognized from their open-eye phenotype. For studies of pancreas in newborn mice, animals were killed by decapitation and the pancreata dissected. For histological analysis, embryos were collected at E12.5, 13.5, 14.5, 15.5 and 16.5, and for pancreatic organ cultures at E12.5. For cell proliferation experiments, pregnant animals or pups, newborn to P11, were injected intraperitoneally with bromodeoxyuridine (BrdU; 100 µg/g animal mass; Sigma) 2 hours before hot start (80°C for 3 minutes) and addition of nucleotides. For genotyping, the embryonic tissue was digested with 100 µg/ml proteinase K (Sigma) in lysis buffer (100 mM Tris-HCl buffer, pH 8.5, containing 200 mM NaCl, 5 mM EDTA and 0.2% SDS) at 55°C in a shaking water bath overnight. After phenol/chloroform and chloroform extraction and isopropanol precipitation, the pellet was washed with 70% ethanol, air dried and dissolved in TE (1 mM EDTA in a shaking water bath overnight. After phenol/chloroform and chloroform extraction and isopropanol precipitation, the pellet was washed with 70% ethanol, air dried and dissolved in TE (1 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0). For PCR analysis, the genomic DNA (1-2 µg) was mixed with reaction buffer (for EGF-R 3′: 60 mM Tris-HCl, pH 9.5, 15 mM (NH₄)SO₄, 3.0 mM MgCl₂ with 5% DMSO, and for PGK 3′: 60 mM Tris-HCl buffer, pH 9.5, 15 mM (NH₄)SO₄, 4.5 mM MgCl₂ with 10% DMSO) and 40 pmoles each of 5′ mouse EGF-R primer (5-AGG CAC AAG TAA CAG GCT-3) and either EGF-R 3′ primer (5-TCC TTT GCA CAT AGG TA-3; for EGF-R (−/−) identification) or PGK 3′ prime (5-CTA CCC GCT TCC ATT GCT CAG C-3; for EGF-R (+/+) identification) in a total volume of 45 µl before hot start (80°C for 3 minutes) and addition of nucleotides. The samples were then denatured at 94°C for 2 minutes prior to 35 cycles of amplification (1 minute each at 94°C, 53°C and 72°C for EGF-R 3′ and at 94°C, 58°C and 72°C for PGK 3′) using a MJR-100 thermocycler. The final extension was 7 minutes at 72°C. A sample of the reaction mixture was then analyzed on a 2.5% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5). Homozygous embryos were identified by the absence of a 123 bp band (this band is generated from wild-type and heterozygote embryos) and heterozygotes by the presence of a 359 bp band. To exclude false PCR negative samples, all EGF-R (−/−) samples were confirmed by Southern analysis (Miettinen et al., 1995). The study was approved by the Haartman Institute Ethical Committee for Animal Studies.

Blood glucose levels

Random blood glucose recordings were obtained from newborn animals at the time they were killed using a portable glucose meter (MediSense Inc., Waltham, USA). Most animals had milk in their stomach at this time. Glucose tolerance tests were not feasible due to the premature death of the EGF-R (−/−) pups.

Histological analysis

Samples were fixed either in 4% paraformaldehyde (embryo series) or in Bouin’s fixative (newborn pancreata and organ cultures) and serial paraffin sections cut using routine procedures. For general morphology, deparaffinized sections were stained with Haematoxylin-Eosin. The sections were then examined by light microscopy and photographed, or morphometrically analysed directly by light microscopy.

Pancreatic organ cultures

Pancreatic rudiments from E12.5 embryos were microdissected together with the stomach anlagen. The appearance of the vaginal plug was noted as day E0.5. The tissues were cultured by a technique originally designed for embryonic kidney (Saxén and Lehtonen, 1987). Briefly, tissue explants of pancreas/stomach were cultured on Nucleopore filters (1.0 µm pore size; Costar) on metal grids in serum-free I-MEM (Improved Eagle’s Minimum Essential Medium; Gibco) supplemented with transferrin (30 µg/ml), penicillin (100 i.u/ml) and streptomycin (100 µg/ml). For the longer cultivation needed for the gelatinase and caseinolysis assays, the culture medium was also supplemented with 10% heat-inactivated fetal calf serum. The medium was changed every second day. After 5-6 days in culture, the explants were fixed in Bouin’s solution for 4 hours and processed for paraffin embedding. The number of ductal branches was manually calculated under the light microscope from the serially sectioned pancreatic explants (n=8 for EGF-R (−/−) and n=7 for wild-type embryos).

Immunohistochemistry

Tissues were fixed in Bouin’s fixative or in 4% paraformaldehyde at room temperature (RT) for 4 hours. After rinsing with 50% alcohol, tissues were stored in 70% ethanol prior to dehydration and paraffin embedding. For quantitative morphometric analysis of endocrine cell types, the entire tissue block was sectioned for immunostaining. Every fifth section was stained with the same primary antibody. Deparaffinized, rehydrated sections were incubated in 3% normal goat serum (ZYMED) in PBS (phosphate-buffered saline, pH 7.4) at RT for 2 hours to block nonspecific binding sites and incubated with the primary antibody (mouse anti-human E-cadherin, Transduction Laboratories; mouse anti-pancytokeratin, Sigma; guinea pig anti-porcine insulin, rabbit anti-human glucagon, rabbit anti-human somatostatin, goat anti-human pancreatic polypeptide, DAKO) diluted in PBS containing 3% normal goat serum overnight at 4°C. All antisera were pretested for optimal dilution and staining.
conditions. After rinsing several times with PBS, the sections were incubated with biotinylated-goat anti-rabbit IgG (ZYMED) for 30 minutes at RT, rinsed and incubated with peroxidase-conjugated streptavidin (ZYMED), diluted in PBS. The sections were finally developed with the AEC substrate (3-amino-9-ethylcarbazole), which produces a red reaction product, and rinsed with PBS. Light counterstaining was performed with Haematoxylin. For simultaneous detection of insulin-, glucagon- and cytokeratin-positive cells or insulin- and E-cadherin-positive cells, fluorochrome (FITC, TRITC and AMCA)-conjugated secondary antibodies were used (Jackson Immunolaboratories).

For double staining of hormone- and BrdU-positive cells, the staining protocol was continued from the one described above. The slides were treated with 0.1% pepsin in 0.1 M HCl at RT for 30 minutes to reveal antigenic sites, rinsed in aqua and in PBS, and incubated in 95% (v/v) formalde 0.15 M sodium citrate at 70°C for 45 minutes. Double staining was performed using the Vectastain® ABC-kit (Vector, Burlingame, CA, USA). The sections were incubated with horse blocking serum at RT for 2 hours and incubated with mouse monoclonal anti-bromodeoxyuridine antibody diluted in PBS containing horse serum, overnight at 4°C. After rinsing with PBS, the sections were incubated with biotinylated secondary antibody for 30 minutes, washed, incubated in Vectastain ABC- alkaline phosphatase (AP) reagent for 30 minutes, washed again, and developed with the AP substrate (3-amino-9-ethylcarbazole), which produces a red reaction product, and rinsed with PBS. Light counterstaining was performed with Haematoxylin. A similar procedure was carried out using the terminal dideoxynucleotidetransferase (Tdt)-mediated ddUTP nick end labelling (TUNEL) procedure (Boehringer Mannheim, Mannheim, Germany). The sections were permeabilized by microwave treatment (5×3 minutes in 10 mM citric acid), followed by preincubation in 5 mM CoCl₂ TdT buffer for 10 minutes and the digoxigenin-conjugated ddUTP labelling of the nicked DNA ends by Tdt (5 mM CoCl₂, 5 mM Tdt-Buffer, 0.23 buffer for 10 minutes and the digoxigenin-conjugated ddUTP labelling (TUNEL) procedure (Boehringer Mannheim, Mannheim, Germany).

Image analysis

The Bouin-fixed pancreata were processed as described above. Every fifth section was stained with the same hormone antibody. Only pancreatic tissue was included in the computerized image analysis, which was carried out using KS400 (version 2.00) Image Analysis software. The area covered by cells stained by each antibody was measured and expressed as a percentage of the total pancreatic area.

Detection of apoptosis

Deparaffinized sections were stained using the terminal dideoxynucleotidetransferase (Tdt)-mediated ddUTP nick end labelling (TUNEL) procedure (Boehringer Mannheim, Mannheim, Germany). The sections were permeabilized by microwave treatment (5×3 minutes in 10 mM citric acid), followed by preincubation in 5 mM CoCl₂ TdT buffer for 10 minutes and the digoxigenin-conjugated ddUTP labelling of the nicked DNA ends by Tdt (5 mM CoCl₂, 5 mM Tdt-Buffer, 0.23 mM ddATP, 0.13 mM dig-ddUTP, 0.58 U/ml Tdt) at 37°C for 20 minutes. To detect the labelled cells, the sections were treated with 2% blocking reagent (Boehringer Mannheim) in 150 mM NaCl, 100 mM Tris-HCl buffer, pH 7.5, and then treated with anti-digoxigenin Fab fragments (0.19 U/ml in blocking buffer) at 37°C for 60 minutes. Finally, the reaction products were visualized by incubation with a peroxidase dye, NBT/BCIP, in 67% DMSO, for up to 60 minutes. Nuclei were counterstained with Haematoxylin. A similar procedure without the Tdt-treatment was used as a negative control for every sample. The cell specificity of apoptosis was confirmed by insulin immunocytochemistry following TUNEL staining.

To detect DNA fragmentation at the tissue level, DNA was purified from neonatal pancreata using a commercial kit (Apopotic DNA ladder kit, Boehringer Mannheim). The purified DNA samples were nick-end labelled with digoxigenin-ddUTP. Gel electrophoresis was performed and the DNA blotted onto a Hybond N+ membrane (Amersham). The membrane was then incubated with an anti- digoxigenin-alkaline phosphatase antibody and further with CSPD, a chemiluminescent substrate for alkaline phosphatase (Boehringer Mannheim).

RNA analysis

Total RNA from the EGF-R (−/−) and normal mouse pancreata was prepared by guanidinium thiocyanate extraction followed by CsCl gradient centrifugation (Sambrook et al., 1989). mRNA was purified from total RNA preparations with oligo(T)-coated magnetic microbeads (Dynabeads, Dynal AS, Oslo, Norway). mRNA (1.2 μg/lane) was fractionated on a 1.2% formalin-agarose gel and transferred to a nylon membrane (Hybond-N, Amersham) by capillary blotting. The cDNA probes were 32P-labeled by a random priming method (Prime-A-Gene Labeling System, Promega). Mouse EGF-R cDNA and rat erb-b-2 cDNA corresponded to the extracellular domains of the receptors. Human erb-b-3, erb-b-4 cDNAs and mouse 72-kDa type IV collagenase (Reponen et al., 1992) cDNAs represented the whole coding areas of corresponding mRNAs. Mouse β-actin and cyclophilin cDNAs were used as gel loading controls. Hybridizations were carried out in buffer containing 1% SDS, 1 M NaCl and 8% dextran sulfate overnight at 65°C. The blots were washed at 65°C in 1× SSC and finally in 0.5× SSC. Hybridization signals were visualized using a Bio-imaging analyzer (Fuji Photo Film Co., Ltd).

Gelatin zymography

Analyses for gelatinase activity were carried out as previously described (Chin and Werb, 1997). Briefly, E17.5 and newborn pancreata were dissected and digested with collagenase (4 mg/ml in Hanks buffer) in a shaking water bath (37°C for 5 minutes). Tissues were then cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 10 mM Hepes, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine for 3 days. Samples of conditioned medium were then collected and diluted in non-reducing 2× Laemmli sample buffer (0.125 M Tris, 4% SDS, 20% glycerol, 0.002% Bromophenol Blue, pH 6.8). Equal amounts of protein were loaded and separated under non-reducing conditions in 10% polyacrylamide Zymogram READY GEL (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis the gels were washed twice with 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM CaCl₂, 1 μM ZnCl₂, 2.5% Triton X-100 for 15 minutes to remove SDS, followed by brief rinsing in washing buffer without Triton X-100 and then incubated in substrate buffer (50 mM Tris-HCl buffer, pH 7.6, containing 5 mM CaCl₂, 1 μM ZnCl₂, 1% Triton X-100 and 0.02% NaN₃) overnight at 37°C. The gels were then stained with Coomassie Brilliant Blue R250, destained with 10% acetic acid and 10% methanol, dried and photographed. Zymograms were then densitometrically scanned and the gelatinolytic bands were quantified by NIH Image-analysis software. Conditioned medium from human fibrosarcoma HT-1080 cells containing gelatinases A and B was used as a mobility marker.

Zymographic and reverse zymographic assays

Caseinolytic-in-agarose assays were carried out as described (Saksela, 1981). Briefly, the caseinolytic gels (total volume 30 ml) contained 1 U plasminogen (Cromogenix), casein (1.5% milk powder) and 1.2% agarose (LITEX) in 0.1 M Tris-HCl buffer, pH 8.0. The medium samples were first subjected to SDS-PAGE, under non-reducing conditions. After removal of SDS by washing (see below), the gels were placed on caseinolytic gels and incubated at 37°C until lysis zones were observed. The positions of plasminogen activators (tPA and uPA) in SDS gels were determined as lysis zones in the indicator zymography gels.

To analyse the levels of plasminogen activator inhibitor-1 (PAI-1) levels, the polypeptides of the medium samples were separated by SDS-PAGE. SDS was removed by washing with 2.5% Triton X-100 in PBS, and human u-PA (2 U/ml) was added for the last 30 minute rinse. u-PA induces plasminogen-mediated digestion of the casein, except at the zones containing PAIs, which can be seen as lysis-resistant bands.

Statistics

The number of ducts is expressed as mean ± s.d. Statistical significance was assessed by the unpaired two-tailed t-test.
RESULTS

Islet morphogenesis is disturbed in newborn EGF-R (−/−) mice

Random blood glucose levels did not vary significantly between the controls and EGF-R (−/−) newborn mice, although the values tended to be lower in the EGF-R (−/−) mice. Neither was there any gross macroscopic abnormality in the EGF-R (−/−) pancreata. Although all four major endocrine cell types were found in the EGF-R deficient mice, a striking difference in the cell localization was observed, particularly in the newborn animals. In the controls, the endocrine cells formed rounded aggregates close to, but mostly separate from pancreatic ducts. In contrast, in the EGF-R (−/−) pancreata the endocrine cells were mainly found in streak-like structures directly next to ductal epithelial cells (Fig. 1A-D). However, both in the EGF-R (+/+) and (−/−) mice the insulin-producing β-cells were surrounded by a peripheral rim of glucagon-producing α-cells (Fig. 1A-D). Furthermore, no difference was seen in the aggregation of individual islet cells to each other, or in the intensity of E-cadherin staining (Fig. 1E-H). By quantitative analysis, the insulin-positive cells were in direct contact with duct epithelium twice as often as in the controls (P<0.05; Fig. 2A). The EGF-R (−/−) β-cells did not face the lumen of the duct more often than the control ones.

In vivo proliferation of pancreatic β-cells was quantitated after injecting the newborn animals with BrdU. Statistical analysis of BrdU-positive cells (Fig. 2B,C) revealed that the BrdU-labelling index of β-cells was significantly lower in the EGF-R (−/−) animals both at the age of 1 day (2.6±0.4% versus 5.8±0.9%, P<0.01) and at 7 days (1.4±0.5% versus 4.8±0.6%, P<0.01). A defect in proliferation of similar magnitude was also evident throughout the exocrine pancreas and the intestinal epithelium (data not shown).

We next analyzed whether the rate of apoptosis was changed after the EGF-R inactivation, using newborn pancreata, with both the TUNEL method and DNA fragmentation. The overall rate of endocrine cell apoptosis at this stage was very low; as a maximum, only 1-2 apoptotic β-cells could be identified in each specimen. No differences were evident between the control and EGF-R (−/−) pancreata. Accordingly, no apoptotic DNA fragmentation could be seen in the end-labelled pancreatic DNA of wild-type or EGF-R (−/−) pancreata (data not shown).

Expression of other members of the erbB family

The erbB family consists of erbB1 (EGF-R) and erbB2, 3 and 4 receptor tyrosine kinases, which

Fig. 1. EGF-R deficient endocrine cells form streak-like islets, but aggregate normally. Newborn (P1) and 5-day-old (P5) pancreata from wild-type (+/+) and EGF-R deficient (−/−) animals were either triple stained for insulin- (A-D, green), glucagon- (A-D, blue) and cytokeratin- (A-D, red) like-immunoreactivity or double stained for E-cadherin- (E,F, green) and insulin- (G,H, red) like-immunoreactivities. While the control pancreas contained roundish islets (A,C, arrows), the islet cells in the EGF-R (−/−) pancreata (B,D, arrows) remained significantly more frequently in close proximity to the ducts. Nevertheless, the islet architecture is normal: insulin-positive β-cells are surrounded by a rim of glucagon-containing α-cells in both the wild-type and EGF-R (−/−) islets (A-D). Furthermore, no difference could be seen in cell-cell adhesion within the islets, and the E-cadherin-like immunoreactivity remains unchanged (E,F). Insulin double staining is shown for better visualization of the islets (E-H). Magnification 20× (A-D) and 40× (E-H).
can function both as homodimers and heterodimers (Carraway III and Cantley, 1994). Conceivably, inactivation of erbB1 could also have an impact on the signalling pathways through erbB2-4. Since all the erbB family members are expressed in the pancreas or pancreatic cell lines (Huotari et al., 1998; our own unpublished data), we wanted to know whether their expression levels were affected in the EGF-R (-/-) mice. For this purpose poly(A) + mRNA was isolated and subjected to northern analysis using erbB2, 3 and 4 cDNA as probes. However, the expression levels were similar in normal and EGF-R (-/-) newborn pancreata (Fig. 3). In both cases, the erbB2/neu mRNA expression was relatively high, whereas the erbB4 mRNA expression was barely detectable in the pancreas.

**Characterization of the embryonic pancreata**

Because the EGF-R (-/-) newborn pancreata showed signs of delayed islet cell development, we next evaluated the endocrine pancreatic development at different embryonic stages. Mouse embryos at E12.5 and E16.5 (n=3) were serially sectioned and stained for the four islet hormones, glucagon, insulin, pancreatic polypeptide (PP) and somatostatin. Consecutive sections were then scored for the ratios of positive cells per pancreatic area. In E12.5 embryos, i.e. after the first endocrine cells have appeared but the two pancreatic buds have not yet fused, glucagon-containing α-cells were the only endocrine cell type present in the EGF-R (-/-) embryos, whereas in controls, a few insulin-containing cells were also present. There also tended to be more glucagon-positive cells per pancreatic surface than in the wild-type cells at the same stages (4% versus 1.4%, P=0.13). 4 days later, at E16.5, all islet hormones were expressed in both wild-type and mutant embryos. No significant differences could be seen between insulin, glucagon or somatostatin expression, although the area covered by β-cells tended to be smaller in the EGF-R (-/-) embryos (Fig. 4B,D). Interestingly, the EGF-R (-/-) pancreas contained more PP-positive cells than the wild-type pancreas (4.7% versus 0.9%, P=0.06; Fig. 4E).

As islets are poorly formed by E16.5, total pancreatic area contains mainly ducts and acini and therefore reflects branching of the developing ductular system. The area from consecutive sections was traced manually under the microscope and measured using computer programs. The results show that the E12.5 and E16.5 EGF-R (-/-) pancreata are about half the size (P<0.01) of the wild-type pancreata, suggesting that in addition to the delay in cell differentiation, their branching is also impaired (Fig. 4F).

**Pancreatic development in explant culture reveals delayed β-cell differentiation**

Since pancreatic development seemed to be delayed in the EGF-R (-/-) embryos, we next studied pancreatic differentiation in a more isolated system using whole-mount explants.
organ cultures. In this system the developing pancreas undergoes branching morphogenesis, mimicking the in vivo situation (Ritvos et al., 1995). At E12.5, no size differences could be seen between the EGF-R (−/−) and wild-type embryos. Pancreatic rudiments were dissected from these embryos and cultured in serum-free medium for 6 days. After the culture period, the EGF-R (−/−) explants were generally smaller and contained significantly less ducts than the EGF-R (+/+), suggesting that they had defective branching (number of luminal cross-sections=514±190 versus 1675±358, P<0.005; Fig. 5A-F). All four major islet cell types were present in the explants, showing that cell differentiation occurred in a normal fashion. Since only glucagon-positive cells were present at the onset of culture, all insulin-, somatostatin- and PP-containing cells present at E12+6 had differentiated during the explant culture. The most common cell in the EGF-R (−/−) explants was the glucagon cell (40% of all endocrine cells) and the proportion of insulin-positive cells was significantly lower than in the controls (27±6 versus 48±8%, P<0.01), indicating delayed differentiation of the β-cells (Fig. 5G). Glucagon- and somatostatin-containing cells were equally abundant in the EGF-R (+/+ and −/−) explants while, corresponding to the situation in vivo, there was a tendency for an increase in the number of PP-containing cells in the EGF-R (−/−) explants (21±5% versus 11±3% of all endocrine cells; P=0.11), (Fig. 5G).

Matrix metalloproteinase expression is under EGF-R regulation in the developing pancreas

During islet development the endocrine cells migrate from a ductal position to the intestinal tissue through the extracellular matrix (Miralles et al., 1998a). Besides activation of the cytoskeletal machinery, this process also involves degradation of the extracellular matrix and requires serine proteases and matrix metalloproteinases (MMPs). Of the various MMPs, gelatinase-B (MMP-9) and -A (MMP-2) are expressed in pancreas and these, moreover, are known to be upregulated by EGF-R (Reponen et al., 1992; Kondapaka et al., 1997; van der Zee et al., 1998). Since the EGF-R (−/−) islets stayed predominantly in the vicinity of the ducts and yet no consistent differences were seen in the expression of various adhesion molecules (our own unpublished observations), we sought to determine whether the expression of gelatinolytic MMPs was altered. As no difference was seen in the mRNA expression of MMP-2 by northern analysis of newborn control and EGF-R (−/−) pancreas (data not shown), we next analysed the levels of gelatinase activity. For this purpose medium from 2-day-cultures of newborn and E17.5 control and EGF-R (−/−) pancreata were subjected to zymography. In the marker cell line for gelatinolytic activity, we observed a PMA-induction of 92 kDa gelatinase-B and processing to the intermediate form of 72 kDa gelatinase-A (Fig. 6A, HT-1080 lanes). Gelatinolytic activity of 92 kDa MMP-9 (gelatinase-B) and the 72 kDa inactive form of MMP-2 (gelatinase-A), were detected both in E17.5 and P1 pancreata. Interestingly, the activated (62/64 kDa) form of MMP-2 was detectable only at E17.5 (Fig. 6A, arrow). The total gelatinolytic activity was reduced by one-fifth in the EGF-R (−/−) pancreas, which also expressed about 30% less MMP-9 activity than the wild-type pancreas, based on scanning of the zymograms. No significant differences were seen in the amounts of inactive 72 kDa MMP-2 but interestingly, reflecting the total profiles, the amount of the active (62/64 kDa) MMP-2 was 35% lower in EGF-R (−/−) pancreata, thus making the ratio of active/inactive MMP-2 lower in the EGF-R (−/−) pancreas than in the wild-type one (0.33 versus 0.63; Fig. 6A). In addition, we observed a closely spaced triplet of gelatinolytic activity above the 92 kDa gelatinase. Their expression levels correlated with the expression of gelatinases-A and -B, suggesting similar regulation. The identities of these gelatinolytic proteins were not characterized further.

Serine proteases such as tissue plasminogen activator (tPA) and urokinase (uPA) also regulate cell migration and activate MMPs (Cuzner and Opdenakker, 1999), and their activity is largely regulated by plasminogen activator inhibitor type I (PAI-1). Zymographic and reverse zymographic caseinolysis assays were used to monitor the activity levels of both PAs and PAI-1 in the culture medium of EGF-R (−/−) and control pancreata. In these assays, no differences were observed in the uPA or tPA levels between the control and EGFR (−/−) mice (data not shown). Interestingly, with serum stimulation the EGF-R (−/−) pancreata produced about four times more PAI-1 into the culture medium than the wild-type pancreata (Fig. 6B). This difference was not detected under serum-free conditions, suggesting an EGF-R-mediated modulation of the serum response.

DISCUSSION

The EGF-R (−/−) mice provide a unique model in which to define the role of EGF receptor-mediated signalling in pancreatic islet development. Our study shows that all islet cell types do differentiate in these mice and that the animals are not diabetic at birth. A specific morphological abnormality became evident, however: the islet cells are not able to migrate normally away from the pancreatic ducts, but rather stay as
2623EGF-R is required for pancreatic development

Moreover, there is an apparent delay in islet cell differentiation, with the development of EGF-R (-/-) b-cells occurring at a later stage than in wild-type littermates. This suggests that EGF-R-mediated signalling is specifically required for the b-cells to develop normally.

EGF-R belongs to a family of four receptor tyrosine kinases encoded by the erbB gene family (EGF-R/erbB1, neu/erbB2, erbB3 and erbB4) (Kraus et al., 1989; Plowman et al., 1993). They are all expressed in the developing pancreas in an overlapping manner (M.-A. Huotari et al., unpublished) and their coexpression is likely to explain the relatively mild phenotype of the EGF-R (-/-) pancreata. In addition to functioning as homodimers, the various erbBs can also form heterodimers, further expanding the potential signalling pathways (Carraway III and Cantley, 1994; Riese II et al., 1995). Inactivation of erbB3 and erbB4 by gene targeting results in embryonic lethality (Erickson et al., 1997; Gassmann et al., 1995). Interestingly, pancreatic development is severely disturbed in the erbB3 (-/-) embryos (Erickson et al., 1997).

At E13.5 the pancreata were rudimentary, although endocrine differentiation had proceeded as islet cell markers were detectable. Thus, the overall phenotype resembles the EGF-R (-/-) pancreatic phenotype.

The developing pancreas also expresses many EGF-R ligands (at least betacellulin, EGF, heparin-binding EGF (HB-EGF) and TGF-α) (Kaneto et al., 1997; Miettinen and Heikinheimo, 1992; M.-A. Huotari et al., unpublished results). In humans, TGF-α colocalizes with insulin to fetal b-cells, and in fetal rats HB-EGF is localized to primitive ducts, ductular
cells and islet-like endocrine cell clusters. The expression pattern of HB-EGF is similar to that of the homeodomain protein pdx-1. Pdx-1, an insulin and somatostatin gene transcription factor, is already expressed in the pancreatic bud by the 15-somite stage (Ahlgren et al., 1996). It is essential for pancreatic development, since pdx-1-deficient mice lack a pancreas (Jonsson et al., 1994). These animals form a pancreatic bud, and early insulin and glucagon cells do develop, but further differentiation is arrested. Interestingly, pdx-1 and EGF-R signalling may share common pathways since pdx-1 binds to and activates the HB-EGF promoter (Kaneto et al., 1997). Furthermore, based on sequence analysis, the TGF-α promoter also has a putative PDX-1 binding site. Vice versa, pdx-1 is regulated by betacellulin, but not by EGF or TGF-α, in the glucagon-producing alpha-TC1 cells (Watada et al., 1996). Thus, it is conceivable that EGF-R ligands are already involved in pancreatic development from early stages.

During the last 4 days of gestation, endocrine cells detach from the ducts, increase in number and reorganize to form mature islets (Pictet et al., 1972). Our study shows that without a functional EGF-R the endocrine cells fail in this process. This could either be due to a defect in cell adhesion or an extracellular matrix (ECM)-related problem in cell migration. The important molecules in this context include cadherins, integrins and matrix metalloproteinases. Cadherins are transmembrane calcium-dependent cell adhesion molecules (Takeichi, 1991, 1995) that have been implicated in the formation of trophectoderm (Larue et al., 1994), regulation of cell adhesion during early development (Burdsal et al., 1993) and cell migration (Hermiston et al., 1996). They are expressed in developing pancreas and have been implicated in the formation of islet architecture (Cirulli et al., 1994; Rouiller et al., 1991). Indeed, when a dominant negative mutant E-cadherin is expressed under the insulin promoter, β-cells are dispersed throughout the pancreas as individual cells instead of forming compact islets (Dahl et al., 1996). The cytoplasmic domain of E-cadherin interacts with α-, β- and γ-catenin (Takeichi, 1995). Both β- and γ-catenin bind directly to E-cadherin, while α-catenin links the complex to actin filaments. EGF-R-mediated signalling and cadherin function have been linked together. EGF induces a rapid tyrosine phosphorylation of both β- and γ-catenin without affecting their total levels or the level of E-cadherin (Hazan and Norton, 1998; Hoschuetzky et al., 1994). This phosphorylation disrupts cell-cell adhesion by uncoupling the interaction of E-cadherin with the actin cytoskeleton (Hazen and Norton, 1998). Loss of function of the various cadherin-catenin components may lead to loss of normal epithelial differentiation. Considering the
link between EGF-R and the cadherin-catenin complex, it is conceivable that inactivation of EGF-R expression affects the function of this complex. Although differences in the expression of E-cadherin were not found in the EGF-R (−/−) pancreata, the phosphorylation of β- and γ-catenins could still be reduced, affecting cell-cell adhesion. Nevertheless, this is unlikely since cell-cell aggregation within the islets appeared to be normal in the EGF-R (−/−) mice.

Matrix metalloproteinases are important factors involved in remodeling the extracellular matrix during embryogenesis, tissue repair and various pathological conditions, e.g. cancer invasion and metastasis (Werb, 1997). They are upregulated by EGF-R ligands (Kondapaka et al., 1997; van der Zee et al., 1998), and we have recently found that decreased secretion of MMPs contributes to the abnormal craniofacial development in the EGF-R (−/−) mice (Miettinen et al., 1999). Accordingly, we now find that the gelatinolytic activity is also reduced in the EGF-R (−/−) pancreata, and that this correlates with increased levels of plasminogen activator inhibitor-1 (PAI-1), which is also known to participate in the activation of certain MMPs through a cascade of proteinases. In addition, PAI-1 can affect cell migration by blocking integrin alpha-v-β3 binding to vitronectin (Steffansson and Lawrence, 1996). Elevated pericellular PAI-1 levels might thus affect branching, cell migration and differentiation, regardless of the plasminogen activator levels. EGF-R signalling participates in epithelial-mesenchymal interactions, and in accordance with this, branching is impaired in EGF-R (−/−) lung (Miettinnen et al., 1997), mammary gland (Wiesen et al., 1999) and pancreas (present results). Among many other factors, the MMPs are expressed in the pancreatic mesenchyme (Reponen et al., 1992; Tomita and Iwata, 1997) and could thus be involved in the constant remodeling of the extracellular matrix needed for proper branching morphogenesis. Indeed, MMP-2 has been implicated in islet formation: in rat pancreas, its activation coincides with islet morphogenesis (Miralles et al., 1998a), and upon its inhibition islet morphogenesis is impaired although endocrine cell differentiation proceeds normally. This suggests that MMP-2 is required for the migration of islet cells through the duct basement membrane (Miralles et al., 1998a). Furthermore, inhibition of MMP-9 activity during renal morphogenesis blocks T-shaped branching and further divisions of the ureter bud (Lelongt et al., 1997), and matrix metalloproteinases also regulate the branching of embryonic salivary glands (Nakanishi et al., 1986). Based on these findings, it is conceivable that the reduced MMP-activity is also responsible for the impaired branching of the EGF-R (−/−) pancreata and the defective islet formation.

In conclusion, analysis of pancreatic islet development of EGF-R (−/−) mice shows that EGF-R signalling is required for adequate basement membrane degrading activity and subsequent ductal branching and migration of the developing islet cells through ductal basement membranes. In addition, the absence of EGF-R signalling the differentiation of β-cells is delayed and their proliferation impaired.

This work was supported by grants from the Juvenile Diabetes Foundation International (1996-97), the Sigrid Juselius Foundation, the Foundation for Diabetes Research, the Research Fund of the Helsinki University Central Hospital, the Academy of Finland and the Research and Science Foundation of Farmos (to M.-A. H.). T. Otonkoski is the recipient of a Juvenile Diabetes Foundation International Career Development Award. We also thank Mrs Ulla Kiiski for her valuable help in embryo dissections and Drs Heli Valtanen and Katri Koli for their help with zymographic assays.

REFERENCES


