Embryogenesis and neogenesis of the endocrine pancreas
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CHAPTER 3

Regenerative effect of c-met and c-kit carrying rat neonatal cells on the endocrine pancreas (preliminary data)
ABSTRACT

**Objective:** In the present pilot study we investigated whether c-met and c-kit positive cells may stimulate regeneration of the damaged adult pancreas.

**Research Design and Methods:** Low dose streptozotocin injected animals were infused with c-met or c-kit positive cells obtained from neonatal pancreata.

**Results:** We found large amounts of the infused c-met and c-kit positive cells in the pancreas of infused rats. C-kit and to a much lesser extent also c-met positive cells induced a decrease in the blood glucose levels at 4 days after injection. However, we did not observe any difference in intensity in insulin staining.

**Conclusions:** Our study suggests that pure population of c-met and c-kit carrying neonatal rat cells can contribute to the regeneration of the adult pancreas.

INTRODUCTION

During embryogenesis the pancreas evolves according to a number of strictly controlled and predefined steps. It starts with the epithelial evagination of the foregut endoderm into the surrounding splanchnic mesoderm. This epithelial-mesenchymal interaction is followed by acinar and islet cell differentiation [1]. During this early stage, a multipotential stem cell may differentiate into cells that possess either an endocrine or exocrine phenotype [1]. The endocrine precursor cells develop further by budding from embryonic duct-like cells. This process leads to the formation of primitive islets in the mesenchyme adjacent to the ducts. Much new insight has been gained during recent years in the processes responsible for development of the pancreas in embryogenesis and a number of cell types have been identified that that are being hold responsible for development of the primitive pancreas.

A series of recent reports [2-5] have proposed that such cells that are involved in the development of the endocrine pancreas carry the receptor for hepatocyte growth factor (c-met) and the stem-cell factor (c-kit). These markers have been shown to be associated with stem cells [2-5]. C-met and c-kit positive cells have also been shown in the adult pancreas, especially in situations of pancreatic regeneration. Some authors even suggested that infusion of clonally expanded populations of
these cells may stimulate regeneration of the damaged adult pancreas. To test whether these cells carrying the c-met and c-kit proteins indeed have some efficacy in stimulating regenerative processes in the adult pancreas we infused pure, isolated populations of these cells in adult rat pancreata previously treated with the beta-cell toxin streptozotocin. Since c-met and c-kit positive cells can be found in high quantities in the neonatal pancreas [2,5, this thesis] we isolated these cells from the neonatal pancreas.

**MATERIALS AND METHODS**

*Design of the study*

Since it is suggested that c-met or c-kit positive cells can stimulate regeneration of the damaged pancreas, we choose to use rats in which the pancreas was slightly damaged by a low dose of streptozotocin (STZ), rather than using a high dose of STZ to induce a diabetic state. Rats were treated with 40 mg/kg STZ two days prior to the infusion with c-met or c-kit positive cells. At the day of the infusion the rats were infused with 400,000 isolated c-met or c-kit positive cells from neonatal pancreata which were labelled with the PKH26 Red Fluorescent Cell linker Kit. This labelling was performed to be able to trace back the cells in the pancreata after sacrifice of the rats. The blood glucose levels of the rats were measured at the day of the infusion, and 4 days after infusion (day of termination). We also weighed the rats at these various time points.

At day 4 after infusion the rats were sacrificed and the pancreata were harvested. Using immunofluorescence, we investigated whether the infused c-met or c-kit positive cells could be visualized in the pancreas. We also studied the expression of insulin in the pancreata of c-met (n=5) or c-kit (n=5) injected and non-injected STZ-treated rats using immunohistology. For comparison of insulin in c-met or c-kit injected and non-injected STZ-treated rats, we also stained the pancreata of non-injected, non-STZ-treated rats for insulin.

*Animals*

To obtain neonatal rat pancreata, pregnant rats were purchased from Harlan. These rats were allowed to deliver naturally. Neonatal rats were used 1-2 days after
delivery. All experiments were conducted in accordance with NIH-guidelines for the care and use of laboratory animals. All rats were kept in a temperature- and light-controlled room (lights on from 6 AM to 6 PM).

Female Wistar rats (Harlan; age 3-4 months and weighing ~200 g) were used for the injection of c-met (n=5) or c-kit (n=5) positive pancreatic precursor cells. As controls, we included non-injected STZ-treated rats (control rats) in which we performed sham operations. For c-met injections, we included n=5 and for the c-kit injections we included n=4 control rats.

**Surgery**
The neonatal rat pancreata were obtained from 1-2 days old rat pups. The pups were decapitated and the pancreas was surgically removed. This was done by laparotomy, replacing the stomach aside, and taking the pancreas out by cutting it loose from the spleen, duodenum, and stomach wall. The neonatal pancreata were processed for cell-isolation. For cell-isolation, the pancreata were stored in 5 ml of 0.20\(\mu\)m filtered Krebs-Ringer-Hepes buffer (KRH: 133 mM NaCl, 4.69 mM KCl, 1.18 mM KH\(_2\)PO\(_4\), 1.18 mM MgSO\(_4\).7H\(_2\)O, 10 mM HEPES and 2.52 mM CaCl\(_2\).2H\(_2\)O (pH 7.4)) containing 5% bovine serum albumin (BSA) on ice.

**Cell-isolation**
Neonatal pancreata were digested with collagenase to transform them into single cells. The pancreata were first cut in small fragments of approximately 1 mm. The fragments were then washed once with KRH containing 5% BSA for 5 minutes at 4°C. The pancreas fragments were digested with 5.5 mg/ml Collagenase P (Boehringer Mannheim, Germany) in KRH containing 1% BSA for 12 minutes at 37°C under continuous agitation in a water bath. For the separation of the single cells from the debris, the mixture was centrifuged at 500 rpm for 30 seconds at 20°C. The supernatant (containing the single cells) was aspirated. The single cell suspension was washed 3 times with KRH containing 1% BSA for 5 minutes at 4°C. After washing, the cells were resuspended in KRH containing 1% BSA. Cell viability was determined with Trypan blue dye exclusion, viability was always 90%. After this procedure the single cells were stained for c-met or c-kit in order to be able to sort the cells.
Staining for c-kit and c-met
According to standard methods the following procedure was performed for c-met or c-kit cell-sorting. First the single cells were centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was pre-incubated with undiluted swine serum for 30 minutes. After incubation the cells were washed with PBS containing 0.5% BSA and 0.1% sodium azide. Subsequently, the mixture was centrifuged at 1500 rpm for 5 minutes at 4°C. Next the cells were incubated with the primary antibody anti-c-met (1:100) (H-190, Santa Cruz Biotechnology) or anti-c-kit (1:100) (C-19, Santa Cruz Biotechnology) for 60 minutes. Then the cells were incubated with secondary antibody swine-anti-rabbit FITC (1:50) (DakoCytomation, Denmark) for 30 minutes in the dark. The whole procedure was performed on ice. Positive selection for c-met or c-kit was performed using a Fluorescence Activated Cell Sorter (FACS, MoFlo flow cytometer) (Cytomation, USA). The sorted cells were collected in a tube with sterile RPMI 1640 containing 10% FCS, and 10 mg/ml Gentamycin (GIBCO).

PKH26 labeling for c-met and c-kit sorted cells
Freshly isolated c-met or c-kit positive cells were stained with the PKH26 Red Fluorescent Cell linker Kit (Sigma) according to the manufacturer’s protocol. Briefly, the cells were collected and centrifuged at 1500 rpm for 5 minutes. After centrifuging the cells, the supernatant was aspired leaving no more than 25 μl of supernatant on the pellet. The cells were resuspended in 1 ml of Diluent C. Next 1 ml of diluted PKH26 dye in Diluent C (1:1000) was added to the cells. The sample was mixed by gently pippeting and the sample was incubated for 5 minutes. The staining reaction was stopped by adding 1% BSA to the sample and incubating the sample for 1 minute. To dilute the sample an equal volume of medium, sterile RPMI 1640 containing 10% FCS, and 10 mg/ml Gentamycin (GIBCO), was added and the sample was centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated and the pellet with cells was washed 2 times with medium, sterile RPMI 1640 containing 10% FCS, and 10 mg/ml Gentamycin (GIBCO), and 1 time with medium, sterile RPMI 1640 and 10 mg/ml Gentamycin (GIBCO). After the last wash the cells were resuspended in 150 μl of sterile RPMI 1640 and 10 mg/ml Gentamycin (GIBCO). The whole procedure was performed at room temperature.
**Induction of mild diabetes rats**
The pancreata of the rats were slightly damaged by giving them a low dose of 40 mg/kg STZ (Zanosar) 2 days prior to the infusion. The rats were anaesthetised with a combination of isoflurane, oxygen, and NO\textsubscript{2}. After the rats were anaesthetised, they were injected with 40 mg/kg STZ via the tail vein.

**Injection of c-met or c-kit positive cells via the pancreatic artery**
At the day of infusion the rats were injected with 400,000 isolated c-met (n=5) or c-kit (n=5) positive cells from neonatal pancreata which were labelled with the PKH26 Red Fluorescent Cell linker Kit. The rats were anaesthetised with a combination of isoflurane, oxygen, and NO\textsubscript{2} with a small incision in the abdomen, the abdominal cavity was opened and the pancreas was lifted. Next the pancreatic artery was located near the pancreatic duct and the artery was punctured injected for the infusion of the cells. Sham surgery was performed on control rats which were treated in a similar fashion, except that only medium was injected into the pancreatic artery.

**Suppression of immune system**
Since we used outbred wistar rats, we had to suppress the immune system of the infused and control rats. Therefore, the rats were injected with Ciclosporin A (5 mg/kg) subcutaneously. During the experiment the rats obtained 2 injections of Ciclosporin A, i.e., on the day of injection, and 2 days after the injection.

**Glucose measurement**
The blood glucose levels of the rats were measured by tailsnapping at 2 days before the injection, the day of the injection and 4 days after the injection (day of termination). The measurement was done by using the ACCU-CHEK Advantage blood glucose meter and the accessory glucose strips.

**Isolation of pancreata from injected and control animals**
Pancreata were obtained from c-met and c-kit injected-STZ treated or non-injected/STZ-treated rats. Therefore, these rats were anaesthetised with a combination of isoflurane, oxygen, and NO\textsubscript{2} and the pancreas was removed by laparotomy and subsequently snap-frozen in liquid nitrogen and stored at -80°C until sectioning.
Visualization of c-met and c-kit infused cells
Pancreata of c-met and c-kit injected STZ-treated or non-injected/STZ-treated rats were sectioned at 4 μm and stored at -80ºC until use. Tissue cryosections were air dried and then fixed in acetone for 10 minutes, followed by air drying for 30 minutes. Then the sections were incubated with 4’, 6-diamidino-2-phenylindole (DAPI) (1:2500) (Roche) for 10 minutes and mounted with Citifluor (Agar Scientific). The whole procedure was performed at room temperature. Analysis was performed using the Leica DMRXA fluorescent microscope and Leica Qwin Pro software.

Immunohistological staining for insulin
Pancreata of c-met or c-kit injected STZ-treated and non-injected STZ-treated rats and non-injected non-STZ-treated rats were sectioned at 4 μm and stored at -80ºC. Tissue cryosections were air dried and then fixed in Bouin for 10 minutes and followed by washing with PBS. Next, the sections were incubated with primary antibody anti-insulin (1:750) (Sigma-Aldrich, The Netherlands) for 60 minutes. After three times washing with PBS, The endogenous peroxidase activity was blocked by 30 minutes incubation in PBS with 30 % H₂O₂. Thereafter, the sections were incubated with normal goat serum for 30 minutes. Then the sections were incubated with secondary antibody goat-anti-mouse-IgG1-HRP (1:50) (DakoCytomation, Denmark) for 30 minutes. Peroxidase activity was visualised by applying 3-amino-9-ethyl-carbazole (Sigma, Steinheim, Germany). Background staining was performed with hematoxylin-eosin staining for 3 minutes. The whole procedure was performed at room temperature. The sections were analysed using Leica DMLB light microscope (including Leica DC 300 camera).

RESULTS
As shown in figure 1A, both the infusion of c-kit and c-met positive cells induced a decrease in the blood glucose levels at 4 days after infusion of 400,000 cells. In this graph, we show delta values, due to the large variation in the glycaemic levels after STZ treatment. Figures 1B and 1C show the individual glycaemic levels of the animals. We found as expected a more profound effect on the glucose levels in rats with a high glucose level at the day of injection of the cells. Also in the animals with a less pronounced increase in glycaemia we found a decrease in
glycaemic values after infusion of c-met and c-kit cells, but these effects were less pronounced. This should not be interpreted as a suggestion that the infusion did not contribute to regeneration as we found effects in immunocytochemistry (vide infra). When compared with the rats that received a sham-surgery the difference in basal glycemic levels did not show such clear improvement. In addition, we observed that neither the control groups nor the infused rats show a change in body weight (data not shown).

**C-met and c-kit infused cells in the pancreas**
The fluorescent PKH26 labelling allowed us to study the accumulation of the infused cells in the diabetic pancreas. We found large amounts of the infused c-met and c-kit positive cells in the pancreas, even at 4 days after injection of the cells via the pancreatic artery (Figure 2A). In c-met infused pancreata, PKH26 fluorescent cells were found scattered around in the pancreas (Figure 2B), while also c-met injected cells were found in the islets of Langerhans (Figure 2C). This was quite similar in the STZ-treated c-kit-infused pancreas. Also here we found PKH26 fluorescent cells scattered through the pancreas (Figure 2D), while also c-kit positive cells were found in the islets of Langerhans (Figure 2E).

**Insulin staining**
Since the above mentioned effects may result in increased amounts of insulin in the pancreas we stained slices of the pancreas for insulin. Figure 3 shows a comparison between control non-injected-non-STZ treated animals, sham-treated STZ rats, c-kit, and c-met treated animals. We did not observe any difference in intensity of staining.
Regenerative effect of c-met and c-kit carrying cells on the endocrine pancreas

A

C-kit infusion

Days after infusion

Delta glucose (mM)

B

Control infusion glucose concentration (mM)

Glucose concentration (mM)

control group day -2  control group day 0  control group day 4

C-kit infusion glucose concentration (mM)

Glucose concentration (mM)

infusion group day -2  infusion group day 0  infusion group day 4
Figure 1: Glucose concentrations of c-kit and c-met infused rats before injection of streptozotocin (day -2), 2 days after injection of streptozotocin (i.e. just before injection of c-kit and c-met positive cells; day 0) and 4 days after the injection of c-met or c-kit positive cells (day 4) (A). The delta glucose values. (B and C) Absolute individual glucose values. The delta figure shows that both the infusion of c-kit and c-met positive cells induced a decrease in the blood glucose levels at 4 days after injection. The individual glucose values showed that 3 out of 5 c-kit- and 2 out of 5 c-met infused rats showed a decrease in the blood glucose levels at 4 days after injection of 400.000 c-kit or c-met positive cells (B and C). On the day of injection animals that received an injection of c-kit positive cells had a mean glucose level of 10.56 ± 0.88 mM. After 4 days the mean glucose level was decreased to 7.94 ± 1.83 mM. Animals receiving an injection with c-met positive cells had a mean glucose level of 11.38 ± 1.53 mM. After 4 days the mean glucose level was decreased to 10.66 ± 1.23 mM. Some animals that received a sham-surgery showed some differences in basal glucose levels.
Figure 2: Photomicrographs of pancreata retrieved from c-met and c-kit infused cells in the pancreas. The red staining is the fluorescent staining of PKH, with which the c-met and c-kit positive cells were stained. The staining represents the c-met or c-kit infused cells. Large amounts of the infused c-met and c-kit positive cells in the pancreas were observed at 4 days after injection. Figure A shows a representative of the pancreas of sham operated animals (A). In c-met infused pancreata, the cells were found scattered around in the pancreas (B) and they were also found in the islets of Langerhans (C). Like the c-met infused pancreas, c-kit positive cells were also scattered through the pancreas (D) and were found in the islets of Langerhans (E). Original magnification 200X.
Figure 3: Representative photomicrographs of the non-injected-non-STZ treated rat pancreata and STZ treated rat pancreata after immunostaining for insulin. No difference in intensity of insulin staining was observed. Original magnification 200X

DISCUSSION

Our data suggests that infused, c-met and c-kit carrying cells isolated from the neonatal rat pancreas may contribute to regeneration of the damaged endocrine pancreas. This provides an interesting perspective for the possible future use of these cells in the therapeutical induction of regeneration of the endocrine pancreas. Since the present study is only a pilot experiment, we realise that for a proper interpretation of the data a number of questions still needs to be answered. Our study demonstrates a number of interesting observations that indicate the involvement of cells carrying c-met or c-kit in the regeneration and ontology of the endocrine pancreas. First, we show that after intravenous infusion a large group of cells home back to the damaged pancreas. Obviously the damaged pancreas has a chemo-attractive potential for at least a portion of the c-kit and c-met carrying cells.
Secondly, a striking observation was that we sometimes found accumulation of the cells in structures that had many characteristics of so-called buds. These buds are found in the developing and regenerating pancreas. Small groups of ductal epithelial cells that have recently been identified as cells having precursor cell characteristics. These buds have a strong attraction for the infused c-met and c-kit cells as we found especially around these structures accumulation of the infused PKH26 fluorescent cells.

The specific accumulation around these structures and its absence in the ductal system suggest that the group of cells homing to these structures contribute to regeneration. If the majority of these cells would be precursor cell and contribute to regeneration by direct differentiation to beta-cell we would expect accumulation of the cells in the ductal system. This has recently been identified as the primary site where regeneration occurs. In order to confirm it is mandatory to demonstrate the presence of essential transcription factors.

In conclusion, our study demonstrates that pure population of c-met and c-kit carrying neonatal rat cells can contribute to the regeneration of the adult pancreas. The specific accumulation around budding ductal structures suggests that at least a portion of the cells is involved in guiding or facilitating regeneration of the endocrine pancreas. As our study suggests that this population of cells has an important role in new formation of the endocrine pancreas our future studies will focus on the role of these cells in the developing, regenerating, and growing endocrine pancreas.
REFERENCE LIST
