

Original Article

In Vivo Differentiation of Human Umbilical Cord Blood-Derived Cells into Insulin-Producing β Cells

(diabetes mellitus / insulin / pancreas / β cell / islets / stem cells / umbilical cord blood / differentiation / *in vivo* / radiation/transplantation)

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Abstract. In our study we confirmed the potential of human umbilical cord blood cells to differentiate into insulin-producing cells following transplantation into immunocompromised mice. The average number of C-peptide-positive human cells per animal was 18 ± 13 as assessed by immunofluorescence staining and fluorescence *in situ* hybridization specific for human ALU sequence. Differentiation into insulin-producing cells was further confirmed by reverse transcription-polymerase chain reaction specific for human insulin mRNA. Successful differentiation required sublethal irradiation of xenogeneic recipient at least at a dose of 3 Gy. However, transplantation of human umbilical cord blood cells did not improve hyperglycaemia in diabetic animals. The results of our study show that human umbilical cord blood may be considered as a potential source of stem cells for treatment of diabetes mellitus.

Introduction

Despite advances in the treatment of diabetic patients, diabetes remains one of the most serious health care

problems of our civilization. Clinical islets or pancreas transplantations are the only available therapies able to establish insulin independence and long-lasting normoglycaemia (Shapiro et al., 2000; White et al., 2009). However, the lack of donors limits the application of this therapy for all type 1 diabetic patients in need.

The discovery of stem cells and their successful differentiation into insulin-producing β cells gave a new hope to all diabetic patients. Within last few years various types of stem cells able to give rise to the pancreatic β cells have been identified. They include embryonic and foetal stem cells (Zhang et al., 2005; D'Amour et al., 2006) as well as adult stem cells derived from pancreas, liver, bone marrow and central nervous system (Bonner-Weir et al., 2000; Yang et al., 2002; Ianus et al., 2003).

In addition to these "traditional" sources of adult stem cells, umbilical cord blood-derived stem cells have emerged as a new potential source for cell-based therapies. The main advantages of human umbilical cord blood (HUCB) include plentiful availability, safe and non-invasive procedure of collection, possible expansion and modification of cells *in vitro* and an existing network of umbilical cord blood banks, a large-scale source of cells that allows matching the donor and host human leukocyte antigen (HLA) systems. HUCB, highly enriched for haematopoietic stem cells, has already been successfully applied for the treatment of various blood diseases (Roche et al., 2000; Laughlin et al., 2004). Moreover, several recent reports have shown that some of the HUCB cells are able to differentiate into multiple cell types of non-haematopoietic origin (Kogler et al., 2004; McGuckin et al., 2005). These findings suggest that umbilical cord blood contains multipotent stem cells or primitive progenitors that might have the potential to differentiate into cells of non-haematopoietic phenotype, including pancreatic β cells.

Denner as the first demonstrated successful *in vitro* differentiation of HUCB stem cells into insulin- and C-peptide-producing cells (Denner et al., 2007). Two other groups lately reported similar results using differ-

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Abbreviations: DAPI – 4,6-diamidino-2-phenylindole, EBSS – Earle's balanced salt solution, FISH – fluorescence *in situ* hybridization, HLA – human leukocyte antigen, HUCB – human umbilical cord blood, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, MNCs – mononuclear cells, NK – natural killer, NOD/SCID – non-obese diabetic/severe combined immunodeficient/ β_2 -microglobulin null mice, PCR – polymerase chain reaction, RT-PCR – reverse transcriptase polymerase chain reaction.

ent approaches (Sun et al., 2007; Gao et al., 2008). Sun's group used a specific subpopulation of HUCB cells expressing embryonic markers Oct-4 and SSEA-4. These cells were differentiated by a protocol using only nicotinamide and extracellular matrix proteins laminin and fibronectin. Gao's group worked with HUCB-derived mesenchymal stem cells and employed a more complicated protocol including retinoic acid, nicotinamide, exendin-4 and extracellular matrix proteins. In spite of successful differentiation into insulin-producing cells, the secretion of insulin in response to increased glucose levels was not significantly higher than that at basal conditions. This phenomenon is quite common in case of *in vitro* derived β cells and may be explained by immaturity of this cell type (D'Amour et al., 2006).

In vivo differentiation of HUCB cells into the pancreatic β cells has so far been demonstrated only by Yoshida et al. The presence of human insulin-producing cells in mouse pancreatic tissue after transplantation of T cell-depleted HUCB mononuclear cells (MNCs) into newborn non-obese diabetic/severe combined immunodeficient mice have been reported by the authors. However, the average number of HUCB-derived insulin-producing cells per total number of islet cells was only 0.65%. The low rate of human β cells within mouse pancreas could be explained by a non-diabetic status of the animals (Yoshida et al., 2005). Under diabetic conditions, the demand for neogenesis of insulin-producing cells might be increased and the higher rate of HUCB cell differentiation might represent a compensatory effect in face to a decreased β -cell mass.

In light of these results we decided to investigate the conditions that stimulate *in vivo* differentiation of HUCB mononuclear cells into insulin-producing cells. Survival, homing and differentiation of HUCB cells were studied in athymic nude mice, which do not reject xenografts and thus represent a suitable model for transplantation of human cells. We tested the effect of the whole body irradiation, which had been shown to increase homing and engraftment of human cells in transplanted mice (Becker et al., 2002). Finally, we also examined the possibility to treat the streptozotocin-induced diabetes by transplantation and possible differentiation of HUCB cells into insulin-producing cells.

Here we report that HUCB-derived mononuclear cells convincingly do have the potential to differentiate into a β cell-like phenotype, though, with the use of current protocols, only at a very low rate that still does not reach a therapeutic significance.

Material and Methods

Study design

For the purpose of our study mice were divided into the groups based on the applied radiation dose and eventual induction of diabetes (Table 1). All animals with the exception of the control group were injected with 10^7 unpurified HUCB mononuclear cells into the tail vein. Mice in groups 2, 3, 5 and 6 underwent total body irradiation one day prior to the application of HUCB cells at the dose of 1 (groups 2 and 5) or 3 Gy (groups 3 and 6). In groups 4, 5 and 6 diabetes was induced by streptozotocin three days prior to the application of HUCB cells. In diabetic animals, fed blood glucose was monitored at weekly intervals during the experiment. Animals were sacrificed at the end of 4th week and tissue samples were collected for further analysis.

Isolation of HUCB Cells

Samples of HUCB (40–120 ml) were obtained at the end of physiological delivery. At the admission to hospital, all donors signed an informed consent approved by the Institutional Ethical Committee of the Institute for Clinical and Experimental Medicine and Thomayer Teaching Hospital. Samples of HUCB were collected into standard blood donor bags containing 15 ml of citrate phosphate dextrose (Baxter Healthcare, Deerfield, IL). HUCB was diluted in a ratio 1 : 2 with Earle's balanced salt solution (EBSS) (Sigma-Aldrich, Steinheim, Germany) and centrifuged at 400 g for 20 min at 4 °C on a layer of Ficoll-Hypaque 1.077 (Sigma-Aldrich). Mononuclear cells (MNCs) at the interface of supernatant were washed twice with EBSS. Viability was determined by the trypan blue dye exclusion method. MNCs were resuspended in Iscove's modified Dulbecco's medium (Sigma-Aldrich) containing 20% foetal bovine serum (Biochrom, Berlin, Germany) and cryopreserved with 10% (vol/vol) DMSO (Sigma-Aldrich).

Mice

Female nude athymic mice (CrI:CD1-nu strain, An-Lab, Prague, Czech Republic) were maintained under defined flora with irradiated food and sterile water in sterile cages at the animal facility. All experiments were approved by the Committee for Animal Ethical Treatment of the Institute for Clinical and Experimental Medicine.

Table 1. Groups of animals based on the radiation dose and diabetes induction

	Radiation dose (Gy)	Induction of diabetes	HUCB transplantation
Group 1	0	No	Yes
Group 2	1	No	Yes
Group 3	3	No	Yes
Group 4	0	Yes	Yes
Group 5	1	Yes	Yes
Group 6	3	Yes	Yes
Group 7 (control group)	0	No	No

Induction of diabetes

Athymic mice aged 6–8 weeks were treated with a single intravenous dose of 250 mg/kg streptozotocin (Sigma-Aldrich) freshly dissolved in citrate buffer (0.05 mM, pH 4.5). Mice were considered as diabetic when non-fasting blood glucose levels were > 16 mmol/l on three consecutive days.

HUCB Transplantation

Prior to transplantation, cryopreserved HUCB MNCs were thawed, counted and tested for viability by the trypan blue dye exclusion method. The amount of 10^7 MNCs was injected intravenously into the tail vein of non-diabetic or diabetic mice (8 weeks old). Two groups of diabetic and two groups of non-diabetic mice were conditioned with 1 or 3 Gy of total body irradiation one day prior to the transplantation.

Pancreatic Islet Isolation

Mouse pancreatic islets from the HUCB cell recipients and control animals were isolated using the collagenase digestion method as previously described (Berkova et al., 2005). The pancreases were injected with 1 ml of collagenase at a concentration of 2 mg/ml (Sevapharma, Prague, Czech Republic) and incubated at 37 °C for 15 min in a total of 10 ml of digestion solution under constant shaking. Islets were subsequently washed three times in Hank's balanced salt solution (HBSS) (Biochrom) with bovine serum albumin (BSA) (5 mg/ml) and purified with the use of discontinuous gradients of Ficoll-diatrizoic acid (Sigma-Aldrich). The solution densities of discontinuous Ficoll gradient ranged from 1.034 to 1.1162 g/ml with the densest solution at the bottom of density gradient. During centrifugation, islets migrated to the interface between 1.070 and 1.080 g/ml. The remaining cellular material from the denser layer was also pooled and further processed for gene expression analysis.

FISH and Immunofluorescence Analysis

After the pancreatic tissues were harvested from the recipient mice, the tissues were fixed with Bouin's solution (Sigma-Aldrich) for 2 h at room temperature. The tissues were rinsed with PBS, embedded in OCT mounting medium (Bayer Corp, Pittsburgh, PA), frozen in liquid nitrogen, and stored at -80 °C.

After several washes in PBS, 8- μ m sections of frozen tissue were incubated in a solution containing 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 20 min. Antigen retrieval method for immunofluorescence staining was performed prior to fluorescence *in situ* hybridization (FISH). Slides were heated twice in 0.01 M sodium citrate (Sigma-Aldrich), pH 6.0, in a microwave oven for periods of 4 min at the maximal power setting (900 W) with 120–140 s of boiling. Slides were incubated in blocking solution containing 5% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA). After dehydration in 70%, 90% and 100% ethanol for 2 min each, slides were incubated in 50% formamide/2 \times SSC denaturing solution for 5 min at 75 °C. After denaturation, the slides were incubated overnight at 37 °C with Alexa555-conjugated ALU-species-specific probe diluted in hybridization buffer (50 ng/100 μ l). An Alu-specific probe binds the ALU-sequence that is present only in primate genomes. Alu sequences are about 300 base pairs long and form about 10 % of the human genome. The sequence of the Alu-specific probe is given in Table 2.

After the hybridization, slides were washed three times in 50% formamide/2 \times SSC for 5 min each at 42 °C. For immunofluorescence co-staining, slides were blocked in 5% donkey serum (Jackson Immunoresearch Laboratories) diluted in 0.2% Triton X-100, 0.1 M glycine (Sigma-Aldrich) and PBS for 1 h at room temperature to prevent unspecific binding. Incubation with rabbit anti-human C-peptide antibody (Linco-Research, St. Charles, MO) diluted 1 : 200 was performed in the same blocking solution for 1 h at 37 °C. After intensive washing with PBS, slides were incubated with the secondary antibody Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probe, Eugene, OR) diluted in the blocking solution for 1 h at 37 °C. 4,6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at a concentration 5 μ g/ml was used to label the nuclei for 10 min at 37 °C. After rinsing with PBS, sections were mounted with antifade solution and examined with a fluorescence microscope (BX 41, Olympus, Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction

RNA was isolated from pancreatic islets and remaining pancreatic tissue of the recipient mice using Rneasy Plus Mini Kit (Qiagen, Hilden, Germany). Isolated RNA was treated with DNase using RNase-Free DNase Set

Table 2. Primers and Alu-specific probe sequences used for the RT-PCR and FISH analysis

Primer	sequence	product size (bp)	annealing temp. (°C)	cycle number
human insulin forward	agccgcagcctttgtgaac	141	63	45
human insulin reverse	agctccacctgccccac	141	63	45
mouse insulin forward	ctataatcagagaccatcagcaagc	344	60	35
mouse insulin reverse	gtagaggagcagatgctgg	344	60	35
human GAPDH forward	gagtcaacggattggtcg	141	59	40
human GAPDH reverse	catgggtggaatcatattgg	141	59	40
Alu-specific probe	cctgtaatcccagctactcgggagg ctgaggcaggagaatcgtgaacc		37	

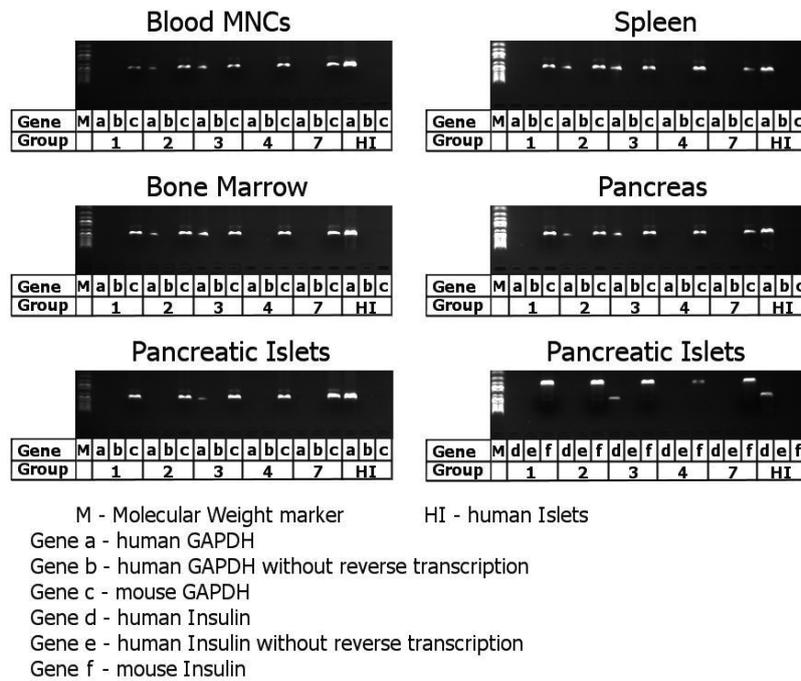


Fig. 1. RT-PCR analysis of gene expression in mouse tissues. Transcripts of human *GAPDH* (a) and insulin (d) were analysed and compared with transcripts of mouse *GAPDH* (c) and insulin genes (f). The products of PCR reaction without reverse transcription served as a negative control (b, e). Human islet RNA was used as a positive control.

(Qiagen) and 1 μ g of RNA was reverse transcribed with Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. cDNA was amplified using HotStar-Taq Master Mix Kit (Qiagen). Gene-specific primer pairs, annealing temperatures, and product sizes are listed in Table 2. All of the primers span at least one of the introns to prevent false-positive results. PCR products were separated and visualized on 2% agarose gel containing ethidium bromide.

Results

Successful HUCB transplantation was confirmed by detection of human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA in samples isolated from mouse blood MNCs 4 h after the transplantation (Fig. 1). Further detection of human cells within mouse tissues was performed four weeks after the application of HUCB cells using the primers specific for human *GAPDH*. The expression of human *GAPDH* was detected only in samples derived from mice exposed to both 1 and 3 Gy radiation doses. Human *GAPDH* was detected in all of the examined tissues (spleen, bone

marrow, blood MNCs and pancreatic tissue). No expression of human *GAPDH* was detected in tissue samples from mice that had not been subjected to total body irradiation (Table 3, Fig. 1).

In order to evaluate the potential of HUCB-derived cells to differentiate into human insulin-producing cells we performed RT-PCR analysis of human insulin gene expression. We used RNA isolated from fresh pancreatic tissue and Langerhans islets of the recipient mice. The expression of human insulin mRNA was observed exclusively in pancreatic tissue derived from mice irradiated with 3 Gy. We did not detect any expression of human insulin gene either in the isolated islets or pancreatic tissue derived from mice irradiated with only 1 Gy or in samples from unirradiated mice. For the detection of human insulin mRNA, we designed the forward and reverse primers that specifically amplified human but not mouse insulin cDNA or human gDNA. The amplified products derived from the recipients' pancreata were clearly seen on agarose gel (Fig. 1). The product size corresponded to the expected size of PCR reaction product amplified by specific primers. These results indicate that human insulin was produced by donor

Table 3. Detection of human cells within examined mouse tissues in the study groups

Tissue	Blood	Spleen	Bone Marrow	Pancreas	Islets	Human β cells
Group 1	-	-	-	-	-	-
Group 2	+	+	+	-	-	-
Group 3	+	+	+	+	+	+
Group 4	-	-	-	-	-	-
Group 5	N/A	N/A	N/A	N/A	N/A	N/A
Group 6	N/A	N/A	N/A	N/A	N/A	N/A
Group 7	-	-	-	-	-	-

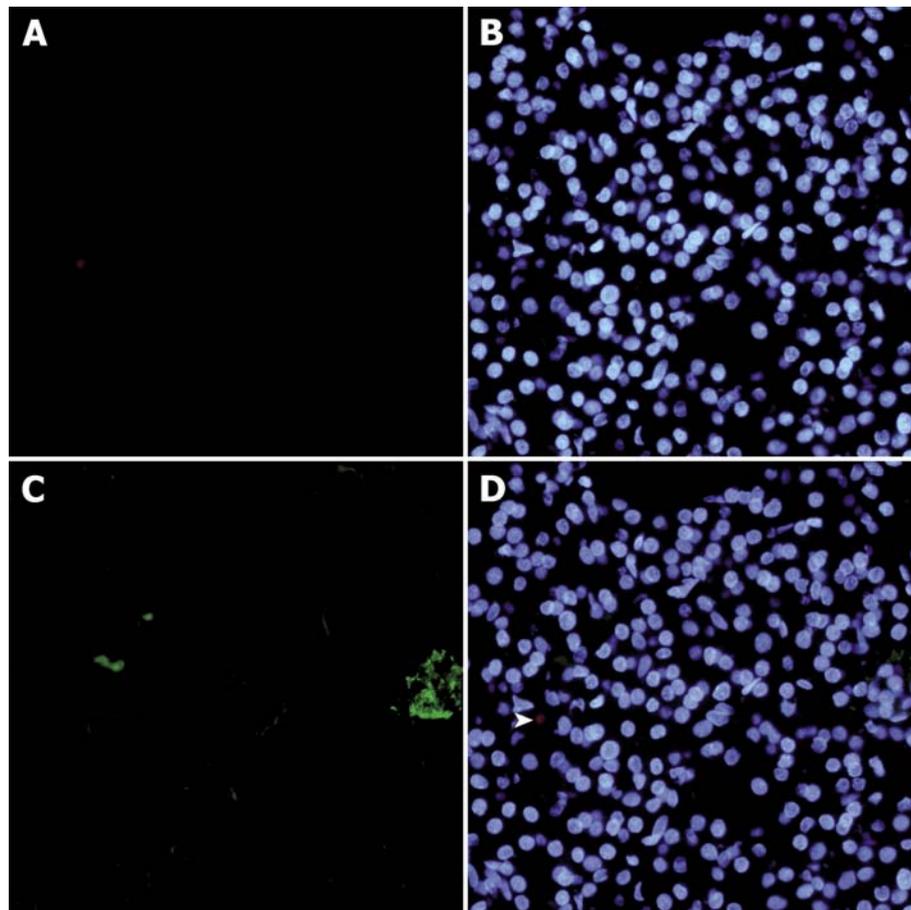


Fig. 2. FISH and immunofluorescence staining of mouse pancreatic tissue four weeks after the irradiation with 1 Gy. (A) FISH for human ALU sequence (red), (B) DAPI staining of nuclei (blue), (C) C-peptide immunofluorescence staining (green) and (D) merged images A–C. HUCB-derived ALU sequence-positive cell is shown (arrowhead).

HUCB-derived cells in the recipient pancreas at the RNA level. Insulin-specific cDNA in the tissue obviously did not originate from passenger haematopoietic cells as this reaction was negative in all starting MNC samples.

The presence of human cells within pancreatic tissue was further confirmed by the fluorescence *in situ* hybridization specific for the human ALU sequence. Rare human cells were detected within the acinar tissue, islets and also in the pancreatic ducts of recipient mice (Fig. 2 and Fig. 3).

In the next step we performed immunofluorescence labelling of human C-peptide in combination with human ALU sequence-specific FISH to confirm the expression of human insulin at the protein level. C-peptide-positive human cells were clearly demonstrated within the pancreatic islets of mice irradiated with 3 Gy, although only in a low number (Fig. 3). The average number of C-peptide-positive human cells per animal was 18 ± 13 . The whole pancreata were cut into 10- μ m sections and the investigation was performed in all of them.

Transplantation of HUCB into severely diabetic animals did not lead to metabolic improvement. All seven animals progressively wasted and died before the end of

the study period. Therefore, the presence of human cells within their tissues could not be studied.

Discussion

For their availability and easy storage, umbilical cord stem or precursor cells have been regarded as a promising source for cellular therapy of diabetes, though the scientific and practical reasons for this hope have still been lacking. The ability to differentiate into the β -cell phenotype undoubtedly depends on selection of the right cell type, on its culture conditions and last but not least, on the post-transplant care of the recipient. Cure or significant improvement of experimental diabetes by HUCB transplantation has not been achieved in any study so far. However, the results of our study demonstrated that the potential of HUCB mononuclear cells to engraft in the host pancreas and to differentiate into insulin-producing cells does exist. The origin of the transplanted cells was confirmed not only by fluorescence *in situ* hybridization staining for specific human DNA sequence in combination with immunofluorescence staining for human insulin in transplanted immunocompromised mice, but also by highly sensitive RT-PCR detection of human insulin mRNA.

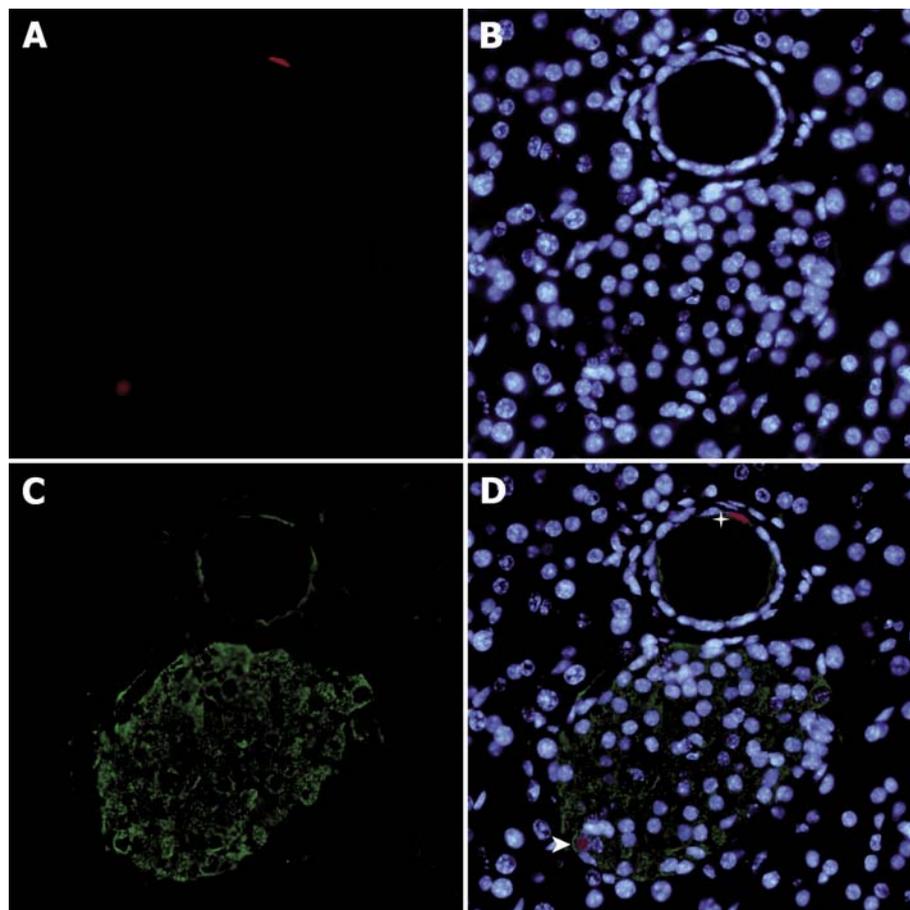


Fig. 3. FISH and immunofluorescence staining of mouse pancreatic tissue four weeks after the irradiation with 3 Gy. (A) FISH for human ALU sequence (red), (B) DAPI staining of nuclei (blue), (C) C-peptide immunofluorescence staining (green) and (D) merged images A–C. HUCB-derived ALU sequence and C-peptide-positive cell in pancreatic islet is shown (arrowhead) and HUCB-derived ALU sequence-positive cell in pancreatic duct is shown (star).

Our results are in agreement with those published by Yoshida's group (Yoshida et al., 2005). They also observed the presence of human insulin-producing cells in pancreatic tissue after the transplantation of HUCB mononuclear cells into the normoglycaemic mice. In their experiment, the rate of differentiation was significantly higher in comparison with our study. The difference between the numbers of differentiated human β cells may be due to different mouse strains used in the studies as well as the type and number of transplanted cells. Yoshida et al. (2005) used non-obese diabetic/severe combined immunodeficient/ β_2 -microglobulin null mice (NOD/SCID/ β_2 m null), which lack mature T as well as B cells and show extremely low activity of natural killer (NK) cells. This profound immunological incompetence obviously enables high engraftment rates of human cells in NOD/SCID/ β_2 m null mice (Ishikawa et al., 2002). In our study we used the CD-1-nu/nu nude mouse strain, which lacks only mature T lymphocytes but still shows functional antibody-producing B and NK cells. The nude mouse strain may provide a lower engraftment potential for xenografts in comparison with the NOD/SCID strain as demonstrated by transplanta-

tion of foetal porcine pancreatic tissue into the NOD/SCID and nude mice (Tuch et al., 1999).

We have therefore decided to use whole-body irradiation in the effort to increase engraftment efficiency of human cells. Without irradiation pretreatment we found neither any human insulin-producing cells nor any human cells in pancreatic or any other examined tissues. Conversely, after a 1 Gy total body irradiation we detected expression of human GAPDH in haematopoietic organs such as spleen, blood and bone marrow using PCR detection. Nevertheless, we did not detect any expression of human insulin gene in pancreatic tissue. Further increase of the radiation dose up to 3 Gy led not only to the engraftment of human cells in pancreatic tissue but also allowed differentiation of human cells into insulin-producing cells. While most of the human cells within the host pancreatic tissue were insulin-negative, we convincingly detected a few insulin-positive human cells in the pancreatic islets. Noteworthy is also the presence of human cells in pancreatic ducts. Since islet cell neogenesis is thought to occur in pancreatic ducts (Slack, 1995), it could be speculated that human cells present in pancreatic ducts may undergo differentiation

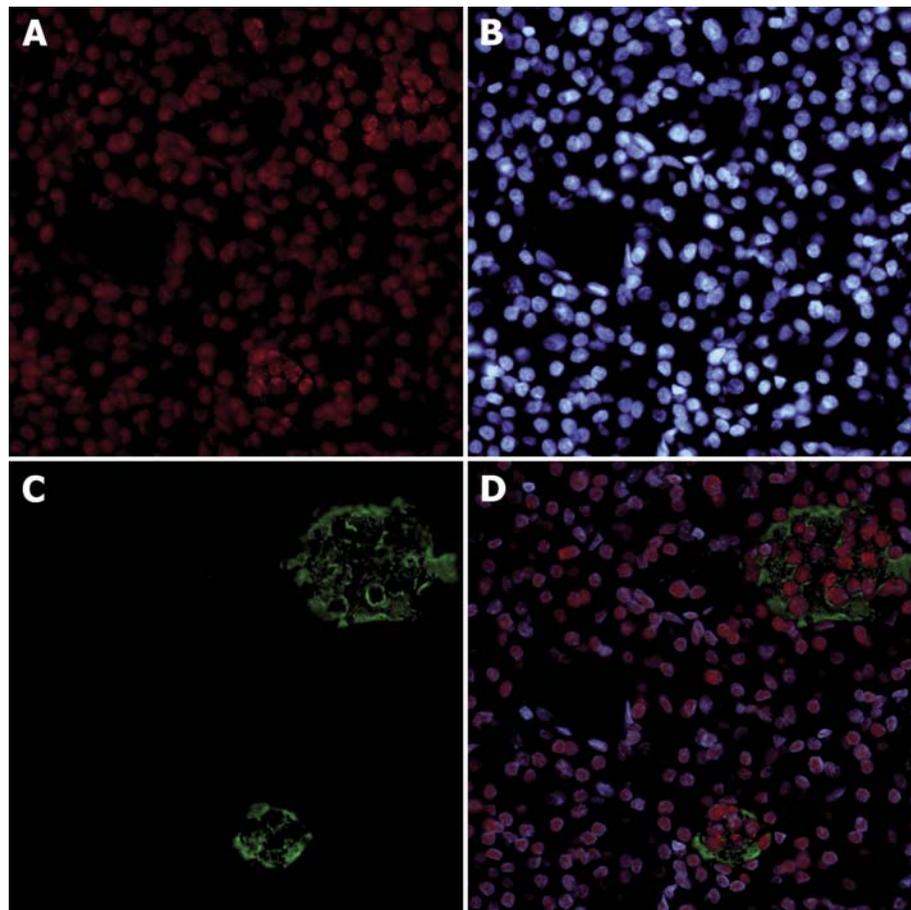


Fig. 4. FISH and immunofluorescence staining of human pancreatic tissue. (A) FISH for human ALU sequence (red), (B) DAPI staining of nuclei (blue), (C) C-peptide immunofluorescence staining (green) and (D) merged images A–C.

into the endocrine cells under the influence of pancreatic ductal niche.

In contrast to the paper of Yoshida et al. (2005), the presence of human insulin-producing cells in the host pancreas and in isolated pancreatic islets was undoubtedly confirmed by specific PCR detection of human insulin mRNA. The lower number of human insulin-positive cells in our study may also be explained by the lower number of transplanted cells. Isolation of HUCB cells from one donor enabled transplantation of 10^7 MNC into 5–10 mouse recipients. Yoshida et al. reported application of 10^7 CD3⁺, CD4⁺ and CD8⁺-depleted MNCs, which represents approximately 35 % of all HUCB MNCs (Pranke et al., 2001). Therefore, we assume that for one mouse recipient they had to use a higher amount of HUCB than we used.

The explanation for successful engraftment and differentiation of HUCB cells in pancreatic tissue after the radiation treatment is not evident from our results. One could speculate that the tissue damage caused by radiation stimulates migration and engraftment of human stem cells into the injured organs. For example, depletion of the host immune system and haematopoietic stem cell pool by radiation-mediated myeloablation led to successful engraftment of donor stem cells in haematopoietic organs (Stewart et al., 1998). The positive ef-

fect of radiation on the engraftment of stem cells and tissue regeneration is not characteristic only for haematopoietic tissue, but also for neural (Marshall et al., 2005) and hepatic tissues (Guha et al., 2001).

Another condition which could have allowed engraftment of human cells is the radiation-mediated myeloablation of the mouse immune system. Depletion of host immune cells caused by myeloablation may have impaired xenograft rejection mediated by the remaining B and NK cells (Yoshino et al., 2000). Although we have no direct evidence how severe the depletion of the mouse immune system caused by irradiation was, we suppose that increasing doses of radiation allowed higher engraftment rate of HUCB cells into haematopoietic tissue with consequent restoration of the impaired immune system. A rather high prevalence of GAPDH-positive cells that we found in the peripheral blood and bone marrow supports this assumption.

An important stimulus for stem cell differentiation into insulin-producing cells might be the diabetes-related hyperglycaemia (Wang et al., 2005). In our study, HUCB administration did not cure or improve streptozotocin-induced experimental diabetes. Previous study conducted by Ende et al. has shown improvement in glycaemia and survival of diabetic mice after the transplantation of HUCB cells (Ende et al., 2004). However,

in their study the dose of HUCB cells was 200×10^6 , 20-fold higher than in our study. In this paper, however, no investigation of insulin-positive human cells had been performed. For further study, a longer time period and milder hyperglycaemia enabling survival will be necessary.

In conclusion, our study confirmed the possibility of human umbilical cord blood mononuclear cells to differentiate into human insulin-producing cells *in vivo*. However, successful differentiation occurred at a rather low rate and required preceding irradiation of the immunodeficient mouse recipient. Further investigation should focus on other potential conditions that might stimulate β -cell differentiation *in vivo* such as hyperglycaemia, administration of incretins, and on identification of the appropriate umbilical cord blood cell type suitable for transplantation.

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