Ex vivo gene transfer of viral interleukin-10 to BB rat islets: no protection after transplantation to diabetic BB rats

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Abstract

Allogeneic and autoimmune islet destruction limits the success of islet transplantation in autoimmune diabetic patients. This study was designed to investigate whether *ex vivo* gene transfer of viral interleukin-10 (vIL-10) protects BioBreeding (BB) rat islets from autoimmune destruction after transplantation into diabetic BB recipients. Islets were transduced with adenoviral constructs (Ad) expressing the enhanced green fluorescent protein (eGFP), α-1 antitrypsin (AAT) or vIL-10. Transduction efficiency was demonstrated by eGFP-positive cells and vIL-10 production. Islet function was determined *in vitro* by measuring insulin content and insulin secretion and *in vivo* by grafting AdvIL-10-transduced islets into syngeneic streptozotocin (SZ)-diabetic, congenic Lewis (LEW.1W) rats. Finally, gene-modified BB rat islets were grafted into autoimmune diabetic BB rats. Ad-transduction efficiency of islets increased with virus titre and did not interfere with insulin content and insulin secretion. Ad-transduction did not induce Fas on islet cells. AdvIL-10-transduced LEW.1W rat islets survived permanently in SZ-diabetic LEW.1W rats. In diabetic BB rats AdvIL-10-transduced BB rat islets were rapidly destroyed. Prolongation of islet culture prior to transplantation improved the survival of gene-modified islets in BB rats. Several genes including those coding for chemokines and other peptides associated with inflammation were down-regulated in islets after prolonged culture, possibly contributing to improved islet graft function *in vivo*. Islets transduced *ex vivo* with vIL-10 are principally able to cure SZ-diabetic rats. Autoimmune islet destruction in diabetic BB rats is not prevented by *ex vivo* vIL-10 gene transfer to grafted islets. Graft survival in autoimmune diabetic rats may be enhanced by improvements in culture conditions prior to transplantation.

Introduction

Islet transplantation represents a potential cure for type 1 diabetes although autoimmune and allogeneic immune responses limit the success in clinical transplantation. More specifically, TH1 cell-mediated immune responses are proposed as the key players in this scenario. Therefore, modulation of the immune response towards a less pathogenic helper T-cell type 2/regulatory T-cell (TH2/Treg)-related immune response may harbour therapeutic utility in preventing islet graft destruction.

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Interleukin-10 (IL-10) is a potent immunomodulatory cytokine that interacts with antigen presenting cells [1]. In addition, IL-10 inhibits production of monokines such as IL-1, IL-6, IL-8 and tumour necrosis factor (TNF)-α [2–5]. In contrast to cellular IL-10 (cIL-10), the Epstein-Barr virus (EBV)-encoded IL-10 homologue (vIL-10) displays these immunosuppressive properties with no stimulatory effects on natural killer (NK) cells and cytotoxic T-lymphocytes (CTLs) [6, 7]. Gene transfer approaches aimed at over-expressing IL-10 in allogeneic transplants may therefore be a promising therapy to suppress effective antigen presentation and to reduce graft immunogenicity and inflammation [8, 9].

Indeed, IL-10 gene transfer led to prolonged graft survival in different allotransplant models [6, 10–13]. Gene transfer in islet transplantation has been performed by several groups [14–21]. In these studies [14–21] the major focus was the mouse transplant model, in particular the non-obese diabetic (NOD) mouse. Both, genetic modification of the transplant recipient by adeno-associated vectors (AAV) expressing IL-10 (*e.g.* by intramuscular injection) [14–19] and local gene transfer of IL-10 into isolated islets [20, 21] were investigated. In the majority of the aforementioned studies, systemic IL-10 expression prevented or delayed diabetes onset in NOD mice [14–19] while local expression of IL-10 did not [20, 21]. However, systemic treatment of transplant patients might not be therapeutically viable, since side-effects of systemic gene transfer strategies must be considered. The genetic modification of islets is an interesting option since they can be cultured for weeks without significant loss of function. The major advantage of this approach is that the transplant recipient will not be challenged with recombinant virus vector thereby minimizing off-target side-effects.

While over-expression of IL-10 was investigated in diabetic mice, its potentially beneficial effects have not been studied in other diabetic animal models, *e.g.* the BioBreeding (BB) rat. We investigated if *ex vivo* gene transfer of vIL-10 to rat islets before transplantation is able to cure type I diabetes in BioBreeding/OttawaKarlsburg (BB/OK) rats. Prior to transplantation, transduction efficiency, induction of apoptosis, cDNA gene array analyses and specific function of gene-modified islets were determined *in vitro*. Additionally, islet function was tested *in vivo* by grafting AdvIL-10-transduced islets into syngeneic strep-tozotocin (SZ)-diabetic congenic Lewis (LEW.1W) rats to exclude islet damage by gene transfer. In this transplantation system neither allogeneic nor autoimmune islet destruction is evident. Furthermore, different culture times for gene-modified islets after transduction were investigated in an attempt to improve graft survival in autoimmune diabetic BB rats.

Material and methods

Animals

Congenic Lewis rats LEW.1W (RT1^{u}) and BB/OK (RT1^{u}) rats were bred in the Animal Laboratories of the University of Greifswald, Germany. Animals were kept under specific pathogen-free conditions [22] in a temperature- and humidity-controlled facility with lights on from 6 a.m. to 6 p.m. Standard rat chow containing 0.6% NaCl (Sniff, Soest, Germany) and sterilized water were provided *ad libitum.*

Prior to transplantation, LEW.1W rats were rendered diabetic by a single injection (i. p.) of 50 mg/kg b. w. SZ (Sigma, Taufkirchen, Germany). Hyperglycaemia remained untreated until transplantation. Diabetes duration was approximately 2 weeks. Diabetic BB/OK rats (age at diabetes onset 100–120 days, diabetes duration 2–3 weeks, plasma glucose >20 mmol/l) were treated once daily with insulin (Lente, NOVO Nordisk, Mainz, Germany) to maintain body weight. Plasma glucose was monitored weekly (Glucometer Elite, Bayer, Leverkusen, Germany). Islet donors of both rat strains were used at an age of 8–12 days.

Experiments were approved by a governmental committee on animal welfare.

Preparation of isolated islets of Langerhans

Donor pancreatic islets were prepared by a modified fractionated Collagenase Pan Plus (Serva, Heidelberg, Germany; EC 3.4.24.3; 1.26 PZU/mg; 0.6 mg/ml) digestion. Isolated islets were separated by centrifugation on a discontinuous Dextran gradient [23] and hand-picked.

Pre-culture of islets

Five hundred islets each were cultured in a final volume of 2.5 ml RPMI 1640 medium containing 10% foetal calf

serum (FCS, Gibco BRL, Life Technologies, Eggenstein, Germany) for 24 hrs at 37°C and 95% O_2/5% CO_2 [24].

Generation of adenoviral vectors

The generation of replication-defective recombinant adenovirus (Ad) type 5 vectors expressing the therapeutic gene vIL-10 has been previously described [13, 25–27]. The Ad vector expressing (α-1 antitrypsin (AAT) was a gift from Dr Mark Kay, Department of Pediatrics and Genetics, Stanford University School of Medicine, CA. The Ad vector expressing enhanced green fluorescent protein (eGFP) was a gift from Dr Alexander Flugel, Max-Planck-Institute for Neurobiology, Department of Neuroimmunology, Martinsried, Germany. AdAAT was used as a second construct since we wanted to transfer a gene the product of which is secreted by the cell such as cytokines.

Transduction of islets

Transduction of islets was performed after 24 hrs of pre-culture. Five hundred islets each were incubated for 90 min in RPMI 1640 medium containing 10% FCS and AdeGFP, AdAAT or AdvIL-10 at multiplicity of infection (MOI) 10, 100 and 1000 at 37°C in siliconized glass tubes. After five washings with Hank's balanced salt solution (HbSS), the islets were again cultured as described.

Fas (CD95) expression and determination of eGFP-positive islet cells

After transduction of islets and a subsequent culture period for 24 or 96 hrs, the islets were harvested and dispersed into single cells by fractionated digestion with Dispase II (Roche, Mannheim, Germany; EC 3.4.24.4, >0.5 U/mg) [28]. Cell number was counted using a Neubauer chamber. A minimum of 0.5×10^5 cells were either used for determination of eGFP-positive (*i.e.* green fluorescent) cells or for Fas expression on islet cells. EGFP-positive cells were measured without any further manipulation on a FACS Calibur (BD, Heidelberg, Germany) [29]. For determination of Fas-expressing cells, the cells were stained with a polyclonal rabbit anti-Fas antibody (StressGene, Victoria, Canada) for 15 min at 4°C in the dark in a final volume of 70 µl phosphate buffered saline containing 0.1% NaN_3 and 10% FCS (PBS). After washing with PBS, the cells were incubated with a FITC-conjugated AffiniPure F(ab′)2 fragment donkey anti-rabbit IgG (H+L) (Dianova, Hamburg, Germany) for 15 min at 4°C in the dark. After two washings with PBS, stained cells were analysed on an FACS Calibur

(BD, Heidelberg, Germany) by means of an air-cooled 488 nm 15 mW argon laser and detectors for forward scatter, 90° light scatter (side scatter), fluorescence 1 (FL1: FITC = green) and fluorescence 2 (FL2: PE = orange). The results were expressed as percentage of cells which were stained positive.

Detection of viral interleukin-10

Viral interleukin-10 (vIL-10) was measured by ELISA using a commercial kit specific for both human and viral IL-10 (Beckman Coulter, Krefeld, Germany). The absorption was determined at 450 nm using an Anthos ht II photometer (Anthos Labtec Instruments, Salzburg, Austria). Cytokine release into the culture medium was calculated per islet.

Measurement of islet insulin content and glucose-stimulated insulin secretion

For insulin content duplicates of five islets each were homogenized by ultrasonication in an acidified solution. Glucose-stimulated insulin secretion was performed after a pre-incubation period of 30 min in HbSS. Five islets each were incubated for 120 min in a modified Krebs-Ringer bicarbonate buffer at 37°C in an atmosphere of 95% O_2 and 5% CO_2 (pH 7.4) in the presence of 1.5 or 20.0 mmol/l glucose [30]. Insulin was measured by ELISA (Mercodia, Uppsala, Sweden) and calculated in pmol/islet.

Transplantation of islets

Two thousand control or transduced islets were grafted either 24 or 96 hrs after transduction under the left kidney capsule of the syngeneic diabetic recipients. SZ-diabetic LEW.1W rats received LEW.1W rat islets while autoimmune diabetic BB/OK rats received BB/OK rat islets. Blood was taken from the tail vein for determination of blood glucose, which was monitored three times weekly for 28 days and then once a week up to day 120. After 120 days of normoglycaemia, islet grafts were removed by nephrectomy and the animals were monitored for redevelopment of hyperglycaemia.

cDNA microarray

The relative expression of immune related genes was analysed with a customized PIQOR^{TM} cDNA microarray (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The PIQOR^{TM} cDNA microarray contains 1222 rat and

mouse genes including six different house keeping genes (ACTA2, TUBB5, HPRT, GAPDH, CYPA and RPS7) as four spot replicates. Detailed information about the microarray technology used including probe generation, experimental protocols, data analysis methods, and data analysis software can be found at the supplier's website (www.miltenyibiotec.com, [31]). Gene expression of three biological replicates from differentially transduced BB/OK rat islet cells per treatment condition was studied. The following conditions were used (1) AdAAT (24 hrs), (2) AdAAT (96 hrs), (3) AdvIL-10 (24 hrs) and (4) AdvIL-10 (96 hrs). Total RNA was isolated using the Mini kit from Stratagene. One μg of each total RNA was used for linear amplification. Total and amplified RNAs (aRNAs) were quality checked on the Bioanalyzer 2100 system (Agilent Technologies, Waldbronn, Germany). aRNA samples showed a Gaussian-like distribution of transcript lengths (ranging between 0.5 and 2 kB in size) and were used for fluorescent labelling. In total, 14 microarray experiments were performed. In 12 experiments, aRNA samples from infected islets were labelled with Cy5, while aRNAs from a mixture of samples from non-infected islets harvested after a 24 or 96 hrs culturing period served as a reference and were labelled with Cy3. In the remaining two experiments non-infected islet cells (24 hrs) as well as non-infected islet cells (96 hrs) were labelled with Cy5 and hybridized against the Cy3 reference as outlined above. Hybridization, scanning and data analysis were performed as described in detail elsewhere [31]. Briefly, image capture and signal quantification of hybridized PIQOR^{TM} microarrays were done with the ScanArrayLite (Packard Bioscience, Billerica, MA, USA) and IMAGENE software Version 4.1 (BioDiscovery, Los Angeles, CA, USA). Local background was subtracted from the signal to obtain the net signal intensity and the ratio of Cy5/Cy3. Subsequently, the mean of the ratios of 4 corresponding spots representing the same cDNA was computed. The mean ratios were normalized using locally weighted linear regression (LOWESS normalization method). For normalization only those spots were used for which the fluorescent intensity in one of the two channels was two times the mean background of all unflagged spots. Only genes displaying a net signal intensity twofold higher than the mean background were used for further analysis. All microarray experiments were performed according to the MIAME guidelines.

Statistical analysis

The results of n experiments are given as mean ± SEM and Student's t-test was used to check statistical significance. GraphPad Prism software was used for linear regression analysis.

Results

Transduction efficiency of islets by Ad-vectors

Islet cells from both LEW.1W and BB/OK rats showed green fluorescence after transduction with AdeGFP in a dose-dependent manner. Figure 1 depicts representative one parameter histograms of islet cells from BB/OK rats, 24 hrs after transduction with MOI 10, 100 and 1000. The percentage of cells which stained positive for eGFP rose from 12.7 to 58.2%. Intensity of green fluorescence (data not shown) increased from 108 logU (MOI 10) over 589 logU (MOI 100) to 1437 logU (MOI 1000). Prolongation of the culture time after *ex vivo* incubation of islets (from 24 to 72 hrs) increased the percentage of eGFP-positive cells from 12.7 to 24.7% using MOI 10. No significant differences in adenoviral vector transduction efficiency were observed between islets from BB/OK or LEW.1W rats (data not shown).

Transduction of islets with increasing MOIs of AdvIL-10 resulted in a linear increase ($r^2 = 0.9997$) of vIL-10 released into the culture medium (Fig. 2). While transduction of islets with AdvIL-10 at MOI 10 did not lead to detectable IL-10 levels in the culture supernatant, AdvIL-10 gene transfer at MOI 100 and 1000 led to about 100 pg vIL-10/islet ($P < 0.05$) and 1000 pg vIL-10/islet ($P < 0.001$), respectively.

Fas expression and islet-specific function after Ad-mediated gene transfer

In order to investigate possible adverse effects of Ad-mediated gene transfer, Fas expression and islet-specific function were investigated. Within non-transduced control islet cells 4.3 ± 0.9 % ($n = 8$) Fas-expressing cells (Fig. 3) were found. No induction of Fas was observed on the surface of islet cells after *ex vivo* gene transfer with AdAAT or AdvIL-10. In contrast, AdvIL-10 gene transfer led to a profound reduction (about 50%) of Fas-expressing islet cells which was independent of the Ad-vector titer used (Fig. 3). The proportion of Fas-expressing islet cells of AdAAT-transduced islets (MOI 1000) was comparable to that of non-transduced control islet cells but was significantly higher than in AdIL-10-transduced islets (MOI 1000). Specific islet function was assessed by islet insulin content and insulin secretion in response to high
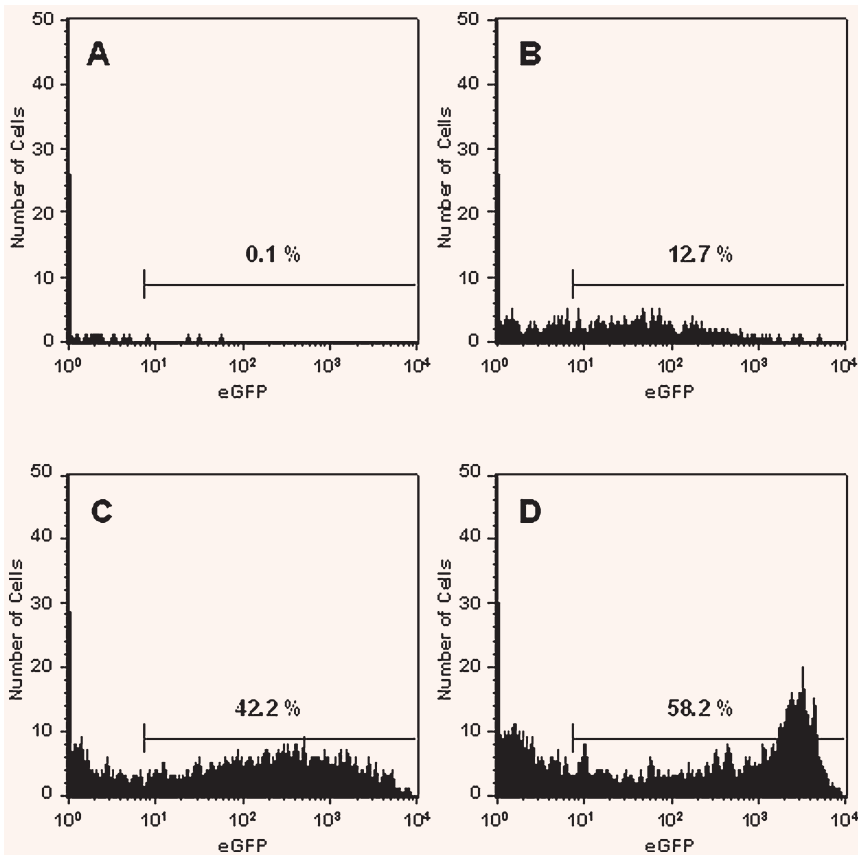
Fig. 1 Representative flow cytometric analysis of the enhanced green fluorescent protein (eGFP) in islet cells from BB/OK rat islets. Islet cells were prepared 24 hrs after transduction of BB/OK rat islets with different titres of an adenoviral construct expressing the eGFP. The results are shown as one parameter histogram of the green fluorescence intensity. (**A**) Control; (**B**) MOI 10; (**C**) MOI 100 and (**D**) MOI 1000.
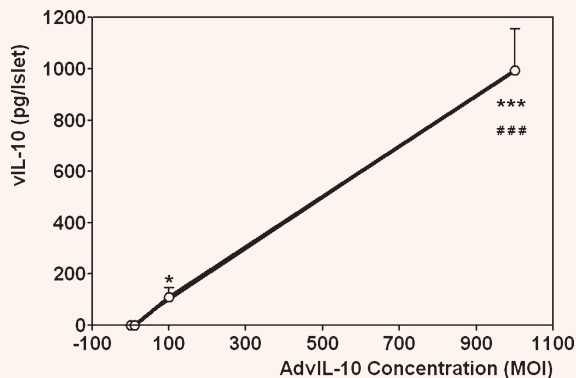

Fig. 2 Cumulative release of vIL-10 from transduced BB/OK rat islets, 24 hrs after gene transfer with different MOI of AdvIL-10. $n = 10$; *$P < 0.05$, ***$P < 0.001$ *versus* control (non-transduced islets); ###$P < 0.001$ *versus* MOI 100.

glucose. As shown in Table 1 islet insulin content of non-transduced islets was 4.13 ± 0.34 pmol/islet. Insulin content of islets after transduction with AdAAT at MOI 10 and at MOI 100 did not differ significantly from that of non-transduced control islets. At MOI 1000 insulin content was reduced ($P < 0.05$). In contrast, transduction of islets with AdvIL-10 had no significant influence on islet insulin content at all MOI used.

Insulin secretion of all islets in response to basal glucose concentration (2.0 mmol/l) revealed expected low values (Table 1). All islet preparations responded well to high glucose. There were no significant differences between non-transduced control and transduced islets even at high MOIs.

Transplantation of LEW.1W rat islets into SZ-diabetic LEW.1W rats

Transplantation of syngeneic non-transduced control islets reduced blood glucose levels in SZ-diabetic

LEW.1W rats to normal values. This effect lasted for more than 120 days in all recipients (Fig. 4A). After removal of the islet grafts, hyperglycaemia recurred rapidly. Transplantation of AdvIL-10-transduced LEW.1W rat islets (MOI 100) into SZ-diabetic LEW.1W rats led to normoglycaemia as observed after transplantation of non-transduced control islets (Fig. 4B).

Transplantation of BB/OK rat islets into autoimmune diabetic BB/OK rats

In six out of eight animals transplantation of syngeneic non-transduced control islets into diabetic BB/OK rats resulted in islet destruction within 12 days (Fig. 4C). In the two remaining animals grafted islets were accepted. AdAAT-transduced islets were destroyed more rapidly than control islets (Fig. 4C) and recipients of AdAAT-transduced islets never showed permanent normoglycaemia (>120 days) after transplantation.

AdvIL-10-transduced islets did not prevent recurrent hyperglycaemia after transplantation into diabetic BB/OK rats (Fig. 4D). Hyperglycaemia recurred within 2 days. Only in one rat (MOI 10) graft destruction occurred within 11 days as observed in recipients with control islets.

In diabetic BB/OK rats the transduced islets were destroyed more rapidly than the non-transduced control islets. This finding is in contrast to the results obtained in SZ-diabetic LEW.1W rats. All transplantation results described above were obtained with islets that had been grafted 24 hrs after transduction. In order to investigate whether an extended culture period after adenoviral gene transfer may improve graft function in BB/OK rats, AdAAT- and AdvIL-10-transduced islets (MOI 100) were grafted 96 hrs after transduction. Indeed, prolonged culture of gene-modified islets resulted in an improvement of graft survival in BB/OK rats. Blood glucose levels were similar in BB/OK recipients of AdAAT-transduced islets (Fig. 5A) and in BB/OK recipients of non-transduced control islets (Fig. 4C). Two BB/OK rats accepted the AdAAT-transduced islets for more than 120 days. Transplantation of AdvIL-10-transduced islets 96 hrs after transduction resulted in an improved graft survival in five out of 10 recipients (Fig. 5B). Islet destruction occurred within 15 days. This was comparable with the survival of non-transduced control islets, although permanent
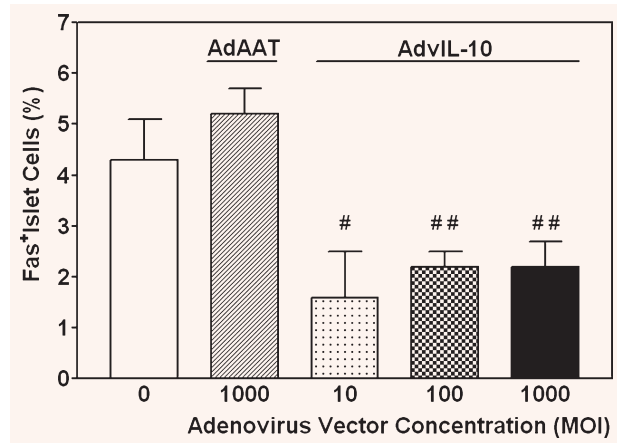

Fig. 3 Expression of Fas (CD95) on the surface of BB/OK rat islet cells of non-transduced control islets ($n = 9$), AdAAT- transduced islets ($n = 4$) or AdvIL-10-transduced islets ($n = 4$). Fas expression was measured by flow cytometry 24 hrs after gene transfer. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ *versus* AdAAT-transduced islets.

islet survival was not observed. In the remaining five BB/OK recipients the prolonged culture time did not improve the survival of AdvIL-10-transduced islets (Fig. 5B).

cDNA microarray of transduced islets

Two-dimensional cluster analysis revealed two prominent subclusters that clearly discriminate the two different time points of islet harvest (24 and 96 hrs) after transduction (Fig. 6). Genes that were highly expressed at 24 hrs after transduction and that were down-regulated after an extended culture period include the chemokine CCL2, CD9, the versican core protein precursor CSPG2-1, connective tissue growth factor CTGF, the osteopontin precursor OPN, the phospholipase A2 precursor PA21 and the pancreatitis-associated protein 2 precursor PAP2. In contrast, expression of the complement subcomponent C1QA was low at 24 hrs after transduction and increased during the following culture period. No significant differences between AdvIL-10- and AdAAT-transduced islets were detected. Table 2 depicts Cy5/Cy3 ratios of clearly up- and down-regulated cDNAs between islets harvested at 24 and 96 hrs after transduction.
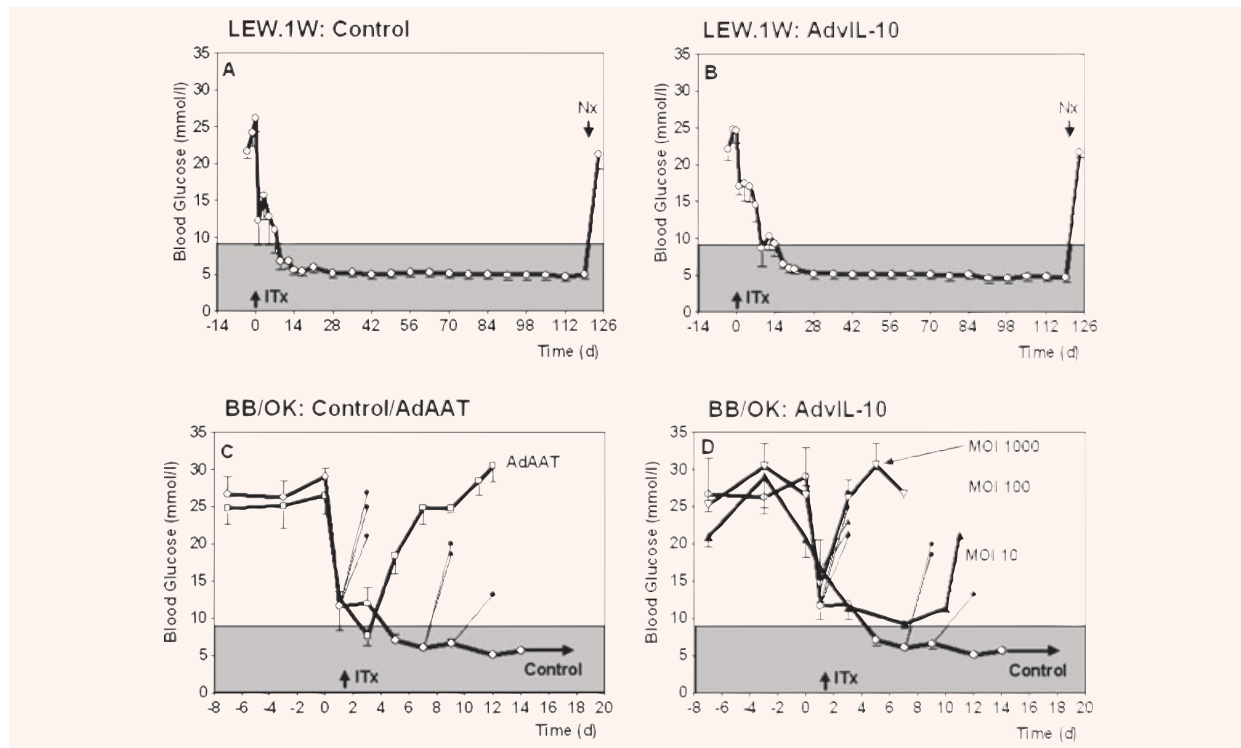
Fig. 4 Blood glucose levels in SZ-diabetic LEW.1W rats (**A** and **B**) or autoimmune diabetic BB/OK rats (**C** and **D**) transplanted with 2000 syngeneic non-transduced control or AdAAT- or AdvIL-10-transduced islets. Transplantation of islets was performed 24 hrs after gene transfer. In C and D individual rats redeveloping hyperglycaemia after transplantation are separately indicated with their first hyperglycaemic value (controls —•; AdvIL-10, MOI 10 —▲). (**A**) LEW.1W rats receiving non-transduced LEW.1W control islets (*n* = 6). (**B**) LEW.1W rats receiving AdvIL-10-transduced LEW.1W islets (*n* = 10). (**C**) BB/OK rats receiving non-transduced BB/OK control islets (*n* = 8) or AdAAT-transduced BB/OK islets (MOI 100, *n* = 4). (**D**) BB/OK rats receiving AdvIL-10-transduced BB/OK islets [MOI 10 (*n* = 3), MOI 100, (*n* = 5), MOI 1000 (*n* = 2)]. Grey area = mean blood glucose of LEW.1W rats with stable normoglycaemia after transplantation of LEW.1W control rat islets + 2SD, ITx = Islet Transplantation, Nx = Nephrectomy.

Table 1 Islet insulin content and glucose-stimulated insulin secretion of non-transduced control islets and AdAAT- or AdvIL-10-transduced islets. Islet insulin content of AdAAT-transduced islets at MOI 1000 was significantly ($P < 0.05$) reduced compared to controls. Regardless whether the islets were transduced or not insulin secretion was significantly higher at 20 mmol/l glucose than at 2 mmol/l glucose ($P < 0.001$). Stimulated insulin secretion at high glucose did not significantly differ between non-transduced control islets and any transduced islets. * *versus* non-transduced control islets

|	MOI	Islet insulin content (pmol/islet)			Glucose-stimulated insulin secretion (pmol/islet)		
		N		P*	N	2 mmol/l Glucose	20 mmol/l Glucose
Non-transduced islets	–	6	4.13 ± 0.34	–	6	0.033 ± 0.007	0.296 ± 0.043
AdAAT-transduced islets	10	7	4.04 ± 0.48	n.s.	6	0.027 ± 0.004	0.309 ± 0.044
	100	7	3.63 ± 0.41	n.s.	6	0.027 ± 0.004	0.275 ± 0.024
	1000	7	3.21 ± 0.25	< 0.05	6	0.038 ± 0.010	0.276 ± 0.060
AdvIL-10-transduced islets	10	6	4.42 ± 0.40	n.s.	6	0.028 ± 0.006	0.292 ± 0.060
	100	6	3.32 ± 0.41	n.s.	6	0.032 ± 0.006	0.273 ± 0.033
	1000	6	4.03 ± 0.46	n.s.	6	0.032 ± 0.007	0.279 ± 0.041
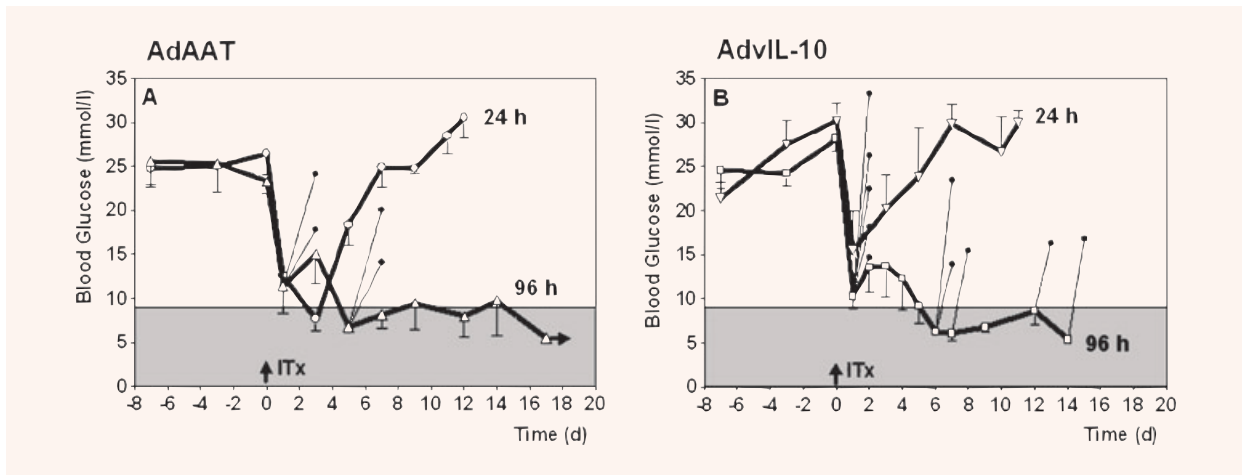
Fig. 5 Blood glucose levels in autoimmune diabetic BB/OK rats transplanted with 2000 syngeneic AdAAT- or AdvIL-10-transduced islets. Transplantation of islets was performed 24 hrs or 96 hrs after gene transfer. Individual rats redeveloping hyperglycaemia after transplantation are separately indicated with their first hyperglycaemic value (—•). (**A**) BB/OK rats receiving BB/OK islets 24 hrs ($n = 4$) and 96 hrs ($n = 6$) after AdAAT gene transfer (MOI 100). (**B**) BB/OK rats receiving BB/OK islets 24 hrs ($n = 5$) and 96 hrs ($n = 10$) after AdvIL-10 gene transfer (MOI 100). Grey area = mean blood glucose of LEW.1W rats with stable normoglycaemia after transplantation of LEW.1W control rat islets + 2SD, ITx = Islet Transplantation.

Discussion

The effect of IL-10 administration by gene therapy in NOD mice depends on the mode of administration (systemic or local) and on the dose of the virus vector expressing IL-10 [14–21]. The use of local gene expression is advantageous over systemic virus vector administration given that it circumvents the off-target side effects that are associated with systemic gene therapy and systemic expression of the therapeutic protein. Therefore, we used the local gene expression approach to investigate whether the destruction of *ex vivo* gene-modified islets expressing the immunoregulatory molecule vIL-10 is prevented following transplantation in autoimmune diabetic BB/OK rats. *In vitro*, we demonstrated that rat islets transduced *ex vivo* with different titres of AdvIL-10 produce the therapeutic gene product in a dose-dependent manner. Insulin secretion of transduced islets in response to basal glucose concentration resulted in expected low values demonstrating that there was no insulin leakage as an indicator of islet destruction. In response to high glucose, transduced islets showed an increased insulin secretion comparable with that of non-transduced control islets. AdvIL-10-transduced LEW.1W rat islets survived permanently in SZ-diabetic LEW.1W rats indicating that adenoviral transduction of islets *per se* had no influence on their function *in vivo*. Unfortunately, the local expression of vIL-10 in islet grafts did not prevent autoimmune islet destruction in diabetic BB/OK rats. Similarly, other groups have also failed to prolong islet survival in autoimmune diabetic recipients (NOD mice) grafted with syngeneic AdIL-10-transduced islets [20, 21].

There may be several reasons for the negative transplantation results in autoimmune diabetic recipients. Possibly, the amount of locally generated vIL-10 by the islets is too low to modulate the immune response towards a less pathogenic helper T-cell type 2/regulatory T-cell (TH2/Treg)-related immune response. It seems that definite serum levels of vIL-10 have to be reached to induce such systemic effects following transplantation. However, it is unclear which serum levels have to be reached to achieve systemic effects. Even in animals with systemic AAV vector-mediated IL-10 over-expression serum levels of IL-10 have not been demonstrated [15]. The results in this study depended on the dose of AAV-IL-10 suggesting that certain IL-10 levels have to be reached in order to afford a protective effect, *e.g.* by inducing regulatory T cells [15].
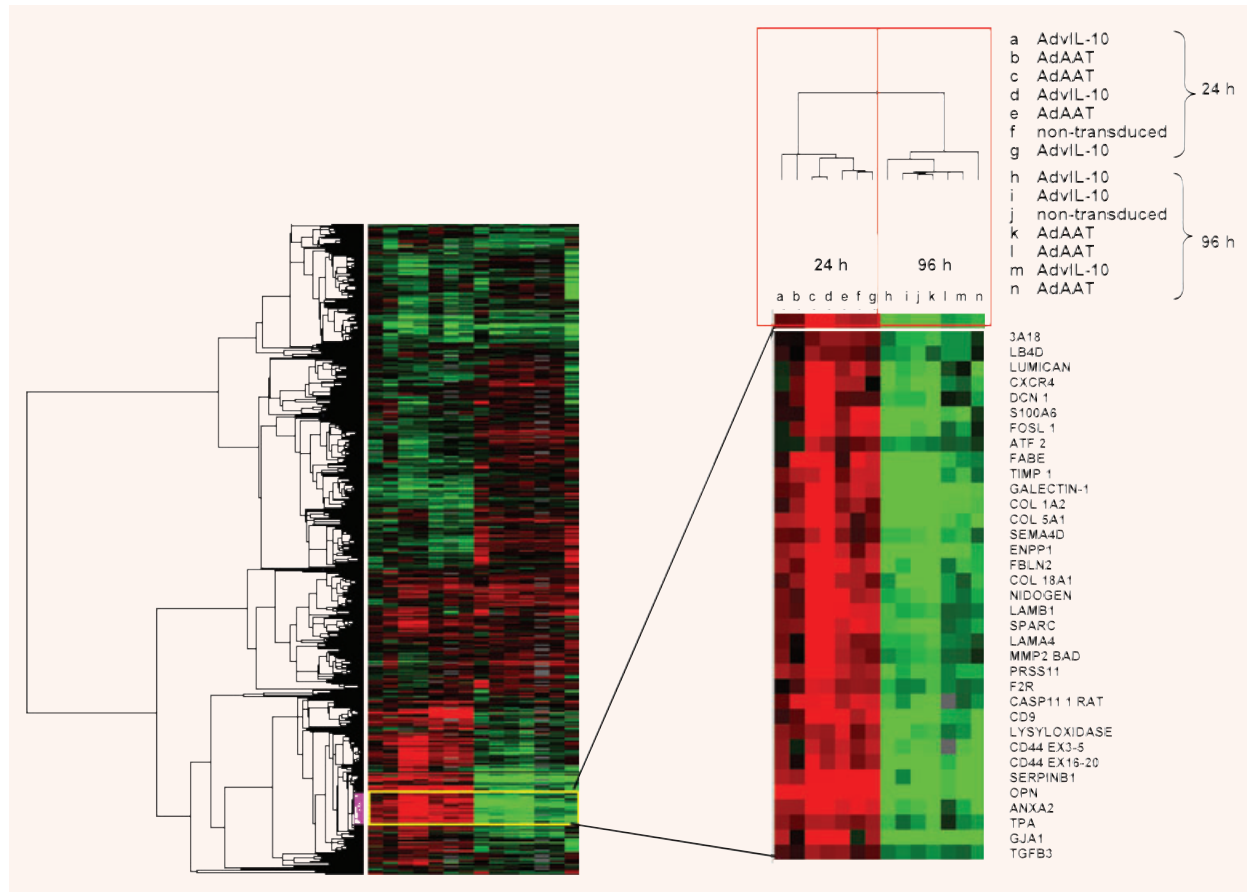
Fig. 6 (**A**) Unsupervised two-dimensional cluster analysis. (**B**) Zoom in section of differentially regulated genes; red = increased expression, green = repressed expression, black = unaltered gene expression, grey = no signal detection. X-axis: different BB/OK rat islet samples; y-axis: gene names. As data input only those genes were used that were detectable in at least 80% of all experiments (representing 742 genes in total). The red rectangles numbered 1 and 2 represent two prominent subclusters of different sets of samples that appear to have similar expression profiles. Cluster 1 represents profiles derived from islet samples harvested 24 hrs after transduction and cluster 2 represents profiles derived from samples harvested 96 hrs after gene transfer.

We used an adenoviral vector because of the favourable transduction efficiency profile of this vector in islet cells. It has been shown that Ad-transduced cells induce both humoral and cellular immune responses [32] which would be a major disadvantage of the vector system used in our study. Fortunately, we did not see any adverse effects of adenovirus-mediated transduction on the islets. Ad-transduced islets were functionally active both *in vitro* and *in vivo*. Furthermore, no induction of Fas expression on islet cells after transduction was observed indicating that there was no enhancement of the death receptor on islet cells and consequently no enhancement of apoptosis.

The demonstrated function of AdvIL-10-transduced islets *in vivo* by syngeneic transplantation to SZ-diabetic rats indicates that immune responses towards the islets or the vector may not contribute to the loss of islets following transplantation in autoimmune diabetic recipients. Immunohistochemical investigations of non-transduced control, AdAAT- or AdvIL-10-transduced islets harvested 48 hrs after transplantation into autoimmune diabetic BB/OK rats did not show marked differences in the proportion of lymphocytes infiltrating the grafts (data not shown). Specially, we looked for leukocytes in general (OX1), helper T-cells (W3/25) and macrophages (ED2). None of these cell types were found predominantly in

Table 2 Cy5/Cy3 ratios of the most prominent cDNAs showing opposite gene expression regulations between BB/OK rat islets harvested at 24 hrs and at 96 hrs after transduction, respectively

Gene	AdAAT	AdvIL-10	AdAAT	AdvIL-10
	24 hrs		96 hrs	
C1QA	0.42 ± 0.14	0.43 ± 0.09	1.41 ± 0.17	1.29 ± 0.18
CCL2	2.21 ± 0.22	1.92 ± 0.79	0.52 ± 0.09	0.63 ± 0.28
CD9	1.81 ± 0.52	1.54 ± 0.33	0.52 ± 0.15	0.52 ± 0.17
CSPG2-1	2.47 ± 0.63	2.53 ± 0.39	0.60 ± 0.25	0.56 ± 0.14
CTGF	1.99 ± 0.23	2.01 ± 0.35	0.87 ± 0.33	0.72 ± 0.02
OPN	2.94 ± 1.02	2.91 ± 0.93	0.37 ± 0.19	0.36 ± 0.15
PA21	4.60 ± 3.83	5.58 ± 5.83	0.72 ± 0.25	0.64 ± 0.10
PAP2	3.73 ± 2.83	3.64 ± 3.00	0.25 ± 0.15	0.29 ± 0.14

the different grafts investigated. These results do not support an immediate local immune response against the viral vector. Possibly, the antiviral immune response occurs later than 48 hrs after transplantation.

On the other hand, it has been shown that adenoviral vector transduction induces the expression of multiple chemokines and chemokine receptors in murine β-cells and pancreatic islets [33]. In addition, in this study transplantation of Ad-vector-transduced islets did not reverse diabetes in SZ-diabetic animals [33]. However, as mentioned above we did not find any adverse effects of Ad-mediated gene transfer on islet function in SZ-diabetic rats. Nevertheless, we feel that the up-regulation of chemokines and their receptors is an important observation which may have an impact on the clinical applicability of our approach. We demonstrated an up-regulation of chemokines such as CCL2 and other genes associated with increased inflammation such as connective tissue growth factor (CTGF), phospholipase A2 precursor PA21 and pancreatitis-associated protein 2 precursor (PAP2) in islet samples harvested 24 hrs after transduction. Therefore, we cultured transduced islets for 96 hrs instead of 24 hrs before transplantation in order to await a reduced expression of chemokine/chemokine receptors and consequently an improved function *in vivo*. Indeed, the highly expressed genes of transduced islets investigated after 24 hrs of culture were found to be down-regulated after the prolonged culture period. Furthermore, transplantation of either AdAAT- or AdvIL-10-transduced islets which were kept in culture for 96 hrs led to a clear improvement in graft function in autoimmune BB/OK rats. However, the improvement of graft function was more convincing with AdAAT-transduced islet grafts. We cannot exclude a positive influence of the produced AAT on islet graft survival. AAT is a serum serine-protease inhibitor that inhibits the activity of several enzymes [34]. Further, AAT is known to inhibit immune cell infiltration and function, *e.g.* prevention of inflammatory cytokine production [34]. Recently, is has been shown that AAT prevents diabetes and prolongs islet allograft survival in NOD mice [35–37]. However, in contrast to our experiments AAT was given systemically to the NOD mice.

Our results indicate that up-regulation of the innate immune response might contribute to the loss of islet graft function in autoimmune diabetic animals. It is possible that the islets may require more time to recover from transduction for a better function in rats with autoimmune diabetes. However, permanent graft survival was not observed with AdvIL-10 transduced islets suggesting that other factors may have contributed to the islet loss in the autoimmune diabetic animals.

Strategies using other therapeutic molecules such as IL-4 [20], IL-12(p40) [21], TGF-β1 [38], CTLA4-Ig [39, 40] or anti-apoptotic molecules [41, 42] for *ex vivo* gene transfer yielded variable results. IL-4 and anti-apoptotic molecules often led to accelerated destruction of transduced islets [43, 44]. Inhibition of IL-12 by local over-expression of the inhibitory subunit of IL-12 (p40) and inhibition of the tumour necrosis factor (TNF) receptor by a construct expressing a soluble type 1 TNF receptor-immunoglobulin(Ig) fusion protein prevented the destruction of syngeneic

or allogeneic islets in diabetic mice [21, 45]. We have previously shown that the application of a soluble TNF receptor inhibitor p55-Ig (TNFRIp55-Ig)-fusion molecule is sufficient to completely block the inflammatory responses mediated by TNF-α and TNF-β [46]. Further a reduction of the early intragraft cellular infiltration and cytokine expression in a syngeneic rat heart transplant model was observed [46]. Therefore, we transduced islets with AdTNFRIp55-Ig in order to block the above mentioned TNF responses. These islets were additionally transduced with AdvIL-10, AdIL-4 and AdIL-12p40 to modulate the immune response towards a TH2/Treg-related immune response. However, preliminary experiments indicated that even this multiple therapeutic gene transfer to isolated islets did not prevent the recurrence of hyperglycaemia after transplantation in autoimmune BB/OK rats (data not shown).

It is not clear why treatment of the islet grafts by local over-expression of vIL-10 and even over-expression of multiple anti-inflammatory molecules does not work in the BB/OK rat. In contrast, treatment of BB/OK islet transplant recipients with either anti-CD25 or anti-CD4 monoclonal antibodies or with lymphocytes obtained from long-term normoglycaemic BB/OK rats prevented autoimmune islet destruction in about 75% of grafted BB/OK rats [29, 47–50].

In summary, we demonstrate herein, that Ad-transduced rat islets produce high amounts of the therapeutic gene product and secrete insulin in response to high glucose *in vitro* as well as *in vivo*. Despite these results, AdvIL-10-transduced BB/OK rat islets were immediately destroyed in autoimmune diabetic BB/OK rats. This rapid destruction of gene-modified islets seems not to be caused by induction of apoptosis due to the gene transfer or by rapid infiltration of the islet grafts with lymphocytes. Furthermore, we demonstrated that a prolonged culture of transduced islets prior to transplantation leads to an improved graft survival in autoimmune diabetic recipients. This improved graft survival might be due to down-regulation of several genes including chemokines and other molecules associated with increased inflammation.

Acknowledgements

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