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Research Article

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Local Expression of Immunoregulatory IL-12p40 Gene Prolonged Syngeneic Islet Graft Survival in Diabetic NOD Mice

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Abstract

Local production of immunosuppressive cytokines will be one of the most suitable therapeutic strategies against organ-specific autoimmune diabetes. To establish such a new therapy, we constructed recombinant adenoviral vectors with inserted mIL-12p40 (Ad.IL-12p40) and mIL-10 (Ad.IL-10). Sufficient amounts of IL-12p40 and IL-10 were secreted by relevant adenovirus-transfected nonobese diabetic (NOD) islets. Shortly after transfection, 400 NOD islets transfected with Ad.IL-12p40 or Ad.IL-10 were transplanted under the renal capsule of a newly diabetic NOD mouse. NOD mice with IL-12p40–producing islet grafts kept normoglycemia in all of 14 grafted mice for over 4 wk after transplantation. In contrast, NOD mice with IL-10–producing islet grafts became diabetic in all of six grafted mice within 2 wk after transplantation. Reverse transcription–PCR analysis revealed that local production of IL-12p40 led to the decrease of interferon-γ and the augmentation of transforming growth factor-β at the graft site. These results suggest that IL-12 plays an important role in the destruction of islet cells at the inflamed site of autoimmunity. Such a local blockade of IL-12 would be a useful gene therapy for human autoimmune diabetes. (*J. Clin. Invest.* 1998. 102:1807–1814.) Key words: type 1 diabetes • transplantation • gene therapy • adenoviral vector • transforming growth factor-β

Introduction

Type 1 diabetes in humans results from a spontaneous T cell–mediated autoimmune destruction of insulin-producing β cells in pancreatic islets. The disease is characterized by hypoinsulinemia and hyperglycemia (1). Autoimmune insulitis and diabetes, which spontaneously develop in nonobese diabetic (NOD)[1] mice, strikingly resemble human type 1 diabetes. This serves as a useful experimental model of the human disease (2, 3). Recent studies on the etiology and pathogenesis of autoimmune diabetes indicate that cytokine imbalance plays a key role in the autoimmune process. Immune responses are regulated by a balance of Th (T helper) 1 and Th2 cytokines (4). Th1 cells, which secrete IL-2 and/or IFN-γ, promote cell-mediated immunity and production of IgG2a antibodies, whereas Th2 cells, which secrete IL-4 and/or IL-10, downregulate Th1 cell activity and stimulate production of IgG1 antibodies (5–7). Th1 cells are considered to be involved in the induction of autoimmune diabetes. For example, diabetogenic T cell clones derived from NOD mice have a Th1 phenotype (8–10). Systemic administration of IL-4, IL-10, or anti–IFN-γ monoclonal antibodies prevents diabetes onset (11, 12). IFN-γ expression in pancreatic islet-infiltrating cells correlates with β cell destruction and diabetes development in NOD mice (13).

IL-12, which is released from macrophages, dendritic cells, and B cells, is a major inducer of Th1 cell differentiation and cytokine production (7, 14, 15). IL-12 induces the production of Th1 cytokines and also stimulates IFN-γ production from natural killer cells and $CD8^+$ T cells, and enhances the cytotoxicity of these cells. Biologically active IL-12 exists as a 70–75-kD heterodimer consisting of disulfide-bonded 40-kD (p40) and 35-kD (p35) subunits. Both subunits are required for IL-12 bioactivity. Interestingly, both IL-12p40 monomer and homodimer have been reported as IL-12 antagonist. Especially, homodimers of the p40 subunit have been shown to bind with high affinity to the IL-12 receptor (15–17). It was demonstrated recently that administration of IL-12 to prediabetic female NOD mice accelerates diabetes onset and decreases IL-4 production by islet-infiltrating lymphocytes (18). It has also been reported that IL-12p40 homodimer inhibits autoimmune diabetes in NOD mice (19, 20). These results suggest that IL-12 may play a predominant role in developing effector Th1 cells.

To establish an effective therapy against insulin-dependent diabetes mellitus (IDDM), islet graft transplantation (Tx) has been attempted on hundreds of human subjects. However, most of these subjects have shown recurrence of diabetes because of the side effects of general immunosuppressants and autoimmune rejection (21). To find the immunoprivileged sites for syngeneic islets, Tx has been performed at different sites in the animal models. Although the renal capsule was postulated to be immunologically and environmentally privileged, Tx of syngeneic islets to this region in diabetic NOD mice could not prevent recurrence of diabetes (22, 23). This phenomenon has shown disease-associated and tissue-specific destruction by cell-mediated immunity (24). Th1 cells have been shown to be correlated with the recurrence of diabetes as well as spontaneous diabetes in NOD mice (25).

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1. *Abbreviations used in this paper:* IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; RT, reverse transcription; TCR, T cell receptor; Th, T helper; Tx, transplantation.

To abrogate organ-specific autoimmunity without systemic immunosuppression, an ideal therapeutic strategy is to deliver the cytokine genes directly at the site. Although the expression of immunoregulatory genes in pancreatic β cells by transgenic technologies has been shown recently to prevent autoimmune diabetes and allograft rejection, application in humans appears to be difficult (26, 27). On the other hand, the recombinant adenoviral vector is characterized by high titers and as having the capacity for efficient gene transfer into a variety of somatic cells in vitro and in vivo (28). Therefore, we added genes for immunosuppressive IL-12p40 and IL-10 by means of a recombinant adenoviral vector in order to inhibit autoimmune rejection of syngeneic islet grafts in NOD mice. The study reported here reveals that local blockade of IL-12 at the graft site can greatly prolong islet graft survival in autoimmune diabetic mice.

Methods

Mice. Male and female NOD/Shi/Kbe mice (H-2g^7) were maintained in a specific pathogen-free facility in the Institute for Experimental Animals, Kobe University School of Medicine. In our NOD colony, insulitis became noticeable in most mice at 5–7 wk of age and was much enhanced with age. Diabetes, which began at 13 wk of age, occurred in female NOD mice. The cumulative incidence of diabetes at 30 wk of age was 65% for female and 5% for male NOD mice. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine.

Generation of the recombinant adenovirus Adex1CA-mIL-12p40 (Ad.IL-12p40) and Adex1CA-mIL-10 (Ad.IL-10). To construct an adenoviral vector that would produce the immunosuppressive cytokine (murine IL-12p40, murine IL-10), these cytokine genes were isolated from activated mouse splenocytes and amplified by PCR. They were cloned into the SwaI site of a shuttle plasmid (pAdex1CAwt), which contained the inverted terminal repeat of the adenoviral genome, encapsidation sequences, and adenoviral sequences necessary for subsequent homologous recombination. The expression cosmid cassette was constructed by inserting the cytokine genes (mIL-12p40, mIL-10) into the E1-deleted region of pAdex1CAwt under the control of CAG promoter, which consisted of cytomegalovirus enhancer, chicken β-actin promoter, and rabbit β-globin poly(A) signal. Both the expression cosmid cassette and the parental adenovirus genome (Ad5-dlx) lacking E1A, E1B, and E3 genes were cotransfected into 293 cells, a human embryonic kidney carcinoma cell line; the homologous recombination between them was performed by calcium phosphate precipitation (29, 30). We generated the replication-defective adenoviral vectors without E1A, E1B, and E3 genes, these being safe and efficient gene transfer vectors; this produced the immunosuppressive cytokine. The final recombinant replication-defective adenoviruses, encoding murine IL-12p40 and IL-10, were termed Adex1CA-mIL-12p40 (Ad.IL-12p40) and Adex1CA-mIL-10 (Ad.IL-10), respectively. As a control, Adex1CA-lacZ (Ad.lacZ) that was previously reported was used in this study (30).

Islet graft preparations and viral infection. Pancreatic islets were isolated from male NOD mice at 6–8 wk of age by stationary digestion of pancreatic tissues with collagenase (Cooper Biomedical Co., Malvern, PA), purified by using Ficoll (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation as previously reported and then hand picked under an inverted microscope (31). Whole islets isolated from young NOD mice were transfected with 10^3 PFU/islet of adenoviral vector (Ad.IL-12p40, Ad.IL-10) and incubated for 1 h in 0.5 ml of RPMI 1640 medium with 10% FCS. To evaluate the distribution of transferred gene in the islet, NOD islets were transfected with Ad.lacZ, and expression of β-galactosidase in the islets was detected with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal). In brief, the islets were embedded in OCT compound, sectioned at 5 μm, fixed in 0.2% glutaraldehyde, and stained with X-gal.

Cytokine production by adenovirus-transfected islets. At 48 h of incubation after recombinant adenoviral infection, the culture supernatants were harvested for the measurement of IL-10, IL-12p40, and IL-12p35/p40. Cytokine contents were evaluated by sandwich ELISA as recommended by the manufacturer (PharMingen, San Diego, CA). In brief, anticytokine mAb-bound ELISA wells were incubated with 100 μl of culture supernatant for 16 h, followed by incubation with relevant biotinylated second mAb. After the binding of avidin-peroxidase, diaminobenzoic acid solution was added to the wells for color development.

Insulin secretion by adenovirus-transfected islets. After 48 h of incubation in RPMI 1640 with 10% FCS, the culture supernatants were removed completely, and unmanipulated and immunosuppressive gene-transferred islets were incubated with HBSS containing calcium magnesium chloride and 1% BSA (glucose concentration 5.6 mM) for 1 h at 37°C in 5% CO_2. After 1 h of incubation, the culture supernatants were harvested, and unmanipulated and immunosuppressive gene-transferred islets were incubated with HBSS containing 11.1 mM glucose for the next 1 h. The culture supernatants were harvested and were replaced by HBSS containing 16.7 mM glucose for the last 1 h of incubation. The insulin content was measured by RIA.

Islet Tx and blood glucose analysis. Female diabetic NOD mice within 1 wk after diabetes onset served as islet graft recipients. Islet donors were male NOD mice, 6–8 wk of age. Similar to procedures used for unmanipulated NOD islets (n = 5), 400 isolated NOD islets transfected with Ad.IL-12p40 (n = 14) or Ad.IL-10 (n = 6) were transplanted under the left renal capsule in each diabetic NOD mouse. Tx was considered successful if the nonfasting blood glucose returned to normal (< 11.1 mM) within 2 d. Tail vein blood glucose was monitored three times a week after Tx using glutestor (Kyoto Daiichi Kagaku, Inc., Kyoto, Japan). Recipients with two consecutive blood glucose levels > 16.7 mM were considered to be diabetic.

Pathohistological evaluation of the grafts. NOD mice, which were engrafted with Ad.IL-10–transfected or Ad.IL-12p40–transfected islets and kept normoglycemia, were killed on day 7 after Tx. In addition, NOD mice, which were engrafted with Ad.IL-12p40–transfected islets and kept normoglycemia, were killed on day 60 after Tx. The grafted islets were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4.5 μm and stained with hematoxylin/eosin. Coded slides were read by light microscopy.

RNA isolation and cDNA synthesis. On day 7 or 60 after Tx, mRNA was extracted from syngeneic islet grafts with a Micro-Fast Track mRNA isolation kit (Invitrogen, NV, Leek, The Netherlands) and 0.1 μg of mRNA was reverse-transcribed with a cDNA cycle kit (Invitrogen), while using oligo-dT primers and AMV reverse transcriptase to generate cDNA for use as a template in PCR amplifications.

Reverse transcription (RT)-PCR analysis of locally produced cytokine mRNA in transplanted islets. The PCR analysis was carried out using cDNA samples for simultaneous analysis of a single cytokine, 20 μM of each primer, and 1.25 U of Ex *Taq* polymerase (Takara Shuzo, Shiga, Japan) in a 50-μl final volume. Samples were amplified with an initial 3-min denaturation at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C, with 10 min at 72°C on the last cycle in a Gene Amp PCR System 9700 (Perkin-Elmer/Cetus Corp., Norwalk, CT). The upstream and downstream primers were for preproinsulin I, for preproinsulin II, for IL-12p40, for IL-12p35, for IFN-γ, for IL-4, for TGF-β, for cyclophilin, and for T cell receptor (TCR) Cβ. The sequences of the specific oligonucleotide primer pairs, 5′ and 3′, are shown in Table I. PCR products were analyzed by electrophoresis in 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

Flow cytometry. To investigate the phenotype of the mononuclear cells infiltrating the islet grafts, NOD mice engrafted with IL-12p40–producing or unmanipulated islets were killed on day 7 after Tx. The islet-infiltrating cells were obtained by mincing and pipetting the islet

Table I. Specific Oligonucleotide Primer Sequences for RT-PCR

PCR product		Primer sequences
Preproinsulin I	Sense	5′ ACATGGCCCTGTTGGTGCACT 3′
	Antisense	5′ TTAGTTGCAGTAGTTCTCCAG 3′
Preproinsulin II	Sense	5′ ACATGGCCCTGTGGATGCGCT 3′
	Antisense	5′ CTAGTTGCAGTAGTTCTCCAG 3′
IL-12p40	Sense	5′ AGATGACATCACCTGGACCT 3′
	Antisense	5′ GCCATGAGCACGTGAACCGT 3′
IL-12p35	Sense	5′ GATGCAGTCTCTCTGAATCATAATGG 3′
	Antisense	5′ GGCACAAAAACAATAGCTTATCAGT 3′
IFN-γ	Sense	5′ TGAACGCTACACACTGCATCTTGG 3′
	Antisense	5′ CGACTCCTTTTCCGCTTCCTGAG 3′
IL-4	Sense	5′ ATGGGTCTCAACCCCCAGCTA 3′
	Antisense	5′ GCTCTTTAGGCTTTCCAGGAAGTC 3′
TGF-β	Sense	5′ AGGAGACGGAATACAGGGCTTTCG 3′
	Antisense	5′ ATCCACTTCCAACCCAGGTCCTTC 3′
Cyclophilin	Sense	5′ GACAGCAGAAAACTTTCGAGC 3′
	Antisense	5′ TCCAGCCATTCAGTCTTGG 3′
TCR Cβ	Sense	5′ GCAAACAAACAAAAGGCTACCC 3′
	Antisense	5′ CCACTTGTCCTCCTCTGAAA 3′

graft in Ca-free, Mg-free HBSS and were stained by antibodies against various cell surface molecules: CD45, CD4, CD8, and B220 for flow cytometric analysis. FITC-conjugated anti–mouse CD45 mAb (30F11.1), phycoerythrin-conjugated anti–mouse CD4 (RM4-5), anti–mouse CD8 (53-6.7), and anti–mouse CD45R/B220 (RA3-6B2) mAbs were purchased from PharMingen (San Diego, CA). In brief, the islet-infiltrating cells were incubated with FITC-conjugated anti–mouse CD45 mAb and phycoerythrin-conjugated anti–mouse mAb at 4°C for 30 min, and analyzed by a FACS® 440 flow cytometer (Becton Dickinson, San Jose, CA).

Statistical analysis. Statistical analysis was performed by the Mann-Whitney U test. All data are presented as the mean±SD.

Results

Cytokine production by adenovirus-transfected islets. Our preliminary experiments determined the effectiveness of transfection with 10^3 PFU/islet of adenoviral vector. To evaluate the cytokine production from the adenovirus-transfected islet graft, supernatants were harvested after culturing the adenovirus-transfected islets for 48 h and concentrations of immunosuppressive cytokines were measured by ELISA. IL-12p40 production from Ad.IL-12p40–transfected islets was detected as 84±13 ng/100 islets/48 h. The active form (p35/p40 heterodimer) of IL-12 was not detected from Ad.IL-12p40–transfected islets. On the other hand, IL-10 production from Ad.IL-10–transfected islets was detected as 52±10 ng/100 islets/48 h (Table II). These results indicate that the adenovirus-transfected islets effectively produced each immunosuppressive cytokine.

Insulin secretion by adenovirus-transfected islets. To determine whether insulin secretion from the adenovirus-transfected islet was disturbed or not by adenoviral infection, we measured insulin secretion at 48 h after adenoviral infection by RIA (Fig. 1). As expected, normal insulin secretion responding to glucose stimulation was observed. These results indicate that the adenovirus-transfected islet grafts maintained normal stimulated insulin secretion against high glucose, without the influence of adenoviral infection. When we performed X-gal staining of Ad.lacZ-transfected islets, gene products (β-galactosidase) were intensively expressed on the margin of the islet. However, the central area of the islets that was rich in insulin-producing cells showed lower expression (data not shown). The distribution of adenoviral transfection might be related to keeping normal insulin secretion from transfected islets.

Prevention of diabetic recurrence by adenoviral vectors. We transplanted unmanipulated NOD islet grafts under the renal capsule in overtly diabetic NOD mice and normoglycemia was disrupted within 14 d after Tx (Fig. 2 *A* and Table III). To prevent diabetic recurrence, we transplanted adenovirus-transfected islet grafts in overtly diabetic NOD mice. As a control, Ad.lacZ-transfected islets were transplanted. As well as unmanipulated islet graft, all four recipients showed recurrence of diabetes within 14 d after Tx (Fig. 2 *B* and Table III). In the case of Ad.IL-10–transfected islet grafts, diabetic recurrence was observed in recipient NOD mice, as well as for unmanipulated islet grafts (Fig. 2 *C* and Table III). These results indicate that locally produced IL-10 did not prolong the synge-

Table II. Cytokine Production by Adenovirus-transfected Islets

Adenoviral vector*	*n*	IL-10‡	IL-12p40‡	IL-12p35/p40‡
			ng/100 islets	
Ad.lacZ	7	< 0.03	< 0.04	< 0.04
Ad.IL-10	8	52±10	< 0.04	< 0.04
Ad.IL-12p40	8	< 0.03	84±13	< 0.04

*Recombinant adenoviruses were transfected to 100 NOD islets (10^3 PFU/islets) for 1 h, and thereafter transfected islets were cultured in RPMI 1640 medium. ‡Culture supernatants were harvested after 48 h of incubation. The contents of IL-10, IL-12p40, and IL-12p35/p40 were measured by relevant ELISA. The results are expressed as the mean±SD.

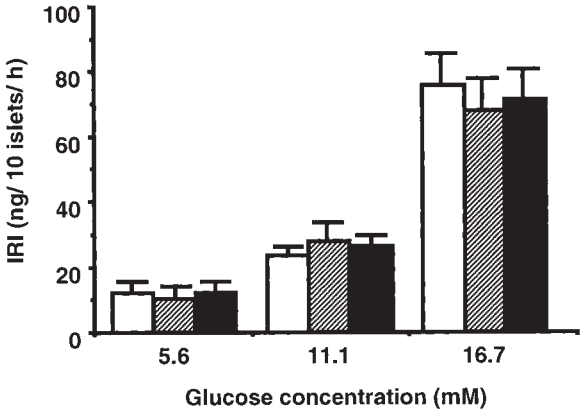

Figure 1. Insulin secretion by adenovirus-transfected islets. 10 islets were incubated with HBSS containing 5.6, 11.1, and 16.7 mM glucose for 1 h at 37°C. The culture supernatants were harvested and the insulin content was measured by RIA. The results of unmanipulated islets (*open bars*), Ad.IL-10–transfected islets (*hatched bars*), and Ad.IL-12p40–transfected islets (*closed bars*) are expressed as the mean±SD (n = 6 for each group).

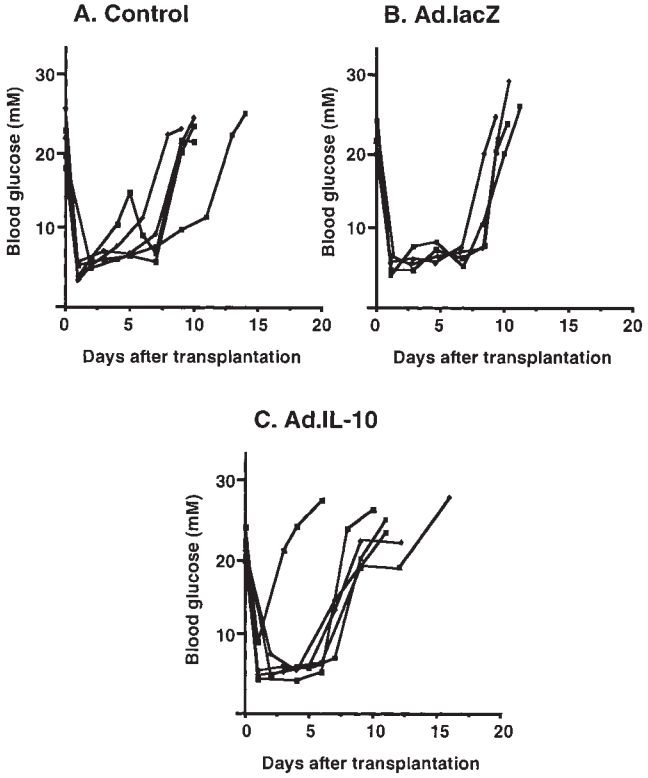

Figure 2. Ad.IL-10–transfected NOD islets failed to prevent diabetic recurrence in NOD mice. 400 unmanipulated (*A*), Ad.lacZ-transfected (*B*), and Ad.IL-10–transfected NOD islets (*C*), freshly isolated from young male NOD mice, were transplanted under the left renal capsule in each female diabetic NOD mouse. Tail vein blood glucose was monitored three times a week after Tx. Recipients with two consecutive blood glucose levels > 16.7 mM were considered diabetic.

neic NOD islet survival. In contrast, in the case of Ad.IL-12p40–transfected islets, blood glucose levels of all recipients remained normal for > 4 wk; significantly, 5 of 14 recipients kept normoglycemia over 3 mo (Fig. 3 and Table III). These observations show that local production of IL-12p40 can prevent the rejection of syngeneic NOD islet grafts. These results suggest that IL-12p40, an antagonist of IL-12, can inhibit autoimmune rejection.

Histological analysis of islet grafts. To evaluate cellular infiltration to the transplanted islet grafts, grafts were removed on day 7 after Tx and examined histologically. In all five cases of IL-10–producing NOD islet grafts, a great number of lymphocytes infiltrated into the grafts and the configuration of islet grafts was mostly destroyed (Fig. 4 *A*). In contrast, in all five cases of IL-12p40–producing NOD islet grafts, only a small number of lymphocytes infiltrated into the grafts and most of the islet grafts remained intact, apparently preserved by the effect of IL-12p40 (Fig. 4 *B*). Moreover, even on day 60 after Tx in two cases where blood glucose level remained normal, IL-12p40–producing NOD islet grafts remained histologically preserved without significant cellular infiltration (Fig. 4 *C*). These histological studies suggest that IL-12 plays an important role in autoimmune inflammation.

Comparison of locally produced cytokine mRNA. To evaluate the biological effects of IL-12p40, local expression of cytokine genes of Ad.IL-12p40–transfected islet grafts and unmanipulated islet grafts was compared using RT-PCR on day 7 or 60 after Tx. Cyclophilin served as an internal control. On day 7 after Tx, expression of preproinsulin I and II was maintained in Ad.IL-12p40–transfected islet grafts, whereas there was decreased expression in unmanipulated islet grafts that suggested the destruction of insulin-producing cells. Moreover, Ad.IL-12p40–transfected islet grafts appeared to show increased expression of IL-12p40 in contrast to an almost unchanged expression of IL-12p35. These results suggest that higher expression of IL-12p40 neutralizes the active form of IL-12 at the site, resulting in inhibition of syngeneic islet graft rejection. When we evaluated cytokine expression at the graft site, IFN-γ expression was diminished in Ad.IL-12p40–transfected islet grafts. To exactly quantify IFN-γ mRNA expressed by the cells infiltrating the Ad.IL-12p40–transfected islet grafts, TCR Cβ was used as an additional internal control. As well, IFN-γ mRNA expression was not detected in Ad.IL-12p40–transfected islet grafts (data not shown). On the other

Table III. Survival of Syngeneic Islet Grafts in Diabetic NOD Recipients

Islet grafts*	n	Diabetes recurrence‡	Mean survival time
		d after Tx	*d*
Unmanipulated	5	8, 9, 9, 9, 13	9.6
Ad.lacZ-transfected	4	8, 9, 9, 10	9.0
Ad.IL-10–transfected	6	3, 8, 9, 9, 9, 9	7.8
Ad.IL-12p40–transfected	14	28, 28, 33, 36, 47 50, 56, > 60, 72 > 90, 98, 102, 185, 248	87.0

*400 NOD islets were transplanted under the renal capsule in each diabetic NOD mouse. Unmanipulated, Ad.lacZ-transfected, Ad.IL-10–transfected, or Ad.IL-12p40–transfected islets were used as grafts. ‡After islet graft Tx, blood glucose was monitored. Recipients with two consecutive blood glucose levels > 16.7 mM were considered diabetic.

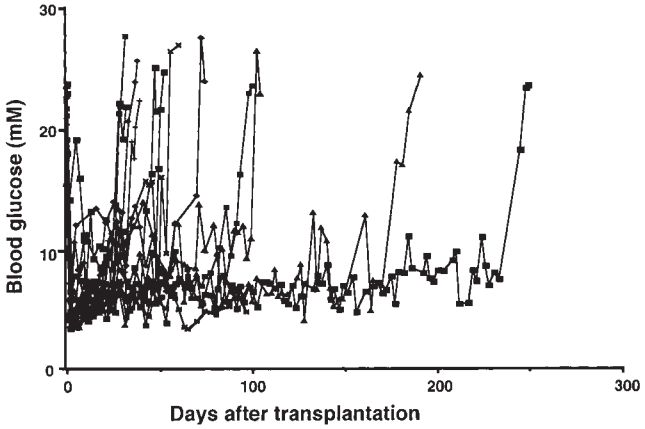

Figure 3. Ad.IL-12p40–transfected islets prolonged graft survival. 400 Ad.IL-12p40–transfected NOD islets were transplanted under the left renal capsule in each female diabetic NOD mouse. Tail vein blood glucose was monitored three times a week after Tx. Recipients with two consecutive blood glucose levels > 16.7 mM were considered diabetic.

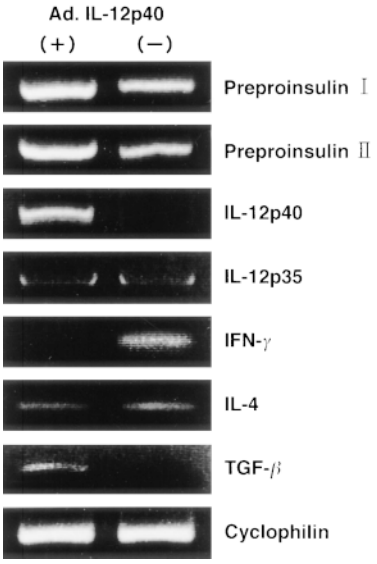

Figure 5. RT-PCR analysis of locally produced cytokine mRNA in transplanted islets on day 7 after Tx. On day 7 after Tx, mRNA was isolated from Ad.IL-12p40–transfected and unmanipulated islet grafts, and RT-PCR for preproinsulin I, preproinsulin II, IL-12p40, IL-12p35, IFN-γ, IL-4, TGF-β, and cyclophilin as an internal control was performed using specific primers, respectively.

hand, there was no change in IL-4 expression between Ad.IL-12p40–transfected and unmanipulated islet grafts. On day 60 after Tx, local production of cytokine in Ad.IL-12p40–transfected islet grafts was shown (see Fig. 6). The increased expression of IL-12p40 on day 7 after Tx as shown in Fig. 5 was not observed, suggesting that transduced genes could not keep in the graft so long. On the other hand, IFN-γ mRNA expression was not detected on day 60 as well as on day 7 in Ad.IL-12p40–transfected islet grafts. There was no change in expression of IL-12p35 and IL-4 in Ad.IL-12p40–transfected islet grafts between days 7 and 60 after Tx. Interestingly, TGF-β expression emerged and stayed in Ad.IL-12p40–transfected islet grafts on days 7 and 60 after Tx, although TGF-β mRNA was almost not detected in unmanipulated islet grafts on day 7 after Tx (Figs. 5 and 6). These results suggest that local production of IL-12p40 inhibited IFN-γ–producing cells and drove TGF-β–producing cells without enhancing IL-4–producing cells, resulting in prevention of β cell destruction.

Flow cytometric analysis of mononuclear cells infiltrating IL-12p40–producing islet grafts. To examine whether locally produced IL-12p40 alters the subsets of the islet-infiltrating mononuclear cells at the graft site, the islet-infiltrating cells were isolated from the Ad.IL-12p40–transfected islet grafts or unmanipulated islet grafts on day 7 after Tx. The cell surface markers were examined by flow cytometry. Since CD45 molecules were expressed on all lymphocytes, the lymphocyte subsets of islet-infiltrating cells were evaluated by absolute number of $CD45^+$ cells and percentages of $CD4^+$, $CD8^+$, and $B220^+$ cells (Table IV). Absolute number of $CD45^+$ cells surrounding Ad.IL-12p40–transfected islet grafts was much reduced, compared with unmanipulated islet grafts. In Ad.IL-12p40–transfected islet grafts, the percentage of $CD4^+$ T cells seemed to be increased, whereas that of $CD8^+$ T cells seemed to be decreased, compared with unmanipulated islet grafts. Since lymphocyte subsets were varied in each islet graft, no significant difference between Ad.IL-12p40–transfected and unmanipulated islets was observed. On the other hand, the percentage of B lymphocytes was not changed. These results

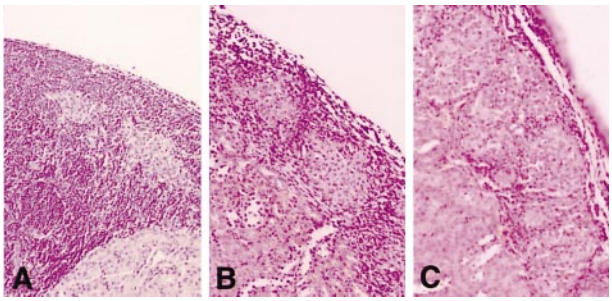

Figure 4. Histological analysis of islet grafts after Tx. NOD mice engrafted with IL-10– or IL-12p40–producing islets were killed on day 7 or 60 after Tx. The grafted islets were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4.5 μm, and stained with hematoxylin and eosin. On day 7 after Tx, IL-10–producing NOD islet grafts (*A*) and IL-12p40–producing NOD islet grafts (*B*) were shown at a magnification of 400. On day 60 after Tx, IL-12p40–producing NOD islet grafts (*C*) were shown at a magnification of 400.

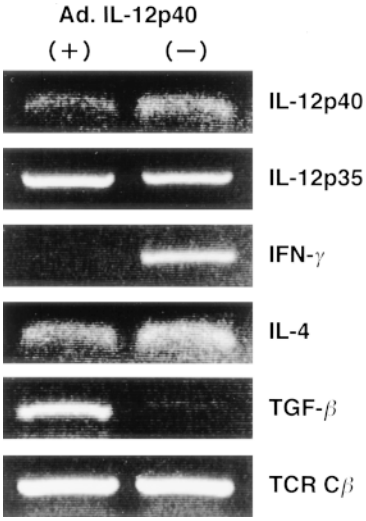

Figure 6. RT-PCR analysis of locally produced cytokine mRNA in transplanted islets on day 60 after Tx. On day 60 after Tx, mRNA was isolated from Ad.IL-12p40–transfected islet grafts, and RT-PCR for IL-12p40, IL-12p35, IFN-γ, IL-4, TGF-β, and TCR Cβ as an internal control was performed using specific primers, respectively (*left*). To compare cytokine mRNA expression, RT-PCR products of unmanipulated islet grafts on day 7 after Tx were shown in the right lane.

Table IV. Lymphocyte Subsets of the Cells Infiltrating the Islet Grafts

Source of islet grafts*	*n*	Number of CD45^{+}‡	CD45^{+}§		
			CD4^{+}	CD8^{+}	B220^{+}
		$\times 10^6$		%	
Unmanipulated	5	1.21±0.11	48.1±7.9	35.3±11.4	10.2±4.5
Ad.IL-12p40–transfected	7	0.19±0.05‖	58.3±8.7	20.8±10.7	8.8±3.1

*IL-12p40–producing or unmanipulated islet grafts were collected on day 7 after Tx. The islet-infiltrating cells were obtained by mincing and pipetting the islet grafts. ‡Absolute number of CD45^{+} cells/graft was determined by flow cytometric analysis. ‖$P < 0.01$ compared with unmanipulated islet grafts (Mann-Whitney U test). §Percentages of CD4^{+}, CD8^{+}, and B220^{+} cells in CD45^{+} cells were determined by using two-color fluorescent staining and were evaluated by flow cytometric analysis. No significant difference of lymphocyte subsets between unmanipulated and IL-12p40–transfected islet grafts was observed (Mann-Whitney U test). All data are expressed as the mean±SD.

suggest that local production of IL-12p40 reduces mononuclear cells infiltrating islet grafts and shifts cytokine balance without change of the percentages of CD4^{+}, CD8^{+}, and B220^{+} cells at the graft site.

Discussion

One of the most suitable therapeutic strategies against organ-specific autoimmune diseases has been proposed to prevent the specific autoimmune inflammatory process without systemic immunosuppression. To establish such an ideal therapy, NOD mice have been used commonly as a spontaneous organ-specific autoimmune diabetes model. There have been attempts to prevent an antigen-specific autoimmune response in NOD mice by oral or nasal administration of putative autoantigens such as insulin (32, 33). So far, such trials using putative autoantigen peptides to induce immunological tolerance have provided incomplete prevention, and treated NOD mice have shown diabetes, albeit with lower incidence. In contrast, gene therapy using IL-10–transduced islet-specific Th1 lymphocytes in NOD mice has been demonstrated recently to be useful (34). However, the isolation of autoreactive T cells from autoimmune subjects is not so easy, and expansion to obtain sufficient amounts of T cells for prevention in human subjects seems difficult at this time. In this study, we demonstrate for the first time that the transduction of the IL-12p40 gene, an antagonist of active IL-12, into islet grafts can be an effective way to inhibit autoimmune graft rejection in diabetic NOD mice. This implies a clinically applicable and attractive immunotherapy for human autoimmune diabetes, because pancreatic islet Tx has been performed unsuccessfully for hundreds of IDDM patients.

It has been suggested that CD4^{+} T cells are divided into two types, Th1 and Th2 cells. Th1 cells produce IL-2 and IFN-γ, which induce the cellular immune response, whereas Th2 cells produce IL-4 and IL-10, which take part in the humoral immune response. Th1 and Th2 cells are also known to cross-regulate by the release of IFN-γ and IL-10 (5–7). In allogeneic Tx, Th1 cells appear to be the key regulatory immune cells that promote cytotoxic T lymphocytes and delayed-type hypersensitivity responses, resulting in the rejection of allografts (35). Although hundreds of IDDM patients have received islet or pancreatic grafts, most of the recipients have shown the recurrence of autoimmune diabetes even in the case of an identical twin donor (21). Like human subjects, NOD mice have shown autoimmune inflammation against syngeneic islet grafts, resulting in the recurrence of diabetes. Similar to the allograft rejection, Th1 cells are supposed to play a key role in syngeneic islet graft rejection. The islet grafts, as well as pancreatic islets, have shown the predominant infiltration of Th1 cytokine (IFN-γ)–producing cells in NOD mice (13, 25). Various immunomodulations such as CFA or insulin can cause a shift from predominance of Th1 cells to Th2 cells, resulting in the suppression of spontaneous diabetes and diabetic recurrence in NOD mice (13, 25, 36, 37).

IL-12 has been known as a major inducer of Th1 cell differentiation and cytokine production (15). IL-12 mRNA expression in NOD pancreatic islets was reported as having a correlation with destruction of insulin-producing cells during the development of autoimmune diabetes (38). In syngeneic islet Tx in diabetic NOD mice, IL-12 expression level is also related to autoimmune graft rejection (39). It was demonstrated recently that administration of IL-12 to young NOD mice accelerates diabetes, which correlates to higher production of IFN-γ and lower production of IL-4 (18). In contrast, systemic administration of homodimeric IL-12p40, an antagonist of the active form of IL-12, has been shown to suppress spontaneous and cyclophosphamide-induced diabetes (19, 20). From these results, IL-12p40 appeared to be an attractive candidate for our therapeutic strategy to prevent autoimmune process in syngeneic Tx. The study presented here revealed that IL-12p40 production in islet grafts could suppress Th1 cell activity and prolong islet graft survival. Unlike systemic administration of IL-12p40, serum IL-12 could not be detected in normoglycemic NOD mice with Ad.IL-12p40–transfected islet graft (< 0.06 ng/ml). This may suggest that local production of IL-12p40 can prevent the autoimmune process in islet grafts without systemic influence on recipient mice.

Another attractive candidate for the local immunosuppression on the grafted site is IL-10, which is a Th2 cytokine and can inhibit the activation of Th1 cells and production of IFN-γ (40). Like that of IL-12p40, systemic administration of IL-10 or IL-10/Fc fusion protein can prevent spontaneous diabetes of NOD mice (12, 41). To our surprise, the survival of islet grafts could not be prolonged by IL-10 gene transfer. IL-10–producing islet sites have shown massive infiltration of mononuclear cells in contrast to the minor cellular infiltration of IL-12p40–producing site (Fig. 4). The failure of graft survival of IL-10–producing islets may imply two possibilities. One possible explanation may be insufficient production of IL-10 in the islet site because in vitro IL-10 production is relatively lower compared with IL-12p40 production. However, 52 ng during 48 h culture of 100 islets is almost equal to 26 U, which is based on the calculation of the biological activity of recombinant IL-10 produced by several manufacturers. They appeared to be sufficient to induce biological activity in vitro. However, local IL-10 production in the graft site might be much lower to prevent the graft rejection. Another possible explanation is that activated memory Th1 cells, which can be present in the diabetic NOD mice, might not be suppressed by IL-10 in vivo. Many

reports suggest that the effects of IL-10 may be paradoxical. Transgenic NOD mice that produce IL-10 in the islet site have been reported as showing accelerated diabetes, in contrast to inhibition of diabetes by systemic administration of IL-10. This suggests that the timing of IL-10 administration or production in the autoimmune process may reflect the biological behavior of IL-10 in vivo (42, 43). Further studies may be required to elucidate the role of IL-10 in vivo at different times in the process of autoimmune diabetes.

In this study, to transduce cytokine genes into islet grafts we used an E1-deleted adenoviral vector, which was replication deficient and showed transient expression of cytokines. Furthermore, adenoviral vectors have been reported as having the capacity to express the partial epitope of adenovirus so that cells transfected by adenoviral vectors are cleared $\sim$ 3–8 wk after Tx (44, 45). In fact, when RT-PCR analysis of three Ad.IL-12p40–transfected islet grafts was performed on day 60 after Tx, the higher expression of IL-12p40 mRNA recognized on day 7 after Tx was not observed on day 60 after Tx, although three recipient NOD mice kept normoglycemia. In this study, transfectants by adenoviral vectors appeared to be cleared at least within 2 mo. In spite of such a relatively short-lived transgene expression, 5 of 14 IL-12p40 gene-transferred NOD mice stayed normoglycemic for $>$ 3 mo and grafted islets remained normal in configuration with a small amount of lymphoid infiltration on day 60 after Tx. Thus, our results suggest that immunological tolerance might be induced after Tx of IL-12p40–producing islet grafts.

To our interest, TGF-β expression was detected in Ad.IL-12p40–transfected islet sites without higher expression of IL-4 on days 7 and 60 after Tx (Figs. 5 and 6). Prolonged islet graft acceptance in autoimmune diabetic mice seems to be accounted for by the induction of TGF-β–producing cells in addition to the suppression of IFN-γ–producing cells. In fact, it seems that blood glucose concentrations fluctuate within 3 wk after Tx and then stabilize at normoglycemic levels in IL-12p40–recipient mice (Fig. 3). This observation might be in support of the induction of TGF-β–producing cells. Another supportive observation that has been reported is that systemic administration of anti–IL-12 antibody in vivo enhances TGF-β but not IL-4 production by antigen-specific T cell stimulation (46, 47). Our study suggests that neutralization of IL-12 at the islet graft site may drive TGF-β–producing immunoregulatory T cells. TGF-β is known to downregulate IFN-γ production by Th1 cells (47). No detection of IFN-γ production without significant change of the lymphocyte subsets at the islet graft site may be due to TGF-β production in addition to abrogation of IL-12 activity that promotes IFN-γ secretion from Th1 cells, $CD8^+$ T cells, and natural killer cells (47). Furthermore, oral administration of insulin to NOD mice has shown the prevention of diabetes associated with generation of TGF-β–producing T cells that directly suppress diabetes transfer (32). It has been demonstrated that an islet-reactive $CD4^+$ T cell clone that secretes TGF-β can inhibit spontaneous diabetes and diabetes transfer (48). Taken together, TGF-β–producing T cells may prolong islet graft survival after abrogation of adenoviral transgene expression. On the other hand, it has been reported recently that systemic administration of anti–IL-12 antibody can induce T cell apoptosis in peripheral lymphoid tissue (46). To elucidate the reasons for the longer survival of IL-12p40–producing islet graft, further experiments are now being undertaken.

In conclusion, local expression of IL-12p40 gene at the islet graft site was found to prevent autoimmune rejection effectively for an unexpectedly long time. Furthermore, it has been reported that IL-12p40 production using a retroviral vector suppresses Th1 response and prolongs survival of allogeneic transplanted myoblast (49). Taken together, these results suggest that permanent expression of immunoregulatory genes such as cytokines in and around the target organs would be attractive and useful. IL-12p40 would be one of the most effective cytokines for such an immunotherapy. In future, this novel gene therapy might be an ideal strategy, applicable not only in the consideration of autoimmune diabetes, but also to other organ-specific autoimmune diseases in humans.

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