Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 340 (2006) 236–243
BBRC
www.elsevier.com/locate/ybbrc

Immune responses to an encapsulated allogeneic islet β-cell line in diabetic NOD mice

Sasha P. Black [a,*], Ioannis Constantinidis [b], Hong Cui [c], Carol Tucker-Burden [c], Collin J. Weber [c], Susan A. Safley [c]

[a] Charles River Laboratories, Pre-clinical Services Montreal, Senneville, Que., Canada H9X 3R3
[b] Department of Medicine, Division of Endocrinology, University of Florida, Gainesville, FL 32610, USA
[c] Department of Surgery, Emory University School of Medicine, Atlanta, GA 30322, USA

Received 18 November 2005
Available online 9 December 2005

Abstract

Our goal is to develop effective islet grafts for treating type 1 diabetes. Since human islets are scarce, we evaluated the efficacy of a microencapsulated insulin-secreting conditionally transformed allogeneic β-cell line (βTC-tet) in non-obese diabetic mice treated with tetracycline to inhibit cell growth. Relatively low serum levels of tetracycline controlled proliferation of βTC-tet cells without inhibiting effective control of hyperglycemia in recipients. There was no significant host cellular reaction to the allografts or host cell adherence to microcapsules, and host cytokine levels were similar to those of sham-operated controls. We conclude that encapsulated allogeneic β-cell lines may be clinically relevant, because they effectively restore euglycemia and do not elicit a strong cellular immune response following transplantation. To our knowledge, this is the first extensive characterization of the kinetics of host cellular and cytokine responses to an encapsulated islet cell line in an animal model of type 1 diabetes.

Keywords: Alginate; Allogeneic cells; Animal model; Cell encapsulation; Cytokines; Immune response

Type 1 diabetes mellitus (T1DM) results when pancreatic β-cells are destroyed or absent, leading to a decrease in insulin production and secretion [1]. The long-term consequences of T1DM may include cardiovascular disease, retinopathy, neuropathy, renal disease, limb loss, and other debilitating chronic symptoms [2]. One goal of treating T1DM is to control the disease symptoms associated with hyperglycemia, ideally keeping the blood glucose level between 80 and 200 mg/dl. Islet cell transplantation is one option for treating T1DM. Since development of the "Edmonton Protocol" [2], transplantation of human islets has been clinically successful, however long-term freedom from the need for exogenous insulin has not been achieved in the majority of cases [3]. Furthermore, the supply of available human islets is inadequate to treat all of the new cases of T1DM diagnosed annually. As alternatives, allogeneic β-cell lines or porcine islet xenografts potentially may be used.

A major challenge in the application of cell therapy to T1DM is to prevent immunological rejection by the recipient. In addition to allograft rejection, transplanted islets are also subjected to autoimmune rejection, since spontaneously diabetic hosts are already primed to destroy islet β-cells [1]. To protect transplanted islets from host immune responses, immunoisolation has been employed, using encapsulation in alginate-PLL microcapsules that reduce direct contact with host effector T cells and anti-graft antibodies [4]. The selective membrane permeability of our alginate-PLL microcapsules excludes larger molecular weight proteins, such as host immunoglobulin, but allows free diffusion of insulin [4].

Encapsulated porcine islets have been used experimentally and may be promising for the treatment of T1DM in the future [5]. Porcine islets normalize blood glucose

* Corresponding author. Fax: +1 416 241 8301.
E-mail address: Sasha.Black@ca.crl.com (S.P. Black).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.180

when implanted into diabetic animals but unfortunately, transplantation between widely discordant species is complicated by the problem of xenograft rejection [6] and remains controversial because of the potential risk of introducing new animal pathogens into the human population [7]. Allogeneic cell lines may be used experimentally as an alternative to freshly isolated islets to model the functions of the pancreas. An advantage of cell lines is that they have the ability to proliferate allowing easy amplification in culture to provide a sufficient number of cells for transplantation [8]. However, the potential for uncontrolled growth is problematic for encapsulated cell systems, because without growth regulation, transformed cells grow continuously developing large clusters of cells, resulting in expansion and eventual rupture of the capsules [9]. In addition, if encapsulated islet β-cell lines overgrow in transplant recipients, the production of high levels of insulin may cause hypoglycemia, a more serious clinical condition than hyperglycemia.

In order to prevent problems associated with excess proliferation of transplanted cells, a mechanism is required to prevent cellular overgrowth [9]. For this reason, Efrat et al. have developed growth-regulated conditionally transformed allogeneic β-cell lines (βTC-tet) derived from the expression of the SV40 T antigen oncoprotein under control of the bacterial tetracycline-resistance operon regulatory system [10]. In the presence of tetracycline, growth arrest is induced because production of the oncogene is repressed [10]. The effects of growth regulation on the long-term metabolic and secretory characteristics of alginate-PLL encapsulated βTC-tet cells were evaluated in vitro for 68 days [11]. The results showed that the growth of alginate-encapsulated βTC-tet cells could be controlled in a dose-dependent manner by continuous exposure to tetracycline in vitro. The authors concluded that if a sufficient concentration of tetracycline could be delivered to the encapsulated cells to regulate their growth, the regulation was not detrimental to their insulin secretory function [11]. After transplantation into syngeneic mice with streptozotocin-induced hyperglycemia, replication of βTC-tet cells was inhibited by administration of tetracycline in drinking water [9]. The growth-arrested cells responded to hyperglycemia by a significant increase in insulin secretion, showing that growth-arrested βTC-tet cells maintain insulin production and glucose-induced insulin secretion in vivo [9]. However, to our knowledge no previous studies have measured serum concentrations of tetracycline in the recipient and correlated them with regulation of βTC-tet cell growth in vivo to determine the effective dose of the conditioning agent to administer to the recipient.

Therefore, while in vitro and in vivo studies of βTC-tet cells have been previously reported [9,11], we are the first to evaluate the correlation between serum tetracycline levels and proliferation of this conditionally transformed β-cell line, as well as its ability to normalize blood glucose levels in vivo. In addition, we are the first to extensively characterize the kinetics of the cellular and cytokine immune responses in spontaneously diabetic NOD mice transplanted with encapsulated allogeneic βTC-tet cells.

Materials and methods

Animals. Eight-week-old adult NOD/LtJ mice (H-2^{g7}) (Jackson Labs, Bar Harbor, ME) were housed under specific pathogen-free conditions. The mice were screened weekly for diabetes using urine glucose strips (Roche Diagnostics, Indianapolis, IN) and subsequently treated with insulin until transplantation [5]. Forty-one mice were deemed diabetic (blood glucose levels exceeded 250 mg/dl for three consecutive days). Thirty-seven of the mice were transplanted with encapsulated βTC-tet cells, and four mice received non-encapsulated βTC-tet cells. Ten additional NOD mice were used as sham-transplanted controls, and five more NODs were used as controls for serum tetracycline levels in normal, untreated mice. All procedures were conducted according to the Guidelines of the Committee on Care and Use of Laboratory Animals, Institute of laboratory Animal Resources (National Research Council, Washington, DC).

βTC-tet cells. Murine βTC-tet cells (H-2^k) were kindly provided by Shimon Efrat (University of Tel Aviv, Israel) [10]. The cells were cultured at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) containing 20 mM glucose, 15% (v/v) equine serum (Hyclone, Logan, UT), 2.5% (v/v) fetal bovine serum (Hyclone), 100 U/ml penicillin, 100 ng/ml streptomycin, and 6 mM L-glutamine (Sigma, St. Louis, MO). Cells from earlier passages were chosen for use in experiments, and when the cells had been passaged 55 times, they were discarded. For encapsulation, the adherent cells were removed from the flasks using 0.25% trypsin with EDTA (Mediatech, Herndon, VA), resuspended as single cells, washed to remove all serum, and counted.

Encapsulation and transplantation. βTC-tet cells were encapsulated in alginate beads using 2% low viscosity sodium alginate (ISP Alginates, San Diego, CA) with a guluronic acid content of approximately 41%. The beads were gelled in 1.1% $CaCl_2$ and then coated with double PLL walls. These microcapsules are about 900 μm in diameter and exclude IgG (MW = 146,000–165,000) but not hemoglobin (MW = 68,000) [4]. Recipient NOD mice ($n = 37$) were transplanted i.p. with 9×10^6 encapsulated βTC-tet cells in a volume of 0.5 ml via midline celiotomy. Transplanting this relatively large number of insulin-secreting cells resulted in prompt reversal of diabetes within 2–3 days. Four of these mice were sacrificed on day 1 post-transplantation as controls for surgery-related inflammatory responses. Ten additional NOD mice received Hanks' balanced salt solution (HBSS) i.p. and were sacrificed 12–14 days later as sham-treated controls. Non-encapsulated βTC-tet cells (8×10^6) were transplanted under the renal capsule ($n = 2$) or i.p. ($n = 2$) and were sacrificed 17–27 days later as controls for the effectiveness of encapsulation. Beginning on Day 2 post-transplantation, mice were given drinking water ad lib containing 4 mg/ml tetracycline (Sigma) with 2.5% (w/v) sucrose. The tetracycline water was replenished twice weekly.

Serum tetracycline assay. Groups of NOD mice were sacrificed at day 0 and then at weekly intervals for 8 weeks following transplantation. The numbers of mice per group were: 5 control mice (Day 0), 6 mice (Days 7–9), 4 mice (Day 14), 3 mice (Days 19–22), 4 mice (Days 26–29), 4 mice (Days 33–34), 4 mice (Days 39–42), 4 mice (Day 49), and 4 mice (Days 50–56). Blood was drawn via intracardiac puncture, centrifuged at 1200 rpm for 10 min, and serum was removed for the tetracycline assay. A tetracycline sensitive strain of *Bacillus cereus* (ATTC, Manassas, VA) was prepared as previously described and used in an agar-well diffusion assay to determine the concentration of tetracycline in the serum samples [12]. The *B. cereus* stock suspension was seeded into nutrient agar (Difco, Spark, Maryland) and poured into Petri dishes (Kirby–Bauer, 150 × 15 mm, Fisher Scientific, Pittsburgh, PA). After solidification, 4.5 mm wells were punched in the agar using sterile technique, and serial dilutions of tetracycline (0.12–6.25 μg/ml) were added to the wells for use as standards. Undiluted serum from mice was added to separate wells. The plates were incubated at 37 °C for 16–22 h, and zones of inhibition of *B. cereus* growth were measured around the wells [12]. The concentration

of tetracycline in murine serum was determined by comparing zones of inhibition around test samples with the zones of inhibition around known tetracycline concentrations.

Histology and immunohistochemistry. Encapsulated βTC-tet cells were harvested from the peritoneal cavities of mice by peritoneal lavage, and a sample of the capsules was placed in a small Petri dish with Hanks' balanced salt solution (HBSS) and visualized by light microscopy to evaluate capsule integrity and host cell attachment. The remaining microcapsules were fixed overnight in 10% neutral buffered formalin (Fisher Scientific), transferred to 70% ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (Sigma) for histological evaluation.

Flow cytometry. Peritoneal cells (PEC) were pre-incubated with Mouse Fc-Block (Pharmingen, San Diego, CA) to prevent non-specific binding of antibodies. All PEC were bone marrow-derived as judged by staining with antibody to the CD45 leukocyte common antigen. The PEC were stained with PE-conjugated anti-CD11b mAb (MAC-1) (Pharmingen) in combination with FITC-conjugated antibodies to CD3, CD4, CD8, CD19 or Ly6G (Pharmingen). Conjugated matched isotypes served as negative controls. Anti-CD11b was used in combination with anti-Ly6G (Gr-1) to identify neutrophils [13]. Macrophages were characterized by their expression of MAC-1, high autofluorescence, and their phagocytic activity. The antibodies were diluted in staining buffer (PBS, pH 7.4, containing 1% BSA and 0.1% sodium azide) to <1 μg/50 μl/10^6 cells. The cells were incubated for 30 min on ice, in the dark, washed three times in staining buffer, and fixed in 1% paraformaldehyde. Analysis was performed on a FACScan Cytometer using Cellquest software (Beckton–Dickinson, San Jose, CA) and the data were further analyzed using FlowJo software (Tree Star, San Carlos, CA). Forward angle and side light scatter were used to exclude dead cells.

Cytokine ELISA. To collect the peritoneal fluid, mice were injected i.p. with 10 ml HBSS containing 0.06% BSA, 10 mM Hepes, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 U/ml heparin. Fluids were aspirated from the peritoneal cavity and centrifuged (1200 rpm, 7 min) to remove PEC and encapsulated cells. Supernatant fluids were stored at −20 °C until cytokine ELISAs were performed. Peritoneal fluids were assayed for cytokines using paired mAbs specific for IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ (PharMingen), IL-6, IL-12, and activated TGF-β (R&D Systems, Minneapolis, MN). Biotinylated Abs were added and detected with avidin peroxidase (Vector Laboratories, Burlingame, CA) plus 2,2 azino-di-[3-ethyl-genzthiazoline sulfonate] substrate containing H_2O_2 (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The colorimetric reaction was read at 450 nm using an automatic microplate reader (Molecular devices, Menlo Park, CA). The concentrations of the cytokines were calculated from the standard curve of the appropriate recombinant cytokine.

Statistical analysis. All data were analyzed using the GraphPad InStat program (GraphPad Software, San Diego, CA). The statistical significance of differences in quantitative variables between groups was analyzed by unpaired (two-tailed) Mann–Whitney *t* tests with Welch correction. The statistical significance of differences among more than two groups was analyzed by the Tukey–Kramer Multiple Comparisons Test (parametric ANOVA). *P* values $\leqslant 0.05$ were considered significant.

Results

Restoration of euglycemia in diabetic NOD mice transplanted with encapsulated βTC-tet cells

Spontaneously diabetic NOD mice were transplanted i.p. with encapsulated βTC-tet cells, and graft function was monitored by measuring random blood glucose concentrations. Prior to transplantation, the NOD mice were hyperglycemic, but within 24–48 h following transplantation, blood glucose levels were normalized. The blood glucose levels remained within normal ranges (80–200 mg/dL)

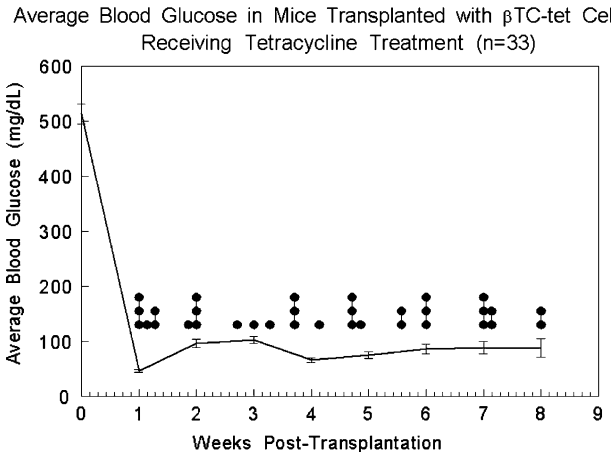

Fig. 1. Blood glucose levels of NOD mice transplanted with encapsulated βTC-tet cells i.p. NOD mice ($n = 33$) were transplanted i.p. with encapsulated βTC-tet cells and continuously treated with 4 mg/ml tetracycline in the drinking water beginning on day 2 post-transplantation. Random blood glucose levels were monitored to determine graft function. Solid round symbols represent individual mice sacrificed on a given day. All mice were normoglycemic at sacrifice.

until the time of euthanasia in all mice, indicating that the βTC-tet cell allografts were functioning (Fig. 1). None of the mice rejected their grafts during the 8 weeks of the study. In contrast, when NOD mice were implanted with non-encapsulated βTC-tet cells either under the renal capsule or i.p., the mice remained hyperglycemic until they were sacrificed 17–27 days later with non-functioning grafts.

Effect of tetracycline on βTC-tet cell growth in vivo

The purpose of this experiment was to measure the concentration of tetracycline in the serum of recipient mice after administration of 4 mg/ml tetracycline in drinking water. This experiment was also designed to determine whether exponential growth of the encapsulated cells could be inhibited by these levels of tetracycline in the serum. Thirty-three mice were transplanted with encapsulated βTC-tet cells and groups were sacrificed each week for eight weeks. At the time of sacrifice, microcapsules were harvested for histological evaluation, and the concentration of tetracycline was measured in the serum. Insignificant levels of tetracycline were detected in the serum of control mice ($n = 5$) given no tetracycline water (Fig. 2). When less than 4 mg/ml tetracycline was administered to the mice, the levels in the serum were undetectable by our methods (data not shown). However, when mice were given 4 mg/ml tetracycline in drinking water continuously, the tetracycline levels in serum of individual mice ranged from 120 to 300 ng/ml. There were no significant differences in tetracycline concentrations in the serum in relation to the number of weeks the animals were involved in the study (Fig. 2).

To determine whether the levels of tetracycline in the serum correlated with inhibition of growth of the encapsu-

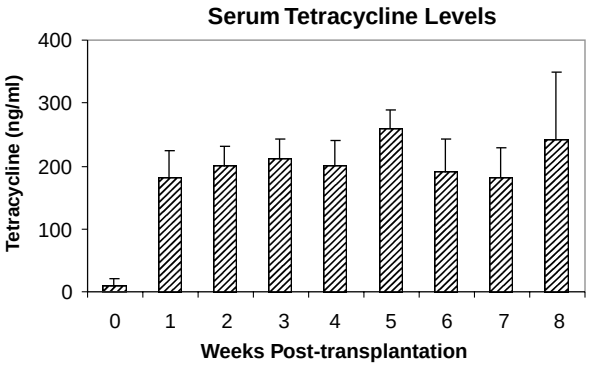

Fig. 2. Serum tetracycline levels in 33 transplanted NOD mice and five non-transplanted controls at time of sacrifice. Groups of mice were sacrificed at day 0 and then at weekly intervals for 8 weeks following transplantation. The numbers of mice per group were: 5 mice (Day 0), 6 mice (Days 7–9), 4 mice (Day 14), 3 mice (Days 19–22), 4 mice (Days 26–29), 4 mice (Days 33–34), 4 mice (Days 39–42), 4 mice (Day 49), and 4 mice (Days 50–56). Blood was collected via intracardiac puncture and centrifuged at 1200 rpm for 10 min. The serum was removed and tetracycline concentrations were measured as described in Materials and methods. Serum tetracycline concentrations (ng/ml) were plotted against time (weeks) of administration of tetracycline to the mice in drinking water. Bars represent mean values ± SD.

lated βTC-tet cells, the microencapsulated cells were analyzed by light microscopy and by histology. At all time points from Day 0 until week 8, the majority of capsules were intact without significant evidence of breakage, and the morphology of the βTC-tet cells appeared normal by light microscopy. Colony growth was observed during the first two week post-transplantation by light microscopy (Fig. 3) and by histological analysis (Fig. 4). After the first two weeks, there was insignificant additional growth in most capsules, suggesting that proliferation of the cells was inhibited by serum levels of tetracycline in the range of 120–300 ng/ml. However, at later time points a few rare colonies of cells could be detected that continued to grow in spite of tetracycline treatment (Fig. 4, Day 56, inset). The majority of capsules showed little or no evidence for fibrosis or overgrowth by host peritoneal cells (Figs. 3 and 4), but occasionally capsules were found with some host cell attachment (Fig. 4, Day 19, inset). These rare incidents of capsule overgrowth by host cells did not have a detrimental effect on the function of the grafts or the health of recipient NOD mice.

Evaluation of peritoneal cellular infiltration and cytokine secretion following transplantation of encapsulated βTC-tet cells

The purpose of this experiment was to characterize the peritoneal cellular infiltrate following i.p. transplantation of encapsulated βTC-tet cells in diabetic NOD mice treated with tetracycline. For these experiments, groups of transplanted diabetic NOD recipients were sacrificed each week for 8 weeks after transplantation and peritoneal cells and fluids were removed by lavage. The phenotypes of the

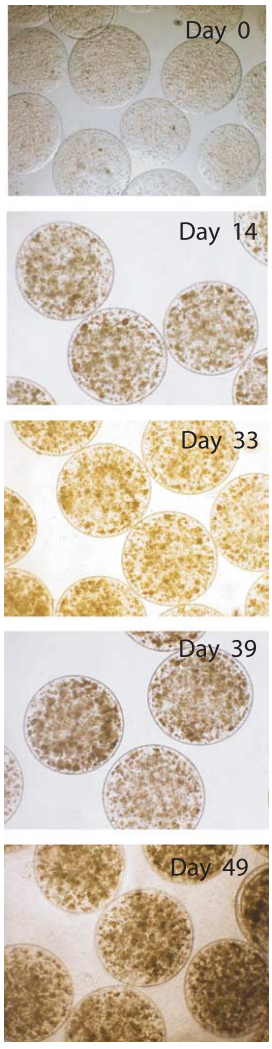

Fig. 3. Representative light micrographs of encapsulated βTC-tet cells retrieved from the peritoneal cavity of transplanted diabetic NOD recipients. Four NOD mice were transplanted simultaneously with 9×10^6 βTC-tet cells from a single encapsulation procedure. Freshly microencapsulated, non-transplanted βTC-tet cells from the same encapsulation procedure are shown as Day 0. The 4 mice were sacrificed at various time points post-transplantation (Days 14, 33, 39, and 49), and all microcapsules were harvested and visualized by light microscopy (40×).

peritoneal cell populations were determined by flow cytometry, and the numbers of $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, macrophages, neutrophils, and eosinophils were quantitated. Controls included peritoneal cells from 10 NOD mice given sham surgery and sacrificed on days 12–14 post-transplantation and four NOD mice transplanted with encapsulated βTC-tet cells and sacrificed on day 1 post-transplantation.

Significantly elevated numbers of peritoneal cells were found in recipients of encapsulated βTC-tet cells on day 1 post-transplantation ($15.1 \pm 2.8 \times 10^6$) compared to sham-operated controls on days 12–14 ($3.5 \pm 2.6 \times 10^6$, $P < 0.001$) and transplant recipients during weeks 1–2 ($3.7 \pm 1.4 \times 10^6$, $P < 0.001$), weeks 3–4 ($6.4 \pm 2.3 \times 10^6$, $P < 0.05$), and weeks 5–6 post-transplantation ($3.0 \pm 0.6 \times 10^6$, $P < 0.001$). During the first 24 h after transplantation,

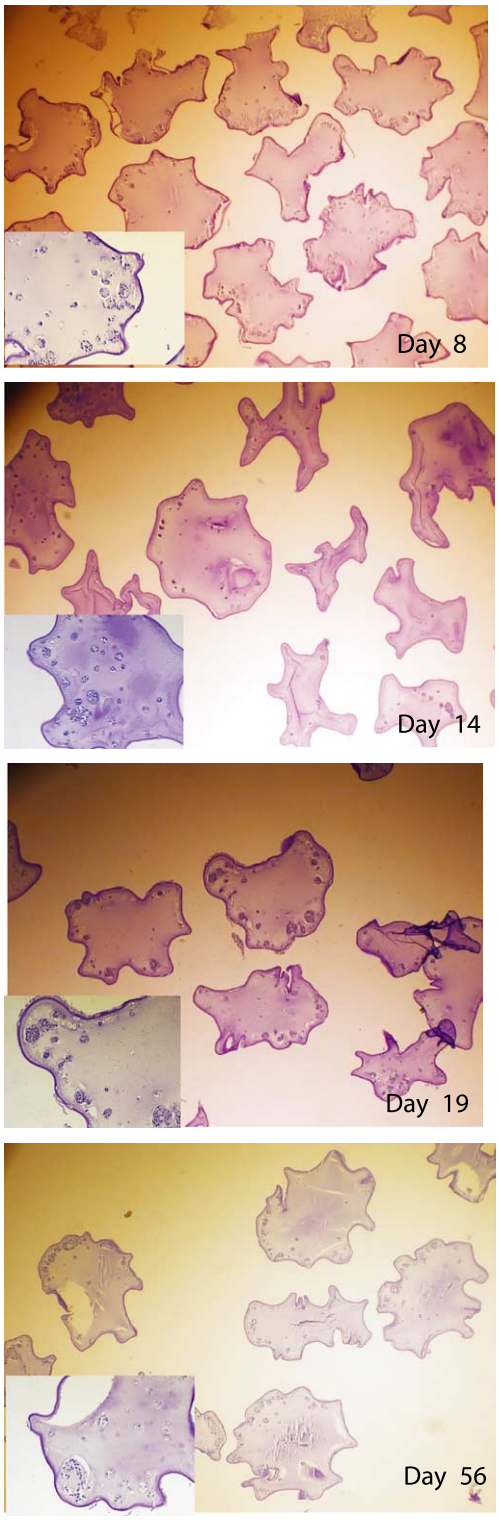

Fig. 4. Histological analysis of representative encapsulated βTC-tet cells retrieved from the peritoneal cavity of transplanted diabetic NOD recipients. Four NOD mice were transplanted simultaneously with 9×10^6 βTC-tet cells from a single encapsulation procedure. The mice were sacrificed at various time points (Days 8, 14, 19, and 56), microcapsules were harvested, prepared for histology, stained by hematoxylin and eosin, and visualized by light microscopy (40× and 100× inset).

large numbers of host macrophages and neutrophils were recruited to the peritoneal site, most likely as a result of tissue injury during surgery (Table 1). However, by two weeks post-transplantation, relatively low levels of macrophages and neutrophils (comparable to levels in sham-operated controls) were found in the peritoneal cavities of transplanted mice (Table 1). Peritoneal populations of $CD3^+$ T cells, $CD19^+$ B cells, macrophages, neutrophils, and eosinophils remained at normal levels during the 8 weeks of study following transplantation, with the exception of slightly elevated numbers of $CD4^+$ T cells and $CD8^+$ T cells at three to four weeks post-transplantation (Table 1).

The levels of 10 different cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFNγ, TNFα, and TGFβ) in peritoneal fluids of NOD mice transplanted with βTC-tet cells were measured over the course of 8 weeks post-transplantation. In recipient NOD mice, significant levels of IL-2, IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-12, and TNFα were not detected at any time point. Very low levels of IL-1β (85 ± 43 pg/ml) were found in the peritoneal fluid during weeks 1–2 post-transplantation, when neutrophils and macrophages were present, but IL-1β decreased thereafter, becoming undetectable by weeks 7–8. TGFβ was detected in peritoneal fluid (300–400 pg/ml) each week until the end of the study, but these levels were not significantly different from control mice sacrificed on day 1 post-transplantation.

Discussion

The data presented here represent a thorough characterization of the peritoneal cellular and cytokine milieu in diabetic NOD mice for 8 weeks following i.p. transplantation with a microencapsulated allogeneic β-cell line, βTC-tet. Other groups have studied a different mouse insulinoma cell line (MIN6), characterizing its glucose metabolism and glucose-stimulated insulin secretion [14] and ability to restore normoglycemia in chemically diabetic mice after transplantation in various types of alginate microcapsules that were not found to be overgrown with host cells in vivo [15]. However, to our knowledge, a comprehensive study of host immune responses to encapsulated allogeneic transformed cells has not been previously reported. One of the most significant findings of our study is that the peritoneal cavities of the recipients appeared to be normal. This result suggests that transplantation of diabetic recipients with microencapsulated allogeneic cell lines may be successful in the future with little or no immunosuppression.

Our results demonstrate that diabetic NOD mice treated with 4 mg/ml tetracycline in drinking water had detectable serum levels of tetracycline ranging from 100 to 300 ng/ml. This concentration of tetracycline effectively controlled the growth of encapsulated βTC-tet cells in vivo for the duration of the experiment, up to 58 days, so that the microcapsules did not burst or release allogeneic cells into the peritoneal cavity of recipient mice. During this time period, all diabetic NOD recipients remained normoglycemic,

Table 1
Kinetics of peritoneal cell infiltration in diabetic NOD mice transplanted with encapsulated βTC-tet cells

Groups	CD3 × 10^6 [a]	CD4 × 10^6	CD8 × 10^6	CD19 × 10^6	MAC × 10^6	NEU × 10^6	EOS × 10^6
Sham controls	0.20 ± 0.04	0.10 ± 0.02	0.04 ± 0.01	1.49 ± 0.31	1.52 ± 0.29	0.02 ± 0.01	0.01 ± 0.00
Day 1	0.20 ± 0.05	0.01 ± 0.01	0.07 ± 0.02	0.34 ± 0.06	5.99 ± 1.43[d]	6.83 ± 1.26[e]	0.01 ± 0.00
Weeks 1–2	0.08 ± 0.06	0.06 ± 0.03	0.01 ± 0.01	0.33 ± 0.25	2.80 ± 2.13	0.06 ± 0.03	0.09 ± 0.09
Weeks 3–4	1.04 ± 0.61	0.61 ± 0.36[b]	0.32 ± 0.17[c]	2.69 ± 1.47	1.15 ± 0.38	0.31 ± 0.14	0.10 ± 0.09
Weeks 5–6	0.24 ± 0.09	0.15 ± 0.06	0.06 ± 0.02	0.95 ± 0.28	1.56 ± 0.51	0.70 ± 0.44	0.01 ± 0.00
Weeks 7–8	0.30 ± 0.17	0.20 ± 0.09	0.08 ± 0.05	1.09 ± 0.34	1.79 ± 0.49	0.41 ± 0.13	0.01 ± 0.00

[a] Values shown are mean number of cells ($\times 10^6$) of each phenotype ± SE. The number of cells with each phenotype was calculated by multiplying the percentage of cells (determined by flow cytometry) by the total number of peritoneal cells harvested from each mouse.
[b] For $CD4^+$ cells, ANOVA: $P = 0.0358$; Tukey–Kramer Multiple Comparisons Test: $P < 0.05$ for Weeks 3–4 versus sham-operated controls, Day 1, and Weeks 1–2 post-transplantation.
[c] For $CD8^+$ cells, ANOVA: $P = 0.0141$; Tukey–Kramer Multiple Comparisons Test: $P < 0.05$ for Weeks 3–4 versus sham-operated controls and Weeks 1–2 post-transplantation.
[d] For macrophages, ANOVA: $P = 0.0212$; Tukey–Kramer Multiple Comparisons Test: $P < 0.05$ for Day 1 versus sham-operated controls and Weeks 3–4, 5–6, and 7–8 post-transplantation.
[e] For neutrophils, ANOVA: $P < 0.0001$; Tukey–Kramer Multiple Comparisons Test: $P < 0.05$ for Day 1 versus sham-operated controls and Weeks 1–2, 3–4, 5–6, and 7–8 post-transplantation.

suggesting that exposure to tetracycline prevented overgrowth of the cells that could have resulted in the production of excess insulin and thus the onset of hypoglycemia in recipients. The normal blood glucose values detected in recipient mice throughout our study validate previous reports by others proving that continuous administration of tetracycline controls the growth of βTC-tet cells in vitro [11] and also allows animals to maintain normal glucose levels following transplantation of βTC-tet cells in vivo [8–10]. Our study shows that a period of time is required for tetracycline levels to be effective in controlling proliferation of this allogeneic cell line in vivo, since colony growth was observed during the first two weeks post-transplantation. However, once tetracycline began to regulate cell growth, further proliferation of the cells was inhibited for the majority of colonies. The presence of a few colonies of βTC-tet cells that continued to grow in spite of chronic tetracycline treatment suggests that non-clonal cells with slightly different phenotypes may develop over time in vivo. Knaack et al. [16] reported that clonal βTC-tet cell lines isolated before passage 20 in culture demonstrated a greater stability of phenotype and function than cell lines isolated after passage 20. This finding suggests that conditionally transformed cell lines must be carefully characterized before use in tissue engineered constructs in vivo to assure safety and efficacy.

Simpson et al. [11] recently showed that continuous exposure to tetracycline initiated within a day of encapsulation resulted in dose-dependent growth regulation in vitro. Specifically, in the absence of tetracycline the βTC-tet cells grew exponentially and the unregulated cultures had to be discarded by day 40 because excessive cell growth caused some of the capsules to burst. In contrast, exposure to 1 ng/ml tetracycline prevented cell growth for 15 days, but afterwards the βTC-tet cells began to grow within the alginate beads. A dose of 10 ng/ml resulted in an effective delay in cell growth for about 40 days, while a dose of 30 ng/ml resulted in a stable metabolic activity and minimal growth as evidenced by histological evaluation. Exposure to 100, 300, or 1000 ng/ml caused a 50% reduction in metabolic activity within the first 3 weeks of culture. This reduced metabolic activity was maintained for the remainder of the experiment (80 days). Our study compliments that of Simpson et al. in that we have evaluated the effect of tetracycline on βTC-tet cell growth within microcapsules in vivo. We found that a minimal serum level of 120 ng/ml tetracycline required at least two weeks before becoming effective in controlling the growth of βTC-tet cells. (Lower levels of serum tetracycline could not be detected in our system, and hence could not be evaluated.)

The finding that insulin-secreting βTC-tet cells maintained normoglycemia in diabetic NOD mice throughout the 8 weeks of the study suggests that the mice did not mount an inflammatory response to this encapsulated allogeneic cell line. However, when non-encapsulated βTC-tet cells were implanted i.p. or under the renal capsule, the NOD recipients remained hyperglycemic, suggesting that the allogeneic cell lines were quickly rejected. Many studies have shown that non-encapsulated allografts are rejected by a complex immune response involving $CD4^+$ T cells, cytolytic $CD8^+$ T cells, and macrophages [17,18]. By contrast, none of the encapsulated βTC-tet allografts were rejected during the 8 weeks of our study, although macrophages and neutrophils briefly infiltrated the peritoneal graft site within 24 h after surgery. Normal levels of $CD4^+$ T cells, $CD8^+$ T cells, macrophages, neutrophils, and eosinophils were found at all time points post-transplantation. The small elevation of T cells at 3–4 weeks after transplantation was not sufficient to provoke hyperglycemia or graft rejection in any recipients. In addition, dramatically elevated levels of products of immune cells known to be toxic for islet β-cells, such as TNFα, IL-1β, and IFN-γ [19], were not elicited by encapsulated βTC-tet cells transplanted i.p., and none of the other cytokines studied were elevated above the levels found in sham-operated controls. These findings provide further evidence that

encapsulated allogeneic cell lines do not evoke a significant immune response in diabetic recipients. Although we do not know whether the surface antigenicity of βTC-tet cells changes with long-term culture, we do not believe that the long-term function we observed in NOD mice is due to loss of antigenicity with time, because this conditionally transformed βTC-tet cell line has been reported to remain phenotypically stable up to 60 passages in culture [9]. Furthermore, the immediate rejection of non-encapsulated βTC-tet cells which we observed suggests that these cells are highly antigenic.

Studies in diabetic NOD mice are required to address autoimmune issues, as there is no other animal model for human type 1 diabetes, except the Bio-Breeding (BB) rat. As in humans, spontaneous diabetes in NOD mice and BB rats is the result of humoral and $CD4^+$ and $CD8^+$ T cell-mediated destruction of insulin-producing pancreatic β-cells [20]. NOD mice exhibit a number of immune abnormalities including defective macrophage function [21], defects in the $CD4^+$ T cell response to superantigens [22], low levels of natural killer cell activity [23], deficiencies in regulatory $CD4^+CD25^+$Foxp3 T cell function [24], and absence of hemolytic complement [25]. In spite of these deficiencies, NOD mice vigorously reject non-encapsulated allogeneic murine islets [4], and most investigators agree that the NOD mouse is an important tool allowing researchers to gain insights that will lead to effective therapies for type 1 diabetes and other human diseases [26].

Recently, a reversibly immortalized, glucose-responsive human pancreatic β-cell line (NAKT-15) has been developed that does not senesce after multiple passages in vitro [27]. This human β-cell line corrected hyperglycemia for longer than 30 weeks in streptozotocin-diabetic mice with severe combined immunodeficiency [27]. These promising results suggest that a clinically relevant source of β-cells for transplantation into diabetic human patients may be developed in the near future. We conclude that although care must be exercised in selection of a phenotypically stable cell line and in administering the conditioning agent to achieve optimum results without adverse side effects, such as hypoglycemia, encapsulated conditionally transformed allogeneic cell lines may be useful for the treatment of diabetes and other diseases in human patients in the future, since they do not elicit a strong immune response in the recipient.

Acknowledgments

This work was supported in part by funding from the Georgia Tech/Emory Center (GTEC) for the Engineering of Living Tissues, an ERC Program of the National Science Foundation under Award Number EEC-9731643. It was also funded by NIH Grants DK56980 and DK47858. We acknowledge Emanuel Wani for excellent care of diabetic NOD mice and Sean Cauffiel for outstanding technical assistance and for graphing Fig. 1.

References

[1] S. Efrat, Cell replacement therapy for type 1 diabetes, Trends Mol. Med. 8 (2002) 335–338.
[2] J. Shapiro, J.R. Lakey, E.A. Ryan, G.S. Korbutt, E. Toth, G.L. Warnock, N.M. Kneteman, R.V. Rajotte, Islet transplantation in seven patients with type 1 diabetes mellitus using glucocorticoid-free immunosuppressive regimen, N. Engl. J. Med. 343 (2000) 230–231.
[3] E.A. Ryan, B.W. Paty, P.A. Senior, D. Bigam, E. Alfadhli, N.M. Kneteman, J.R. Lakey, A.M. Shapiro, Five-year follow-up after clinical islet transplantation, Diabetes 54 (2005) 2060–2069.
[4] C.J. Weber, S. Safley, M. Hagler, J. Kapp, Evaluation of graft-host response for various tissue sources and animal models, Ann. N. Y. Acad. Sci. 875 (1999) 233–254.
[5] S.A. Safley, L.M. Kapp, C. Tucker-Burden, B. Hering, J.A. Kapp, C.J. Weber, Inhibition of cellular immune responses to encapsulated porcine islet xenografts by simultaneous blockade of two different costimulatory pathways, Transplantation 79 (2005) 413–416.
[6] S. Safley, J.A. Kapp, C.J. Weber, Proliferative and cytokine responses in CTLA4-Ig-treated diabetic NOD mice transplanted with microencapsulated neonatal porcine ICCs, Cell Transplant. 11 (2002) 695–705.
[7] J.A. Fishman, C. Patience, Xenotransplantation: infectious risk revisited, Am. J. Transplant. 4 (2004) 1383–1390.
[8] D. Milo-Landesman, M. Surana, I. Berkovich, A. Compagni, G. Christfori, N. Fleischer, S. Efrat, Correction of hyperglycemia in diabetic mice transplanted with reversibly immortalized pancreatic β-cells controlled by the tet-on regulatory system, Cell Transplant. 10 (2001) 645–650.
[9] N. Fleischer, C. Chen, M. Surana, M. Leiser, L. Rossetti, W. Pralong, S. Efrat, Functional analysis of a conditionally transformed pancreatic β-cell line, Diabetes 47 (1998) 1419–1425.
[10] S. Efrat, D. Fusco-DeMane, H. Lemberg, O.A. Emran, X. Wang, Conditional transformation of a pancreatic β-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene, Proc. Natl Acad. Sci. USA 92 (1995) 3577–3579.
[11] N.E. Simpson, N. Khokhlova, J.A. Oca-Cossio, S.S. McFarlane, C.P. Simpson, I. Constantinidis, Effects of growth regulation on conditionally transformed alginate-entrapped insulin secreting cells in vitro, Biomaterials 26 (2005) 4633–4641.
[12] J.V. Bennet, J.L. Brodie, E.J. Benner, W.M.M. Kirby, Simplified, accurate method for antibiotic assay of clinical specimens, Appl. Microbiol. 14 (1966) 170–174.
[13] E. Lagasse, I.L. Weismann, Flow cytometric identification of murine neutrophils and monocytes, J. Immunol. Methods 197 (1996) 139–150.
[14] H. Ishihara, T. Asano, K. Tsukuda, H. Katagiri, K. Inukai, M. Anai, M. Kikuchi, Y. Yazaki, J.I. Miyazaki, Y. Oka, Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets, Diabetologia 36 (1993) 1139–1145.
[15] S. Sakai, T. Ono, H. Ijima, K. Kawakami, MIN6 cells-enclosing aminopropyl- silicate membrane templated by alginate gels differences in guluronic acid content, Int. J. Pharmaceut. 170 (2004) 65–73.
[16] D. Knaack, D.M. Fiore, M. Surana, M. Leiser, M.E. Laurance, D. Fusco-DeMane, O.D. Hegre, N. Fleischer, S. Efrat, Clonal insulinoma cell line which stably maintains correct glucose responsiveness, Diabetes 43 (1994) 1413–1417.
[17] C.D. Heidecke, J.W. Kupiec-Weglinski, P.A. Lear, M. Abbud-Filho, J.L. Araumo, D. Araneda, T.B. Strom, N.L. Tilney, Interactions between T lymphocyte subsets supported by interleukin 2-rich lymphokines produce acute rejection of vascularized cardiac allografts in T cell deprived rats, J. Immunol. 133 (1984) 582–588.
[18] R. Bottino, L.A. Fernandez, C. Ricordi, R. Lehmann, M.F. Tsan, R. Oliver, L. Inverardi, Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation, Diabetes 47 (1998) 316–323.

[19] A. Rabinovitch, W. Sumoski, R.V. Rajotte, G.L. Warnock, Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture, J. Clin. Endocrinol. Metab. 71 (1990) 152–156.
[20] J.P. Mordes, D.L. Greiner, A.A. Rossini, Animal models of autoimmune diabetes, in: D. LeRoith, S.I. Taylor, J.M. Olefsky (Eds.), Diabetes Mellitus: A Fundamental and Clinical Text, Lippincott Williams and Wilkins, Philadelphia, 2004, pp. 591–610.
[21] D.V. Serreze, J.W. Gaedeke, E.H. Leiter, Hematopoietic stem-cell defects underlying abnormal macrophage development and maturation in NOD/Lt mice: defective regulation of cytokine receptors and protein kinase C, Proc. Natl. Acad. Sci. USA 90 (1993) 9625–9629.
[22] J. Radons, V. Burkart, H. Kolb, MHC class II-dependent abnormal reactivity toward bacterial superantigens in immune cells of NOD mice, Diabetes 46 (1997) 379–385.
[23] L. Shultz, P.A. Schweitzer, S.W. Christianson, B. Gott, I.B. Schweitzer, B. Tennent, S. McKenna, L. Mobraaten, T.V. Rajan, D.L. Greiner, E.H. Leiter, Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice, J. Immunol. 154 (1995) 180–191.
[24] B. Salomon, D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, J.A. Bluestone, B7/CD28 costimulation is essential for the homeostasis of the CD4 + CD25 + immunoregulatory T cells that control autoimmune diabetes, Immunity 12 (2000) 431–440.
[25] A.G. Baxter, A. Cooke, Complement lytic activity has no role in the pathogenesis of autoimmune diabetes in NOD mice, Diabetes 42 (1993) 1574–1578.
[26] M.S. Anderson, J.A. Bluestone, The NOD mouse: a model of immune dysregulation, Ann. Rev. Immunol. 23 (2005) 447–485.
[27] M. Narushima, N. Kobayashi, T. Okitsu, Y. Tanaka, S. Li, Y. Chen, A. Miki, K. Tanaka, S. Nakaji, K. Takei, A.S. Gutierrez, J.D. Rivas-Carrillo, N. Navarro-Alvarez, H. Jun, K.A. Westerman, H. Noguchi, J.R.T. Lakey, P. Leboulch, N. Tanaka, J. Yoon, A human β-cell line for transplantation therapy to control type 1 diabetes, Nat. Biotechnol. 23 (2005) 1274–1282.