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Inosine Improves Islet Xenograft Survival in Immuncompetent Diabetic Mice

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Abstract
Background: Inflammation is likely to be one of the factors responsible for early islet xenograft rejection. In this study, we have used a novel anti-inflammatory compound, inosine, to investigate the role of anti-inflammatory blockade in protecting rat pancreatic islets from xenograft rejection in diabetic balb/c mice. Inosine is a safe, naturally occuring purine, which appears to be nontoxic to humans, that has recently been shown to be an immunmodulator and anti-inflammatory agent.
Methods: For the transplantation model rat islets were placed under the renal capsule of streptozotocin diabetic balb/c mice and inosine- (200 mg kg^{-1} d^{-1}) or vehicle- treatment started the day of islet tranplantation.
Results: In vehicle-treated mice rat islet xenografts were rejected within 2 - 11 days after transplantation. In contrast, in inosine-treated mice graft survival was markedly prolonged for up to 3 weeks. Furthermore, immunohistochemistry and morphometric analyses revealed that early islet graft infiltration by leukocytes ($CD45^+$) is significantly reduced in the grafts from inosine-treated diabetic mice compared to vehicle-treated mice. As suggested from the graft survival data, inosine-treated mice had also a significantly better preserved ß-cell mass.
Conclusions: These results clearly demonstrate that inosine treatment markdly reduced the early inflammatory reaction elicted by an islet xenograft and substantially prolongs the graft survival. Therefore, such an approach may form an important future component of therapeutic regimens applied in clinical islet xenotransplantation.

Key words: rat islets, xenogenic, transplantation, inosine

Introduction

As has recently been shown [1], long-term normoglycemia of type 1 diabetic patients can be achieved by allogenic human islet transplantation. However, the severe shortage of human islets is one major drawback of this promising therapeutic approach. Therefore, alternative sources of insulin-producing cells have to be found. One option is to use islets prepared from animal tissue. But one of the main obstacles to be resolved before islet xenotransplantation becomes a viable therapy is the antibody-independent inflammatory reaction that xenogenic islets elict when transplanted into the recipient [2]. Recently it has been shown, that inosine, a naturally occuring purine, exerts powerful anti-inflammatory effects in murine endotoxic shock [3], colitis [4], septic shock [5], and severe lung inflammation [6]. Furthermore, it has been shown that inosine protects against the development of diabetes in multiple-low-dose streptozotocin and nonobese diabetic mouse models of type 1 diabetes [7]. In view of the finding that inosine is a potent anti-inflammatory agent, the present study was designed to test the potential effects of inosine in islet xenotransplantation.

Materials and Methods

Isolation of Rat Islets

Isolation of rat islets was performed according to the protocol described previously [8]. Briefly, Sprague Dawley rats (Central Animal Facility, BGFA, University of Bochum) 8 weeks old and of body weight 280 - 330 g, were used as islet donors. Rats were anaesthetised by intraperitoneal pentobarbital administration (60 mg/kg). Then a midline abdominal incision was performed, the pancreas was exposed and injected via the pancreatic duct with Hank's balanced salt solution (HBSS; Gibco BRL, Long Island, NY) containing 1.7 mg/ml collagenase (Serva PanPlus, Heidelberg, Germany). After sacrificing the animal, the pancreatic tissue was surgically removed and incubated for 10 min at 37 °C in the collagenase solution. Mechanical disruption of the digested pancreatic tissue was achieved by further incubation in collagenase solution at 37 °C for 10 min, interrupted every 2 min by shaking for 30 s. Digestion was stopped by addition of 4 °C-cold Hank's balanced salt solution plus 10% fetal calf serum (FCS; Greiner Laboratories, Frickenhausen, Germany). Islet purification was achieved using a discontinuous three-phase Ficoll density gradient (densities: 1.090, 1.077 and 1.040).

Culture Conditions

Islets were cultured in complete medium (RPMI 1640 medium, Biochrom KG, Berlin, Germany) supplemented with 100mg/dl glucose, 10mM HEPES (Greiner Laboratories, Frickenhausen, Germany), 0.2 g/l Glutamax (GibcoBRL, Paisley, Scotland), 10 % fetal calf serum (FCS; Greiner Laboratories, Fricken-

hausen, Germany) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin; GibcoBRL, Paisley, Scotland and 10μg/ml Ciprobay; Aventis, Frankfurt, Germany). After 18-h culture at 37 °C in a 5% carbon dioxide-95% air in a humified atmosphere the islets were used for transplantation.

Animals

Male balb/c mice (Central Animal Facility, BGFA, University of Bochum) 6 – 8 weeks old, were used as recipients. Two weeks before transplantation mice were rendered diabetic via intraperitoneal injections of streptozotocin (250 mg/kg body weight; Sigma, St. Louis, MO) freshly dissolved in citrate buffer. Only animals exhibiting blood sugar concentrations greater than 350mg/dl in four consecutive measurements were used as recipients.

Transplantation of Islets

Transplants were made in a class 100 biological safety cabinet under sterile conditions. Before treatment the balb/c mice were anaesthetized by intraperitoneal administration of ketamine (65mg/kg; Pfizer, Karlsruhe, Germany) and xylazine hydrochloride (13mg/kg; Bayer, Leverkusen, Germany). A total of 450 rat islets were transplanted under the left renal capsule in each diabetic balb/c mice, according a previous described procedure (2). The islet recipients mice were given either vehicle (water; n = 7) or inosine (200 mg kg^{-1} d^{-1}; Sigma, St. Louis, MO; n = 8) by oral gavage beginning the day of islet transplantation. After transplantation blood glucose levels of islet recipients were monitored daily. Blood samples were taken from the tail vein under non-fasting conditions (8 a.m.; standard laboratory chow ad libitum overnight) and determined by using a glucometer (Accutrend sensor, Roche Diagnostics, Germany). The mice were considered to be normoglycemic when the blood glucose levels were lower than 200mg/dl.

Immunohistochemistry and Morphometric Analysis

In a complete seperate set of experiments grafts were examined for insulin content and $CD45^+$ graft-infiltrating leucocytes. For this purpose eight days after transplantation grafts (vehicle vs. inosine; n = 6 per group) were retrieved. Immunohistochemistry of islet grafts was performed as follows: animals were killed by CO2 inhalation, and islet grafts were retrieved from individual animals. After fixing in 10% phosphate-buffered formalin overnight, islet grafts were embedded in paraffin. Consecutive sections (4 μm thick) of paraffin-embedded islet grafts were cut, and consecutive sections were immunostained with rat monoclonal antibody IgG2b (R&D Systems, Wiesbaden, Germany) to mouse total leucocytes ($CD45^+$) and a polyclonal guinea pig anti-insulin antibody (Dako Corporation, Hamburg, Germany) diluted 1:20 in phosphate-buffered saline, respectively. After immunostaining, the sections were examined at x200 magnification in a microscope that was linked to a computerized charge coupled device camera. Microscopic views covering engrafted islets under the kidney capsule that were immunostained by anti-insulin or anti-CD45 antibodies were captured as digitized micrographic pictures using Adobe Photoshop software (Adobe Systems, San Jose, CA). Using the color range section option of Adobe Photoshop [9], insulin- or CD45-positively immunostained color was selected for quantification of the relative intensity per islet graft by densitometry. Using this technique, the relative intensity of insulin or CD45 immunostaining from three sections, on average, per islet graft was evaluated for the determination of the mean values, which were subsequently compared between the two groups (vehicle vs. inosine) of diabetic recipient mice.

Statistics. Values are given as mean ± SE. Statistical significance of differences was calculated with an unpaired Student's t-test (two-sided).

Results

Graft Survival

To examine the effect of inosine treatment on islet graft survival and glycemic control, streptozotocin-induced diabetic balb/c mice were transplanted under the renal capsule with 450 freshly isolated rat islets. Blood glucose levels in individual animals were measured daily posttransplantation (Fig. 1A-B). Diabetic mice transplanted with rat islets and treated with inosine were restored to normal blood glucose levels within 24 hours posttransplantation. The same results were obtained for vehicle-treated control animals. However, vehicle-treated mice remained normoglycemic for only a few days; first graft rejection was detected 2 days posttransplantation and five out of eight animals rejected the rat islet grafts within the first eight days posttransplantation (Fig. 1B). The longest documented graft survival was 11 days in the vehicle-treated group. In contrast in inosine-treated mice, first graft rejection was observed after 12 days posttransplantation in one animal, but most animals rejected their grafts not earlier than 18 days posttransplantation. The longest documented graft survival in the inosine-treated group was 21 days. Together, these data indicate that inosine treatment conferred a beneficial effect on graft survival compared to vehicle-treated mice (17.6 ± 3.0 vs. 7.1 ± 2.9; $p < 0.01$) (Table. 1).

Immunohistochemical studies. To study the effects of inosine treatment on early inflammatory reaction, in a complete separate set of experiments, diabetic recipient mice were killed 8 days posttransplantation, and islet grafts were retrieved for studying the extent of leucocyte infiltration and ß-cell survival. The degree of leucocyte infiltration of the grafts was determined by immunohistochemistry for the leucocyte marker CD45, followed by morphometric analysis. As shown in Fig. 2A, a significant difference in the relative intensity of immunostaining by anti-CD45 antibody was detected between the inosine–treated and the vehicle-terated mice. Mice that were treated with inosine exhibited more than twofold lower CD45-immunostaining intensity than vehicle-treated mice (Fig. 2A). These results indicate that inosine treatment exerts potent anti-

Table 1. Graft function of islets transplanted under the left kidney capsule treated with vehicle or inosine. mean ± SE. * <0.01 vs. vehicle.

Treatment	Individual graft survival (d)	Mean graft survival (d)
vehicle	2, 5, 7, 8, 8, 9, 11	7.1 ± 2.9
inosine	12, 15, 17, 18, 18, 20, 20, 21	17.6 ± 3.0*

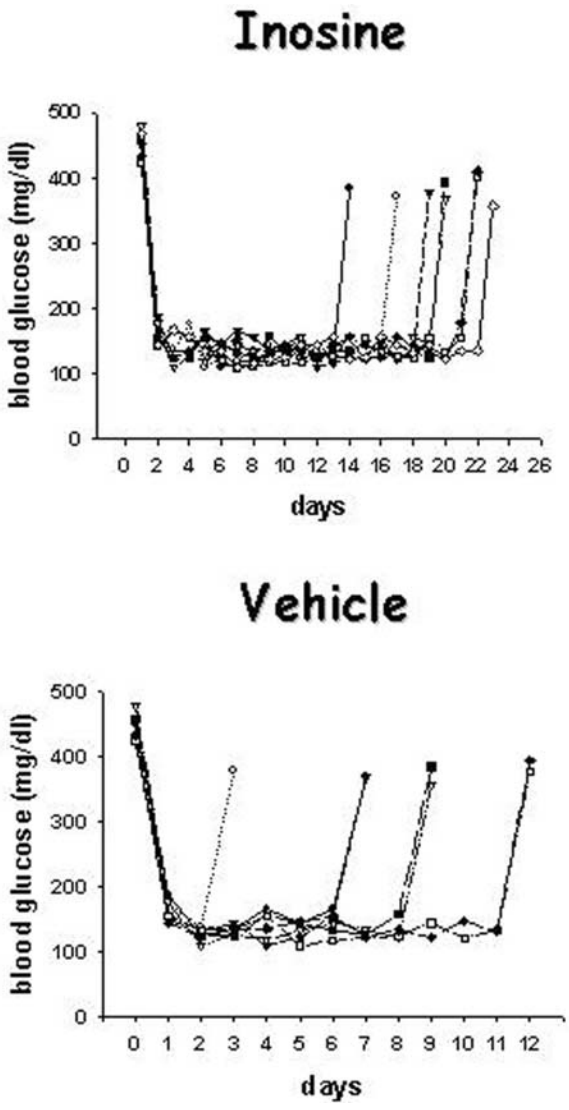

Fig. 1. Effects of inosine treatment on the survival of rat islet xenografts compared to vehicle-treated mice. (A) In the mice that received inosine-treatment (n=8), blood glucose level decreased immediately after transplantation, earliest graft rejection was detected 12 days posttransplantation and longest graft survival was 21 days. (B) In mice that received vehicle-treatment (n=7), blood glucose levels decreased immediately after transplantation but later increased to more than 350 mg/dL, 2 to 11 days after islet transplantation.

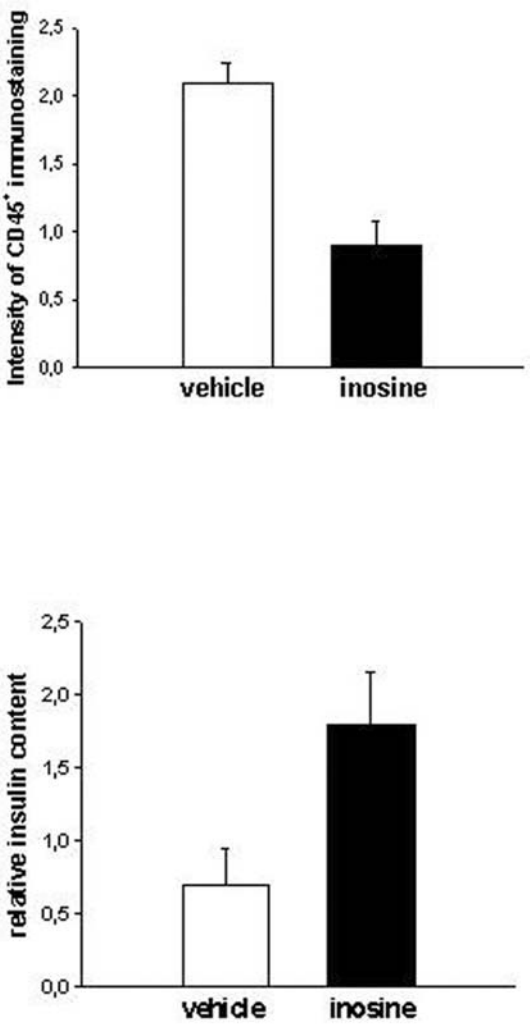

Fig. 2. Anti-CD45 immunostaining intensity (A) and graft insulin content (B) of rat islet grafts transplanted under the left kidney capsule in streptozotocin diabetic balb/c mice treated with inosine or vehicle. The relative intensity of immunostaining with anti-insulin or anti-CD45 antibodies, respectively, was compared between both groups, as determined by morphometric analysis. $^{+}P < 0.01$.

inflammatory proprties in this xenotransplantation setting. To correlate the degree of early inflammatory reaction with the islet mass, the islet grafts retrieved from killed animals were also immunostained for insulin (Fig. 2B). As suggested from the data of anti-CD45 immunohistochemistry, the grafts of inosine-treated animals displayed significantly higher levels of insulin content than control mice (Fig. 2B). This quantitative difference in insulin content of the islet grafts between both groups of diabetic recipient mice correlated well with the graft survival (Fig. 1A-B)

DICUSSION

Recent success in pancreatic islet allotransplantation has raised expectations but has equally highlighted the

acute shortage of donor tissue. The use of xenogeneic tissue would help to address this shortage; however, strong cellular immunity limits the application of this approach. Especially the obstacle of initial severe antibody-independent inflammatory reaction has to be solved before islet xenotransplantation seems to be a viable option. Therefore, the search for drugs with potent anti-inflammatory effects but minimal side effects is an important endeavor. Inosine, a naturally occuring purine, which appears to be nontoxic to humans, has recently been shown to be more than just an "inactive" metabolite of adenosine. Previous reports revealed that inosine had marked anti-inflammatory effects, both in-vitro and in-vivo, in acute inflammatory conditions. Furthermore, it has been shown to prevent glial cell death [10], and protecting against allergic encephalomyelitis [11]. Recently, Mabley et al. [7] found that inosine proteted both the development of diabetes and against the rejection of syngenic transplanted islets. Mabley et al. found that inosine exerts it`s effects by decreasing pancreatic leucocyte infiltration and oxidative stress in addition to switching the cytokine profile from a TH1 to a TH2 profile.

In the present study, we could demonstrate that inosine treatment significantly prolonged islet graft survival in rat to mouse model. Graft survival in inosine-treated mice was twofold longer compared to vehicle treated mice (17.6 ± 3.0 vs. 7.1 ± 2.9; $p < 0.01$). A further significant result from this study is that inosine treatment leads to a markedly reduced number of CD45 graft-infiltrating leucocytes and a better preserved ß-cell mass when compared to vehicle-treated mice. These data are promising since they provide for the first time evidence that islets can be protected against xenogenic rejection for a limited period of time using the non-toxic adenosine metabolite inosine by reducing the amount of graft infiltrating leucocytes. Therefore, these data extend the results of previous reports [3, 4], that inosine has potent anti-inflammatory properties under different experimental settings. It is unclear whether similar results can be achieved in large animal models in which vigorous innate immune responses are likely to comprise a more stringent barrier to xenograft survival. However, our results indicate that inosine can be efficacious in delaying the rejection of xenografts and that such approaches may form an important future component of therapeutic regimens applied in clinical islet xenotransplantation.

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