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Nerve Cells in Transplanted Pancreatic Islets: No Effects of Cyclosporin or Tacrolimus on Immediate Neuronal Survival

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ABSTRACT

Previous experiments have demonstrated that neuronal cells within pancreatic islets survive the isolation procedure and constitute an integral part of transplanted pancreatic islets. The aim of the present study was to investigate to what extent immunosuppressive drugs affects the acute survival of intra-islet neurons after pancreatic islet transplantation. For this purpose, C57BL/6 mice were syngeneically transplanted with 250 islets under the renal capsule. The animals were treated for 7 consecutive days with subcutaneous injections of cyclosporin, tacrolimus or vehicle. After this, the animals were killed and the grafts were removed, fixed and stained for the presence of the neuron-specific protein PGP 9.5. The number of nerves were then morphologically quantitated. No differences between the experimental groups were seen, and the number of nervous elements were approximately 5 per mm^2 in all animals. It is concluded that immunosuppressive treatment does not affect the acute survival of graft neurons after experimental islet transplantation.

INTRODUCTION

Application of the so called Edmonton protocol for islet transplantation in humans has led to much higher success rates, with almost all patients achieving insulin independence [16, 19]. The number of islets implanted when applying this protocol is substantially larger than in previous clinical trials. The need for this high number of transplanted islets, usually exceeding 10.000 islet equivalents per kg body weight may, at least partially, be due to a lack of proper engraftment, and thereby a loss of many of the implanted endocrine cells, in the immediate post-transplantation period [*cf.* 2]. The engraftment is a complex process, encompassing among other things revascularization, reinnervation, ingrowth and/or expansion of stromal connective tissue cells and reorganization of the endocrine cells. In view of the shortage of suitable material for transplantation, any possibility to improve engraftment is of utmost importance.

Initial studies of islet transplant reinnervation, one of the components of engraftment, were mainly focussed on time points several months post-transplantation [7, 8]. The rationale for this was the notion that isolation of islets interrupts all vascular and nervous connections, and it has been assumed that nerves within the islets die

during the subsequent culture period. However, it has been shown that nerves, especially those positive for acetylcholine and VIP, were present immediately after implantation of islet grafts [12, 14]. The functional importance is at present unknown.

It has been suggested that the immunosuppressive drug tacrolimus, which interferes with calcineurin, can stimulate nerve growth [4], whilst no such effects are seen after cyclosporin administration. In view of this, we aimed to investigate if treatment with these immunosuppressive drugs affected the initial survival of neurons within islet grafts.

MATERIALS AND METHODS

Animals: Adult, male C57BL/6 mice (Möllegaard & Bomholtgaard; Ry, Denmark) were used in all experiments. The animals had free access to tap water and pelleted food throughout the studies. All experiments were approved by the local animal ethics committee.

Islet isolation and culture: Pancreatic islets were isolated by collagenase digestion (Collagenase A from *Clostridium histolyticum*; Boehringer Mannheim, Mannheim, Germany), and then hand-picked using a braking pipette as described in detail elsewhere [18]. Groups of approximately 150 islets were cultured free-floating in medium RPMI 1640 (11.1 mmol/l D-glucose) supplemented with 10% fetal calf serum (Sigma-Aldrich Chemicals Co., St. Louis, MO, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml; Boehringer). The medium was changed every second day. Islets were harvested for transplantation after 4 days of culture.

Islet transplantation: A total of 15 recipient mice were anesthetized with an intraperitoneal injection of 20 ml/kg body weight avertin [a 2.5% (v/v) solution of 10 g 97% 2.2.2-tribromoethanol (Sigma-Aldrich) in 10 ml 2-methyl-2-butanol (BDH Chemicals, Poole, UK)]. The left kidney was exposed through a flank incision and a small incision in the renal capsule was made at the lower lobe. By applying a glass probe, a subcapsular space extending up towards the cranial pole was created. Approximately 250 islets were then implanted into this space by means of a braking pipette.

Treatment with immunosuppressive drugs: Beginning immediately after the islet transplantation, the animals received one daily subcutaneous injection of 0.2 ml of Cremophor EL^{R} (vehicle; Sigma-Aldrich), cyclosporin A (Sandimmun^{R}; Sandoz,; Täby, Sweden; 10 mg/kg body weight) or tacrolimus (Prograf^{R}; Fujisawa, Stockholm, Sweden; 0.5 mg/kg body weight).

Morphological examination of the graft: The animals were killed in the afternoon 7 days after transplantation, after receiving a final dose of the immunosuppressive drug or vehicle 2 h earlier. The graft-bearing kidney was removed and fixed by immersion in formaldehyde [10% (v/v)]. The samples were then dehydrated and embedded in paraffin. Sections (5 μm thick) were processed for immunohistochemistry using a mouse monoclonal antibody against the general neuronal marker Protein Gene Product 9.5 (PGP 9.5; NCL-PGP 9.5; Novocastra Laboratories Ltd., Newcastle, UK) [3, 8, 22]. The staining was performed with a high temperature antigen

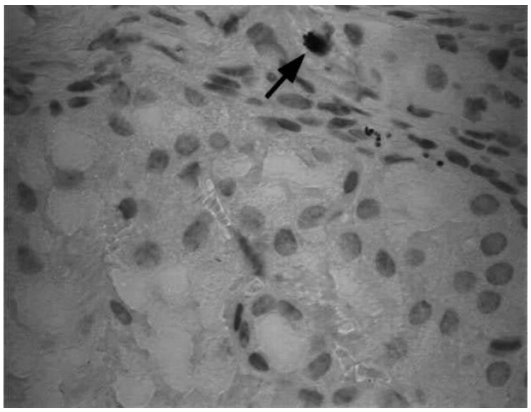

Fig. 1. Nerve cell body (arrow) in an islet transplant under the renal capsule of a mouse 7 days after implantation. The animal had been treated with cyclosporin, and the cell is stained for the presence of PGP 9.5. Magnification: 300X

unmasking technique using sodium citrate buffer according to the manufacturer's instructions. The area of each graft was estimated by point sampling, and the number of nervous structures, *i.e*. nerve cell bodies or nerve fibres stained for the presence of PGP 9.5, per mm^2 was calculated. At least 5 sections from three different parts, i.e. a total of 15 sections, from each islet graft were examined. All evaluations were made by 2 observers unaware of the origin of the sections.

RESULTS

One of the cyclosporin-treated mice was excluded from the study, since no graft was found after 7 days of treatment. In the remaining 14 transplanted mice, islet grafts were easily identified under the renal capsule. No visible difference in graft size could be seen when the groups were compared.

Staining with PGP 9.5 demonstrated the presence of nerve fibres and occasionally a nerve cell body in the islet grafts (Figure 1). When the number of nerve fibres was quantitated, the values were almost identical in the experimental groups (Figure 2).

DISCUSSION

The results in the present study confirm the notion that some nerves survive the islet isolation procedure, and remain within the islet grafts [13]. This may seem surprising, but is probably due to the neurons being enclosed within the islets, forming so called neuro-insular complexes [14]. This means that they are protected from the effects of collagenase. That the observed nerves merely represent transected axons is unlikely, since they would not survive 4 days of culture, followed by one week of transplantation. A degeneration of intra-graft neurons seems to occur with time [13], but in view of the short time span encompassing the present study this could not be evaluated. One reason for survival of nerve cells in grafted islets may be the expres-

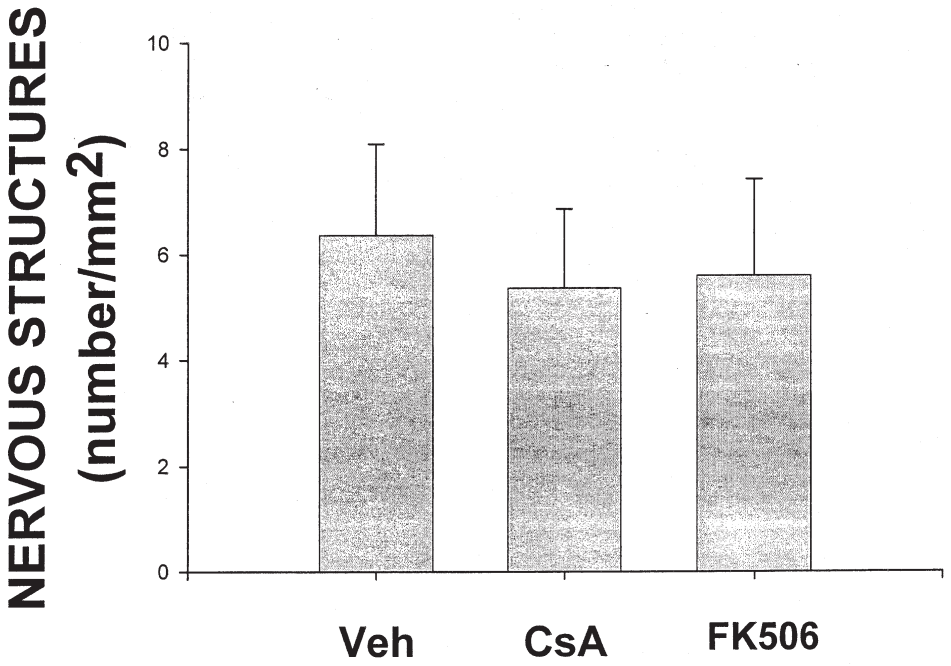

Fig. 2. Number of nervous structures 7 days after implantation in pancreatic islets syngeneically grafted under the renal capsule of C57BL/6 mice. The animals were treated with daily subcutaneous injections of vehicle (Veh; castor oil), cyclosporin (CsA; 10 mg/kg body weight) or tacrolimus (FK506; 0.5 mg/kg body weight). Values are means ± SEM for 4-5 experiments.

sion of neurotrophins in islets when injured [21], which may lead to a stimulation of neuronal survival. Other experiments have confirmed that nerve growth factor may affect beta-cell lines [15] or beta-cells [6]. The production of possible neurotrophic factors from islets is supported by the elegant study of Myrsén and co-workers [11], who observed that only islets with beta-cells became reinnervated, suggesting the presence of specific neurotrophic factors in this cell type [*cf.* 1].

The present study aimed to evaluate if the commonly used immunosuppressive drugs cyclosporin and tacrolimus could affect the survival of intra-islet neurons in the immediate post-transplantation period. The rationale for this approach is that immunophilins, to which both cyclosporin and tacrolimus bind and thereby inhibit calcineurin, have been demonstrated to be important in nerve regeneration [5, 10]. Recent studies have suggested, however, that the effects on nerves also involve processes independent of calcineurin, and that tacrolimus is much more potent than cyclosporin [5, 10, 20]. However, no differences whatsoever in the survival of intra-graft neurons could be seen in the present study. Since the doses used are similar to those used in other investigations in rodents [*e.g.* 17], a binding to immunophilins with its associated actions is likely to occur. The obvious conclusion is therefore that administration of the commonly used immunosuppressive drugs tacrolimus or cyclosporin does not affect the survival of neurons after implantation, either adversely or stimulatory.

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