Streptozotocin is responsible for the induction and progression of renal tumorigenesis in diabetic wistar-furth rats treated with insulin or transplanted with agarose encapsulated porcine islets

Horatiu V. Vinerean,1,* Lawrence S. Gazda,1,2 Richard D. Hall1 and Barry H. Smith1,4,5

1The Rogosin Institute-Xenia Division; Xenia, OH USA; 2The Rogosin Institute; New York, NY USA; 3Bob Evans Farms, Inc.; Columbus, OH USA; 4NewYork-Presbyterian Hospital; New York, NY USA; 5Department of Surgery; Weill Medical College of Cornell University; New York, NY USA

Key words: streptozotocin, porcine islets, xenotransplantation, encapsulation, renal carcinoma

Introduction

Streptozotocin (STZ), a nitrosourea with DNA alkylating properties, has been widely used to induce hyperglycemia by specifically destroying the insulin-producing β-cells of the islets of Langerhans in experimental models of Type I diabetes. STZ’s known carcinogenic properties, however, raise concerns about its suitability for long-term studies. We conducted a formal study of STZ’s carcinogenic effects in long-term surviving diabetic Wistar-Furth rats. To determine if insulin therapy or islet transplantation exacerbated tumorigenesis, rats were randomly assigned to one of four experimental groups: normal animals with no treatment (Group 1, n = 12); normal animals that underwent peritoneal implantation of porcine islets encapsulated in a double layer of agarose to form islet macrobeads (Normal + Islets; Group 2, n = 12); STZ treatment followed by daily exogenous insulin (STZ + insulin; Group 3, n = 18) and STZ treatment followed by the intraperitoneal implantation of porcine islet macrobeads (STZ + Islets; Group 4, n = 14). At 215 days post-STZ induction, no renal proliferative lesions were observed in animals that did not receive STZ (Groups 1 and 2) whereas adenoma incidences of 57% for Group 3 and 34% for Group 4 were observed. By terminal necropsy at day 351, the incidence and severity of renal proliferative lesions increased with tubular carcinoma observed in 67% of Group 3 and 60% of Group 4 animals. We conclude that the STZ-induced diabetic rat model is not suitable for long-term studies because of progressive renal tumorigenesis. Our experiments also demonstrate the safety and effectiveness of porcine islet macrobeads for the treatment of diabetes.
these animals presented with either hepatocellular adenoma or carcinoma. In this situation, it appears that increased intracellular insulin signaling via pathways associated with cell growth and proliferation may be sufficient to trigger carcinogenesis independent from STZ.

Local hyperinsulinemia, however, is not relevant in the case of encapsulated islets that are implanted into the peritoneal cavity and remain free-floating in peritoneal fluid. To alleviate the severe shortage of insulin-producing human islets for transplantation in patients with Type 1 diabetes, animals have been proposed as an alternative source of islets. Porcine islets are likely to be the most suitable source of such animal tissue based on their ability to secrete insulin that is almost identical to human insulin and that the islet macrobeads themselves are safe. We further demonstrate that the development of renal tumors does not interfere with the function of porcine islet macrobeads and that the islet macrobeads themselves are safe.

Results

Diabetes induction. Before STZ induction, all animals had normal blood glucose readings. Animals that received STZ (Groups 3 and 4) became hyperglycemic within three days and were maintained on daily insulin therapy beginning on day five when blood glucose levels reached ≥400 mg/dl.

Insulin requirements. Following the induction of diabetes, average daily insulin requirements were determined during a 28 day pre-implant period; STZ + insulin animals (Group 3) had similar exogenous insulin requirements to STZ + Islets (Group 4) with values of 1.70 ± 0.03 U/day insulin vs. 1.77 ± 0.01 U/day insulin, respectively. Insulin therapy was continued for animals that received STZ + insulin (Group 3) throughout the study (terminal euthanasia group: 1.89 ± 0.50 U/day).

Blood glucose. Normal control animals (Group 1) demonstrated normoglycemia (88.46 ± 0.84 mg/dl) throughout the study (Fig. 1). Normal + islet animals (Group 2) also maintained normoglycemia throughout the study (89.76 ± 0.79 mg/dl) despite receiving a matching number of porcine islet macrobeads as STZ + islets animals (Group 4; Fig. 1).

STZ + insulin animals (Group 3) demonstrated elevated hyperglycemia following STZ induction and throughout the remainder of the study, despite daily insulin therapy, with average blood glucose values of 291.3 ± 3.09 mg/dl (Fig. 1).

The averaged blood glucose concentration of STZ + islets animals (Group 4) initially declined to 128.6 ± 6.04 mg/dl during the first seven days post-macrobead transplantation. Daily blood glucose values then gradually increased over 60 days as these animals became mildly hyperglycemic (236.2 ± 38.4 mg/dl) for the remainder of the study (Fig. 1).

Body weight. At the time of STZ induction, the average body weight of all animals was 238.02 ± 11.11 g. Following diabetes induction, body weight for the animals treated with STZ declined to 227.98 ± 10.54 g but returned to pre-induction body weight one week later. However, at the time of islet macrobead implantation (33 days post-induction), a difference was noted between the body weights of normal animals versus diabetic animals (323.25 ± 16.45 g vs. 288.37 ± 15.09 g, respectively, p < 0.001). Normoglycemic animals maintained average body weights of 370.30 ± 12.48 g for normal control animals (Group 1) and 359.28 ± 5.63 g (macrobead weight subtracted) for normal + islets animals (Group 2). The body weight of diabetic animals was lower compared with the normal counterparts with average values of 335.37 ± 5.02 g for STZ + Insulin animals (Group 3) and 305.43 ± 5.49 g (macrobead weight subtracted) for STZ + islets animals (Group 4; Fig. 2). Regardless of the experimental group, all animals exhibited good body condition for the duration of the experiment indicating that their general health was satisfactory.
Retrieved porcine islet macrobead insulin production. Insulin was detected in the culture media from porcine islet macrobeads retrieved at necropsy from normal + islets animals (Group 2) and STZ + islets study animals (Group 4; Fig. 3). To determine a reliable assessment of insulin production from retrieved macrobeads, insulin was assessed weekly for 8 weeks from macrobead cultures. Islet macrobeads retrieved from Group 2 and Group 4 animals at the time of necropsy had similar insulin production, during both interim euthanasia (12.00 ± 0.49 mU/macrobead/24 hours vs. 13.15 ± 0.49 mU/macrobead/24 hours, respectively, p > 0.05) and study termination (9.89 ± 0.39 mU/macrobead/24 hours vs. 12.76 ± 0.63 mU/macrobead/24 hours, respectively, p = 0.05).

Organ somatic index. The organ-somatic index [OSI] was calculated using the formula: OSI = [organ weight (g)/body weight (g)] x 100. No significant differences in the organ somatic index was observed between groups with the exception that at terminal necropsy renal-somatic index (RSI) was increased for animals receiving STZ (0.44 ± 0.05 RSI for Group 3 and 0.76 ± 0.3 RSI for Group 4) versus animals that did not receive streptozotocin (0.35 ± 0.03 RSI for Group 1 and 0.38 ± 0.02 RSI for Group 2, p < 0.05). This is the consequence of renal carcinogenesis with the development of large nodular tumors.

Macroscopic pathology. At the interim sacrifice, 4 of 7 animals receiving STZ + insulin (Group 3) and 2 of 6 animals receiving STZ + islets (Group 4) were observed to have renal proliferative lesions. By terminal sacrifice, almost all of the remaining animals receiving STZ + Insulin (Group 3) or STZ + islets (Group 4) had renal proliferative lesions (8 of 9 and 4 of 5 animals, respectively). In contrast, animals from normal control (Group 1) or normal + islets (Group 2) showed no evidence of renal proliferative lesions.

Other macroscopic findings at necropsy included areas of minimal to mild chronic inflammation associated with the presence of macrobeads (white plaques on spleen, liver, abdominal cavity).

Microscopic pathology. At interim sacrifice on day 215 post-STZ induction, treatment with STZ + insulin (Group 3) or STZ + islets (Group 4) was associated with renal tubular adenoma. In animals given STZ + insulin (Group 3), there was one observed incidence of renal hyperplasia and seven renal adenomas in seven animals while in those receiving STZ + islets (Group 4), two renal adenomas were observed in six animals.

At study termination on day 351 post-STZ induction, an increase in the occurrence and severity of renal proliferative lesions was observed in those animals treated with STZ + insulin (Group 3) or STZ + islets (Group 4; Table 1). Three instances of renal tubular hyperplasia, six renal adenomas and six renal carcinomas were observed in nine animals treated with STZ and Insulin (Group 3). Two renal adenomas and three renal carcinomas were observed in five animals treated with STZ + islets (Group 4).

When only the most severe renal proliferative lesion was considered for individual animals (carcinoma > adenoma > hyperplasia; Table 2), 57% given STZ + Insulin (Group 3) and 34% given STZ + islets (Group 4) had renal adenomas at the interim sacrifice. At terminal sacrifice, 66% of those animals given STZ + insulin (Group 3) and 60% of animals given STZ + islets (Group 4) had renal carcinomas.

In addition to those lesions shown for the scheduled interim and terminal sacrifice groups, proliferative lesions were also observed in animals euthanized for intercurrent disease (not shown in tables). A renal tubular adenoma and a renal tubular carcinoma were observed in one of two animals euthanized early on day 267 from the STZ + islets group. Also, three renal tubular adenomas were observed in one animal that received STZ + insulin (Group 3) which was euthanized early on day 295. No proliferative kidney lesions were observed in normal animals (Group 1, n = 5) or in normal + islets animals (Group 2, n = 5) euthanized on Day 215. A splenic histiocytic sarcoma was observed at terminal sacrifice (Day 351) in one normal + islets animal (Group 2). This was considered a random biological occurrence and not related to treatment.

Renal tubular hyperplasia was characterized by slightly dilated tubules with epithelial cells that had proliferated to fill the tubular lumen (Fig. 3A). In this study, hyperplasia always involved more than one tubule (often described as atypical hyperplasia). Renal tubular masses greater than 5 tubules in diameter and less than 5 mm in diameter were classified as renal tubular adenomas. Adenomas of the kidney were well-circumscribed and discrete masses of epithelial cells arranged in a papillary pattern with tubules and nests of epithelial cells on a fine fibrovascular stroma (Fig. 3B). The surrounding renal
We have demonstrated that the use of STZ to induce diabetes in Wistar-Furth rats leads to the early development of renal hyperplasia and adenomas which then progresses to renal carcinoma over the next 4.5 months. Because renal carcinoma was only observed in long-term STZ-induced animals, and not in normal animals that received islet macrobeads, we conclude that macrobead treatment does not augment the development of renal carcinoma. Further, because insulin-treated animals developed renal tumors at an equivalent rate and prevalence, we conclude that neither islet macrobeads nor insulin treatment increases renal tumorigenesis in this model of STZ-induced diabetes.

**Discussion**

tissue was slightly compressed by the growth of the adenoma. In adenomas, clustered cells often formed new lumens (minilumen formation), with minimal debris within the lumens. Mitoses were rare in adenomas. Renal tubular masses of 5 mm or greater in diameter were classified as renal tubular carcinoma. Carcinomas were comprised of eosinophilic epithelial cells arranged on a fine fibrovascular stroma in papillary or tubular patterns ([Fig. 3C and D]). Carcinomas had prominent lumens and/or cystic spaces containing cellular debris. Tumor cells were minimally pleomorphic with a vesiculate nucleus and one or two small nucleoli. Cytoplasm was eosinophilic with indistinct cell borders. The largest carcinoma was 2.2 cm in diameter.

**Figure 3.** Micrographs of renal hyperplasia, adenoma and carcinoma following the intravenous injection of streptozotocin. (A) Renal tubular hyperplasia (atypical) from the kidney of an animal 351 days post-STZ injection. In the central tubules, tubular epithelial cells fill the renal tubules. A few of the cells are highly vacuolated (arrow). Hematoxylin and eosin stain. Bar = 50 microns. (B) Renal tubular adenoma of an animal 215 days post-STZ injection. This adenoma is a well-circumscribed, discrete, approximately 0.5 mm mass comprised of epithelial cells arranged in tubules and nests on a fine fibrovascular stroma. The surrounding renal tissue is slightly compressed by the growth of the adenoma. Hematoxylin and eosin stain. Bar = 0.1 mm. (C) Renal tubular carcinoma from the kidney of an animal 351 days post-STZ injection. This carcinoma is approximately 17 mm in diameter. Tumor cells are arranged in a papillary pattern on a thin fibrovascular stroma. Much of the mass is comprised of areas of necrotic debris (pink areas). The tumor compresses the adjacent renal cortical tissue (arrow). Hematoxylin and eosin stain. Bar = 1 mm. (D) Higher magnification of the renal tubular carcinoma shown in Figure 3C. Tumor cells are somewhat pleomorphic, occasionally vacuolated and are arranged in a papillary pattern on a thin fibrovascular stroma (arrow). Necrotic debris occupies intervening spaces. Hematoxylin and eosin stain. Bar = 100 microns.
recently shown to induce long-term hyperglycemia (56 and 82%, respectively) but mortality was higher in mice receiving freshly prepared STZ (36 vs. 7%). In this same study, de la Garza-Rodea et al. went on to report diabetes induction in 8 of 8 mice receiving STZ that had been stored in the dark at 4°C for 40 days and also noted that STZ was previously demonstrated to be stable for days at room temperature in acidic buffer solution by Oles in 1978. Thus, the timing of STZ administration in relation to its preparation is likely to impact both the success and the morbidity and mortality of this procedure. It is unknown what affect the use of stored STZ, as opposed to freshly prepared STZ, would have on the long-term development of renal proliferative disease.

Alloxan could be used for the chemical induction of diabetes in order to avoid the development of renal or liver tumors in long-term studies of diabetes, as well as other reported side effects of STZ induction including heart and corneal toxicity. Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) was first reported to induce islet necrosis in rabbits by Dunn et al. in 1943, and can be administered intravenously, intraperitoneally or subcutaneously. Alloxan specifically destroys the insulin-producing beta cells of the islets of Langerhans while preserving the glucagon-secreting alpha cells, which is analogous to the islet pathology of Type I diabetes. In rats, a typical dose of alloxan is 65 mg/kg body weight when given intravenously and

Table 1. Total number of proliferative kidney lesions by treatment group

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Multiple tumors were observed in some animals.

Table 2. Numbers of animals with microscopic proliferative kidney lesions by treatment group and sacrifice interval

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<td>3 (60)</td>
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Only the most severe diagnosis (carcinoma > adenoma > hyperplasia) was attributed to a given animal; parentheses = % affected. Animals euthanized moribund are not included in this table.
Federiuk et al. (2004) have recently reported an optimized protocol to induce diabetes in the rat using an intraperitoneal injection of alloxan that results in 80% of the animals developing diabetes with a survival rate of 90%. Kidney tumors were not found in rats surviving for as long as 16 months and other oncogenic associations with alloxan have not been reported. In a review of various induced animal models for the study of diabetes, Etuk has very recently concluded that alloxan is the most reliable and easy method for experimentally induced diabetes.

As previously reported by our group, xenografts of porcine or rat islets encapsulated in agarose-agarose macrobeads controls blood glucose in spontaneously diabetic rodents as well as in streptozotocin-induced diabetic animals. Importantly, the animals in these studies were insulin-free throughout the study periods in the absence of immunosuppression. Blood glucose levels gradually increased and islet macrobead recipients became mildly hyperglycemic. Recipient animals continued, however, to demonstrate improved control of their diabetic state as evidenced by weight gain, lack of ketotic episodes, lack of polyuria and improved responses to glucose loading. As mentioned above, no macroscopic renal pathology has been detected at study termination in spontaneously diabetic BB rats treated with porcine islet macrobeads. However, renal tumors were observed in 75% of STZ-induced diabetic Wistar Furth rats at 352 days post-STZ induction. Together, these studies confirm that the observed renal pathology is a direct result of the injected STZ.

We have established that renal proliferative changes in the rat are common findings in long-term studies of intravenously injected, freshly prepared STZ used for the induction of experimental diabetes. Renal carcinogenesis should be considered at the initiation of long-term studies that are using STZ-induced diabetic animals as the tumorigenic effects of STZ can hasten the death of study animals. Tumor burden is also well known to influence metabolism which can potentially impact the interpretation of any anti-diabetic therapy. Additionally, this study further demonstrates the efficacy and safety of porcine islet macrobeads implanted into the peritoneal cavity of diabetic animals.

Materials and Methods

Animals. Inbred male Wistar Furth rats (WF/NHsd, Harlan Sprague Dawley; Indianapolis, IN) rats, 5–6 weeks old on arrival, were housed in ventilated microisolator poly-sulfone cages, 10.5” wide x 19” deep x 8” high, on α-cellulose bedding (ALPHA-dri, Shepherd Specialty papers, Inc., Kalamazoo, MI) and offered ad libitum rodent chow (Lab Diet 5002 Certified Rodent Diet-Purina Mills International, Brentwood, MO) and provided tap water. Environmental enrichment was provided by adding objects inside of the cages (tubes for tunneling).

The animal holding room was maintained under environmental conditions of fluorescent light with 12:12-hr light:dark cycle (lights on 0700 hr), light intensity of ~200 lux at 1 meter above the floor (HOBO U12 Data Logger, Onset Computer Corp., Bourne, MA), temperature of 20.9 ± 0.8°C, with a relative humidity of 43.7 ± 9.2%, with 14 room air exchanges hourly, 100% fresh air.

This study was reviewed and approved by The Rogosin Institute Animal Care and Use Committee (TRIACUC). All procedures and use of animals were in compliance with the Guide for the Care and Use of Laboratory Animals. Rats were received and maintained at The Rogosin Institute-Xenia Division animal facility which holds Full accreditation awarded by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, Int.).

Experimental groups. After a one week acclimatization period the animals were randomly assigned to one of four groups as follows: normal rats (normal control, Group 1, n = 12); normal rats implanted with porcine islet macrobeads (normal + islets, Group 2, n = 12); STZ-induced diabetic rats maintained on daily insulin therapy (STZ + insulin, Group 3, n = 18); STZ-induced diabetic rats implanted with porcine islet macrobeads (STZ + islets, Group 4, n = 14). Scheduled sacrifices using inhaled CO2 overdose, occurred on Day 215 post-STZ administration (interim euthanasia) or study termination on Day 351.

Diabetes induction. Rats were lightly anesthetized with ether then injected intravenously into the left tail vein with STZ (VWR, West Chester, PA) at 65 mg/kg body weight within 15 min of dissolution in sodium acetate buffer (pH 4.5). Following the administration of streptozotocin, animals exhibited a significant loss of body weight, polyuria, polydipsia and hyperglycemia. Diabetes was clinically confirmed with non-fasting blood glucose values over 400 mg/dl for 2–3 consecutive days. Diabetic rats were treated daily with subcutaneously injection of insulin (PZI insulin 40 U/ml, Blue Ridge Pharmaceuticals, Inc., Greensboro, NC) for 28 days prior to macrobead implantation. Daily insulin requirements were used for the calculation of the number of islet macrobeads required for implantation as previously described in reference 17.

Islet isolation. Donor islets were prepared from Newsham sows obtained from a closed source herd, that were over two years of age and had a history of multiple parities as previously described in reference 34. Islet counts were expressed as equivalent islet numbers (EIN), based on a standard islet size of 150 µm and 500 EIN were encapsulated in agarose-agarose macrobeads.

Microbiology screening of porcine islet macrobeads. Macrobeads and culture media were sent to MicroTest Laboratories, Inc., (Agawam, MA) at ambient temperature via overnight courier for confirmation of sterility using the USP membrane filtration sterility testing methodology in a Class 100 clean room and were examined for the presence of any microbial growth throughout a 14 day period.

In addition, samples of culture media and islet macrobeads were sent to BioReliance (Glasgow, Scotland) for porcine virology testing using validated PCR assays. Samples were considered to be negative for the detection of viral specific sequences of Bornavirus, Bovine viral diarrhea virus, Coronavirus, Cytomegalovirus, Encephalomyocarditis virus, Enterovirus, Hepatitis E virus, Lymphotropic herpes virus type-1, Parvovirus, Pseudorabies virus, Rabies virus, Reovirus, Vesicular disease virus and West Nile virus.
Porcine islet macrobead implantation. During culture, islet macrobeads were assessed weekly for insulin production during a 24 hour static perfusion assay. The day prior to implant, macrobeads were examined for uniformity, aliquoted to 175 ml conical tubes (Nalgene, Rochester, NY) and stored overnight at room temperature (19–27°C) in RPMI medium 1640 (Gibco, Invitrogen, Carlsbad, CA) containing 25 mmol/L HEPES buffer and 2 mmol/L L-Glutamine (Gibco, Invitrogen) + 1% Antibiotic/Antimycotic (Gibco, Invitrogen, USA). Prior to implant, macrobeads were washed three times with RPMI medium 1640.

All animals received an equivalent number of macrobeads from each of 10 islet isolations that were selected. Each animal from Groups 2 and 4 received an average of 52 ± 2 macrobeads with a weight of 14.6 ± 0.7 g and a calculated volume of 13.5 ± 0.6 ml. Average insulin secretion before implant was 35.85 ± 6.36 mU insulin/macrobead (Mercodia Porcine Insulin ELISA, Mercodia AB, Uppsala, Sweden) with an average age of 13.63 ± 4.45 weeks. Therefore, the insulin production theoretically provided 100% of exogenous insulin requirement for the animals at the time of implant. Insulin therapy was discontinued beginning on the day of transplantation and throughout the remainder of the study for the animals receiving islet macrobeads.

The animals from Group 2 received an equivalent number of macrobeads on the day of implant to approximate the number of macrobeads, insulin production, weight and volume of macrobeads received by the animals in Group 4 (STZ and Islets; 52.0 ± 0.6 mg insulin/macrobead (Mercodia Porcine Insulin ELISA, Mercodia AB, Uppsala, Sweden) with an average age of 13.63 ± 0.45 months. Therefore, the insulin production theoretically provided 100% of exogenous insulin requirement for the animals at the time of implant. Insulin therapy was discontinued beginning on the day of transplantation and throughout the remainder of the study for the animals receiving islet macrobeads.

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Acknowledgments

The expert technical assistance of the Diabetes Group, The Rogosin Institute-Xenia Division (Hollie Adkins, Johannah Bailie, Wendy Byrd, Lisa Circle, Steve Harbeck, Robert Holdcroft and Eric Meyer) is appreciated. Laurel Cisler, Heather Davis, Heather Klingensmith, Jennie Puterbaugh and Tracy Walters provided invaluable animal care. Brian Doll and Ashley Blankenship provided excellent administrative support and Deborah Hoffer provided QA oversight during the conduct of the study. We are indebted to Bob Evans Farms Inc., for their continued support and to Metromedia Bio-Science, LLC for ongoing financial support.

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