

Intrauterine Transplantation of Isogenic Pancreatic Islets in Experimental Diabetic Rats

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ABSTRACT. The effect of intrauterine transplantation (IU group) as a potential immunologically privileged site on the diabetic state of the recipient was compared with that of conventional intraperitoneal transplantation (IP group) using Fischer 344 rats. Islets were isolated from the pancreata of normal rats and transplanted into the uterus and peritoneal cavity of the isogenic rats with experimental diabetes, which were treated with estradiol benzoate and progesterone. Although all the rats in both groups became normoglycemic within 4 days after transplantation, all of those in the IU group relapsed into a diabetic state up to the 20th day after transplantation. On the other hand, 6 of 8 rats in the IP group remained normoglycemic throughout the experimental period. Weight gain and diminution of urinary glucose excretion in the IU group were significantly lower than those in the IP group ($P < 0.01$). The glycosylated hemoglobin level in the IU group did not differ significantly from that in the IP group, but the serum level of fructosamine in the IU group was significantly higher than that in the IP group ($P < 0.01$). These results indicate that the response to fluctuations of blood glucose of islets in the uterine cavity is less than that of islets in the peritoneal cavity. Histologically, islets were observed to be aggregated in the uterine cavity, however the number of cells decreased markedly with time. Although this study demonstrated that blood glucose was normalized by transplantation of islets into the uterine cavity of diabetic rats, long-term survival of the islets in this location was not obtained.—**KEY WORDS:** diabetes, immunologically privileged site, pancreatic islets transplantation, rat.

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Diabetic complication is a consequence of poor control of glucose metabolism, and this is the reason why various types of pancreatic transplantation including islets transplantation have been investigated.

In rodents with chemically induced diabetes, pancreatic islets have been successfully transplanted into such diverse sites as the liver [11], peritoneum [2], spleen [1], and beneath the renal capsule [16]. Furthermore, it has been reported that the testis [5], brain [23], and cisterna magna [14] can be immunologically privileged site for islets transplantation.

In earlier studies, maternal immunological tolerance of nidation has been improved [21, 22], and uterus is also established to be “immunologically privileged” site. In order to scrutinize this phenomenon, allo- or xenogenic cells were transplanted into the uterine cavity under treatment of gonadal hormones in rats [15, 24, 25], and these reports also suggested that uterine cavity can provide a suitable environment for the survival of transplanted foreign cells. However, no information is available on the fate of endocrine cells transplanted into the uterine cavity. In this study, we first attempted the transplantation of rat pancreatic islets into the uterine cavity for the purpose of diabetic cure.

The initial objective of the present study was to determine whether isogenic islets can survive in the uterine cavity after transplantation, and whether the insulin secreted into the uterine cavity can regulate peripheral glucose levels. The secondary aim was to compare the effects of intrauterine transplantation with those of intraperitoneal transplantation, which is conventionally used in rodents.

MATERIALS AND METHODS

Animals: Female Fischer 344/Jcl rats (Clea Japan, Inc., Japan) weighing 110–140 g were used as donors of pancreatic islets, and female rats of the same strain, weighing 65–85 g, were used as recipients. All animals were given food (CE-2, Clea Japan, Inc., Japan) and water *ad libitum*, and were maintained in a conventional laboratory condition. The recipient animals were divided into following four groups, intraperitoneal cavity transplants (IP group, $n=8$), intrauterine cavity transplants (IU group, $n=12$), diabetic controls (DC group, $n=7$), and normal controls (NC group, $n=6$).

Induction of diabetes: The recipient rats were made diabetic by a single i.v. injection of 75 mg/kg streptozotocin (Sigma Chemical Co., U.S.A.) dissolved in citrate buffer (pH 4.5). Control animals were injected with citrate buffer only. Diabetes was allowed to develop over a 7–10 day period, and only rats with a fasting plasma glucose concentration consistently above 300 mg/dl were used as transplant recipients.

Monitoring of parameters: Fasting whole blood glucose, 24 hr-urinary glucose, and body weight were measured at 6:00–8:00 pm on each day. Fasting was achieved by keeping the rat in a cage without food for 12 hr. Blood samples were collected via a tail vein puncture, and glucose concentrations were analyzed by the glucose oxidase method using a Dri-Chem 5500V (Fuji Medical System Co., Ltd., Japan).

Failure of transplantation was decided when the fasting blood glucose level of the recipient exceeded 200 mg/dl for two consecutive days. All rats without transplantation failures were sacrificed by exsanguination under the anes-

thetia on the 21st day after transplantation. Whole blood was collected from the heart under the anesthesia for analysis of glycosylated hemoglobin (Hb_{A1c}) and serum fructosamine levels. The amount of Hb_{A1c} was measured by the HPLC method [12] using a HLC-723GHb (Tosoh Co., Ltd., Japan), and the serum fructosamine value by the NBT reduction method [10] using a fructosamine kit BMI (Boehringer-Mannheim, Co., Ltd., Germany).

Isolation of donor islets: Pancreatic islets were isolated by a modified collagenase digestion technique [8]. Animals were anesthetized by an i.p. injection of 50 mg/kg sodium pentobarbital (Nembutal, Abbott Lab., U.S.A.). Approximately 20 ml cold (4°C) Hanks' balanced salt solution (HBSS) containing 0.4 mg/ml collagenase (Sigma Chemical Co., U.S.A.; type V, 680 U/mg) was injected into the common bile duct via a 24-gauge indwelling butterfly needle *in situ*. Digested crude pancreatic tissues, after incubation at 37°C for 40 min, were purified by Ficoll (Pharmacia Fine Chemicals, Sweden) - Conray (Daiichi Pharmaceutical Co., Ltd., Japan) density gradient solution [17]. Subsequently, the purified islets were suspended in supravital dithizone staining solution [9], and picked out manually under an inverted microscope. Islets were cultured in free-floating plastic petri dishes (Falcon 1008, Becton Dickinson and Co., Canada) containing RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% fetal calf serum (ICN Biomedicals Japan Co., Ltd., Australia) for 24 hr until transplantation.

Hormone treatments: Hormone treatment was initiated by an intramuscular administration of 5.0 μ g/day estradiol benzoate (Ovahormon, Teikokuzouki Pharmaceutical Co., Ltd., Japan). From the 4th day, 3 mg progesterone (Luteum, Teikokuzouki Pharmaceutical Co., Ltd., Japan) was also injected intramuscularly daily and the amount of estradiol benzoate was decreased to 1.0 μ g/day thereafter. These hormone treatments were performed in all groups in the same way.

Islet transplantation: Cultured islets were transplanted on the 4th day of hormone treatment. Recipient animals were anesthetized with ether, and a small incision was made through the abdominal wall and peritoneum. In the IP group, 1000 to 1200 islets suspended in less than 20 μ l serum-free RPMI 1640 medium were inoculated into the peritoneal cavity. In the IU group, a 1–2 mm incision was made in the cavity of the right uterine horn near the uterotubal junction and the islet suspension described above was inoculated into the uterine cavity using a glass micropipette. To prevent loss of the inoculum, a ligature was placed near the uterine bifurcation, taking care to preserve the organ's blood supply. In the DC and NC groups, a sham operation was performed on the same day.

Histological examination: At the time of sacrifice, the tail of the pancreas was removed and examined to confirm the absence of native beta-cells, and the omentum, mesentery and fat tissues were also examined to prove the implantation of islets in the IP group. All rats in the IU group, including three sacrificed on each of the 5th, 10th

and 15th days, and in which the fasting blood glucose levels had been confirmed not to exceed 200 mg/dl on the previous day, were subjected to histological examination in order to scrutinize the transplanted islets in the uterine cavity. The contralateral uterine horn was used as a control. All samples were fixed with Bouin's solution and sections of the paraffin-embedded tissue were stained with aldehyde fuchsin trichrome.

Statistical analyses: The mean \pm S.D. was calculated and groups of data were compared using the unpaired Student's *t* test. Differences were considered to be significant at a level of $P < 0.05$.

RESULTS

Reversal of diabetes in recipients: Changes in the fasting blood glucose, body weight, and 24 hr-urinary glucose excretion after transplantation are shown in Fig. 1. All rats in the IP and IU groups became normoglycemic within 4 days after transplantation. In the IP group, two rats relapsed into a diabetic state at 8 and 12 days after transplantation while the other six remained normoglycemic throughout the period of observation. Although 6 rats in the IU group remained normoglycemic up to the 18th day after transplantation, three relapsed into a diabetic state at 6, 8, and 18 days after transplantation.

After induction of diabetes with streptozotocin, all rats in the IP, IU and DC groups lost approximately 10% of their initial body weight. The mean weight of the IP, IU and NC groups then increased by 20.7 ± 3.9 , 8.9 ± 0.8 and 29.2 ± 4.2 g, respectively, while in the DC group, a weight loss of 11.7 ± 3.3 g was observed. The weight gain of the IP group was significantly greater than that of the IU group ($P < 0.01$), but significantly smaller than that of the NC group ($P < 0.01$).

In both the IP and IU groups, the urinary glucose excretion after transplantation was significantly reduced compared to that before transplantation ($P < 0.01$). However, the urinary glucose excretion of the IU group remained significantly higher than that of the IP group after transplantation. Furthermore, in the IU group, the daily urinary glucose excretion did not differ significantly from that of the DC group except on the 13th and 17th days, in contrast to the IP group, whose excretion levels were all significantly lower than those in the DC group after transplantation.

Evaluation of diabetic state by measurement of Hb_{A1c} and serum fructosamine values: The Hb_{A1c} levels and serum fructosamine values in each group are shown in Table 1. The Hb_{A1c} levels in the IU group did not differ significantly from those in the IP group, whereas the values of serum fructosamine in the IP group were significantly lower than those in the IU group ($P < 0.01$). Although the Hb_{A1c} levels in both the IP and IU groups were significantly lower than those in the DC group ($P < 0.01$), the fructosamine values in the IU group did not differ significantly from those in the DC group. When HbF levels were examined, there were no significant

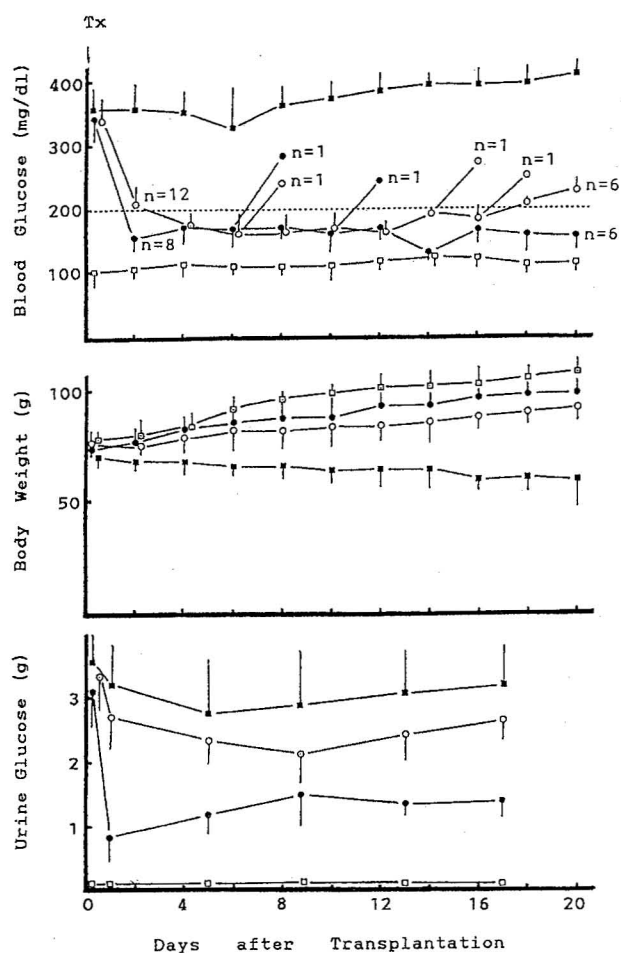


Fig. 1. Fasting whole blood glucose levels, body weight, and urine glucose levels in rats that were given transplants of 1,000–1,200 isogenic islets into the abdominal cavity and uterine cavity, respectively. Data are presented as mean \pm SD for values measured in 8 abdominal cavity transplants (●), 12 uterine cavity transplants (○), 6 diabetic controls (■) and 6 nondiabetic controls (□). In the uterine cavity transplant group, three rats in which the fasting blood glucose level had been confirmed not to exceed 200 mg/dl on the preceding day, were sacrificed on day 5, 10 and 15 for histological examination of transplanted islets in the uterine cavity. In the IP group, two rats relapsed into a diabetic state at 8 and 12 days after transplantation.

differences between the IP, IU and DC groups, indicating that HbF levels did not affect the Hb_{A1c} values of these groups. The values of Hb_{A1c}, HbF and fructosamine in the IP, IU and DC groups were significantly higher than those in the NC group ($P < 0.01$).

Histological examination: Regeneration of native beta cells was not seen on histological examination in six rats of the IP group. Moreover, the presence of well granulated engrafted islets was confirmed in the IP group (Fig. 2).

The islets transplanted into the uterine cavity are shown in Fig. 3. Each figure (A, B) shows the status of the

Table 1. Differences in Hb_{A1c}, HbF levels and the values of serum fructosamine at the time of sacrifice on the 21st day after transplantation

| Group | n | Hb _{A1c} | HbF | Fructosamine (mmol/dl) |
|-------|---|---------------------------------|---------------------------------|---------------------------------|
| | | (% of total Hb) | | |
| IP | 6 | 2.62 \pm 0.15 ^{a)d)} | 0.43 \pm 0.10 ^{c)d)} | 178.3 \pm 9.5 ^{a)b)} |
| IU | 6 | 2.70 \pm 0.14 ^{a)} | 0.48 \pm 0.13 | 223.2 \pm 17.6 ^{c)} |
| DC | 6 | 3.25 \pm 0.29 | 0.47 \pm 0.08 | 236.7 \pm 15.6 |
| NC | 6 | 1.05 \pm 0.14 | 0.20 \pm 0.11 | 157.0 \pm 9.4 |

Values represent Mean \pm S.D.

- a) $P < 0.01$ compared with value for DC group.
 b) $P < 0.01$ compared with value for IU group.
 c) Not significant compared with value for DC group.
 d) Not significant compared with value for IU group.

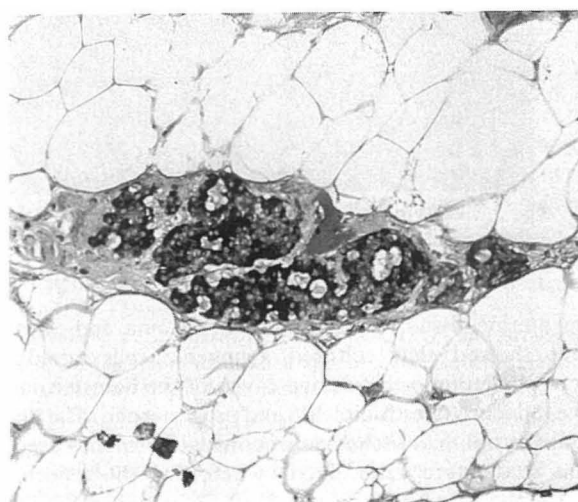


Fig. 2. Well-granulated pancreatic islets in the omental fat tissue at 21 days after transplantation in the IP group. Aldehyde fuchsin, $\times 50$.

uterine cavity in rats sacrificed 5 and 21 days after transplantation. The islets aggregated and were stained blue with aldehyde fuchsin. However, the affinity for aldehyde fuchsin was reduced, and the number of cells decreased markedly as time passed. Implantation of islets was not observed.

DISCUSSION

An ideal site for islet transplantation would be one that allows complete implantation of the transplanted tissue with optimal long-term metabolic function and, if possible, is immunologically privileged. In this study, we clearly demonstrated that intrauterine islet transplants can normalize the fasting blood glucose in rats with experimentally induced diabetes.

The implantation of foreign cells into the endometrium has been investigated by Beer *et al.* [3, 4] and Cochrane and Meyer [6], who recognized the influence of estrogen

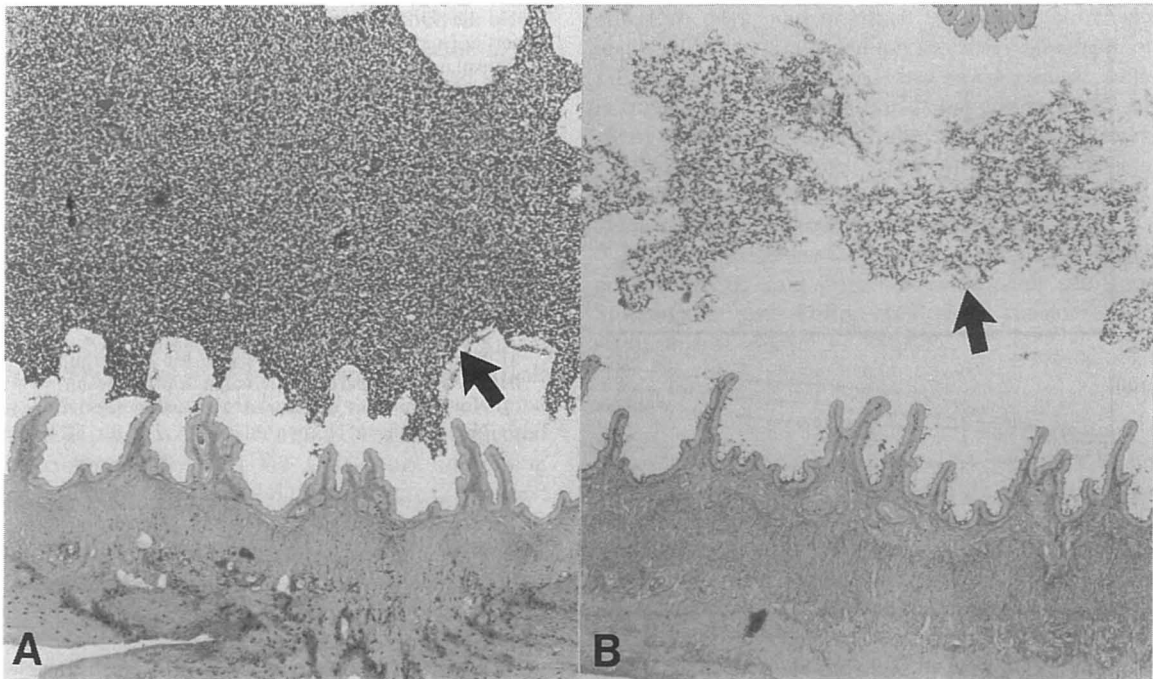


Fig. 3. Islets in the uterine cavity in rats sacrificed on 5th and 21st days after transplantation, shown as A and B, respectively. Islets were observed as aldehyde fuchsin-positive cells (arrow), and degenerated as time passed. Aldehyde fuchsin, $\times 25$.

on uterine tissue. Furthermore, Moriyama and Sugawa [15] showed that cultured xenogenic cells could be transplanted into the uterine cavity of the hamster under the influence of estradiol-17 β and progesterone. The doses and the treatment schedule of gonadal hormones used in this study were determined by referring the reports of Cochrane [6] and Moriyama [15]. Since our preliminary investigation revealed that blood glucose was not normalized in Wistar rats received intrauterine transplantation and treated with either estradiol benzoate or progesterone, an interaction of these two hormones might be an important factor for survival of islets in the uterine cavity.

Transplantation of immunologically privileged sites have been reported in rodents [5, 14, 23], in which delayed responses of transplanted islets to fluctuations in blood glucose have also been shown. Lee *et al.* [14] demonstrated that, using the intraperitoneal glucose tolerance test, more than 4 hr after glucose administration was necessary for the blood glucose to return to the basal value in rats receiving islets transplantation into the cisterna magna. In the present study, although the fasting blood glucose was restored in IU and IP groups after transplantation, another values of parameter; weight gain, urinary glucose excretion, Hb_{A1c} and serum fructosamine of IU group were inferior to those from the IP group, in spite of the same number of islets transplantation. These results indicated that the uterine cavity was a more disadvantageous site for islets transplantation than the peritoneum with regard to response to the fluctuation of blood glucose levels.

We evaluated the metabolic state by measurement of

Hb_{A1c} and fructosamine levels instead of glucose tolerance tests in this study. Since few attempts to measure Hb_{A1c} have been made in the rat [12], we had confirmed that the rat Hb_{A1c} can be measured with the same apparatus which is being used in human. The Hb_{A1c} levels in the IU and IP groups revealed that the metabolic status was ameliorated by the islet transplantation. However, the values of serum fructosamine in the IU group did not differ significantly from those in the DC group. This result further corroborated the inferiority of the uterine cavity as a site of islet transplantation.

Histologically, we could demonstrate islets in the omental tissue, which assured optimal insulin delivery into the portal circulation in the IP group. Using higher magnification, engrafted islets were shown to be well vascularized. But it is presumed that each islets would be loaded by the overstimulation, because the number of transplanted islets might be small in this study. It has been reported that the overstimulation of islets leads the defatigation of each islets [7]. This would be the main reason of the fact that two rats relapsed into hyperglycemia after transplantation in the IP group. Islets in the uterine cavity, however, degenerated and were absorbed without vascularization as time passed. This may also explain the inferiority of the uterine cavity as a transplantation site. The main cause of the grafts' disappearance from the uterine cavity is unknown. Parr *et al.* [18] have shown that a foreign body in the uterine cavity will evoke an inflammatory reaction causing an increase in lysozyme activity. This enzyme or another leukocyte-related enzyme might have led to digestion of the islets.

Although the final objective of this study is diabetic cure for companion animals by transplantation of xenogenic islets, the two problems of donor availability and immunosuppression, as well as expense, may prevent the clinical application of this technique in the future. However, semi-automatic machines for the isolation of islets have been developed [20], and the development of successful islet isolation techniques from the pancreases of animals such as the cow [13] and pig [19], which can be obtained at slaughter, would solve the problem of donor supply. Accordingly, investigation of immunological isolation and so-called "immunologically privileged" sites should be undertaken without delay.

In conclusion, normal blood glucose levels and body weight were transiently restored in rats with diabetes induced by streptozotocin by transplantation of pancreatic islets into the uterine cavity under treatment with gonadal hormones. However, implantation of the islets into the endometrium did not occur. This suggests that intrauterine transplantation is less advantageous than intraperitoneal transplantation. More detailed studies concerning effective hormonal dosages and schedules of administration are required in order to achieve the implantation of islets into the endometrium.

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