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AUGMENTER OF LIVER REGENERATION ENHANCES THE SUCCESS RATE OF FETAL PANCREAS TRANSPLANTATION IN RODENTS[1]

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Background. **Treatment of fetal pancreas (FP) isografts with insulin-like growth factor-I greatly improves the rate of conversion to euglycemia in diabetic rats. Complete knowledge of other factors that may facilitate the engraftment and function of FP in vivo is still embryonic. Augmenter of liver regeneration (ALR) is a newly described polypeptide growth factor found in weanling rat livers. ALR has trophic effects on regenerating liver. We studied the effects of in situ administration of this agent on FP isografts in rats.**

Methods. **Streptozotocin-diabetic Lewis rats (blood glucose >300 mg/dl) received 16 FP isografts transplanted intramuscularly. ALR was delivered from day 1 through day 14, in doses of 40 or 400 ng/kg/d. Animals were followed for 3 months with serial weights and blood glucose monitoring. These animals were compared with those treated with vehicle alone.**

Results. **Of the group treated with ALR at 40 ng/kg/day for 14 days, 89% (eight of nine) were euglycemic (P=0.0003). Of the group treated with ALR at 400 ng/kg/day for 14 days, 88% (seven of eight) were euglycemic (P=0.0007). Of the group treated with vehicle alone, none of the six were euglycemic. Euglycemia is defined here as glucose<200 mg/dl for 3 days. Pathology of the intramuscular transplant site showed patches of islet tissue embedded in fat. These patches demonstrated insulin immunoreactivity.**

Conclusions. **Diabetes was reversed in a significantly greater proportion of FP + ALR-treated recipients than those animals treated with vehicle alone. Local delivery of growth factors may be used as an adjunct to FP transplantation to improve the rate of success. This in situ model may be useful to further evaluate other soluble factors.**

Because of its generative capacity, transplantation of fetal pancreatic (FP*) tissue is an attractive approach to the treat-

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* Abbreviations: ALR, augmenter of liver regeneration; FP, fetal pancreas; IGF-I, insulin-like growth factor-I.

ment of diabetes mellitus (*1–3*). Human FP grafts have been shown to undergo selective endocrine differentiation and to correct diabetes in athymic mice (*4, 5*). Human FP allograft transplantation has been attempted, but only transient function of the grafts has been documented (*6*). These results demonstrate that there is incomplete understanding of the various factors necessary for engraftment, growth, maturation, and function of FP grafts.

Augmenter of liver regeneration (ALR) is a newly described polypeptide growth factor found in the cytosol of weanling rat livers (*7*). Its mechanism of action is still unclear. It has a trophic effect on liver regeneration after hepatectomy, and it augments proliferation in experimental canine portocaval shunt models (*8*). It has no effect on resting livers in vivo, nor does it increase thymidine incorporation in hepatocyte cultures in vitro. Experiments were designed to evaluate the effects of ALR, alone or in combination with insulin-like growth factor-I (IGF-I), on fetal isografts.

MATERIALS AND METHODS

Animals. Inbred Lewis rats were obtained from Harlan Bioproducts for Science (Indianapolis, IN) and were allowed free access to standard rat chow and water. Animal care was administered in accordance with the policies of the Institutional Animal Care and Use Committee at Stanford University Medical Center.

Induction of diabetes. Diabetes was induced in all recipients with a single intravenous injection of streptozotocin (100 mg/kg, Zanosar, Upjohn Co., Kalamazoo, MI). Each graft recipient had at least two serial blood glucose determinations greater than 350 mg/dl before transplantation. Before and after transplantation, diabetic rats received long-acting insulin protamine zinc insulin (NPH Ilentin, Eli Lilly, Indianapolis, IN) in doses of 2–8 units every other day, depending on random blood glucose determinations to prevent weight loss and facilitate FP growth (*9*). Exogenous insulin was not administered if blood glucose was less than 250 mg/dl.

Surgical technique for intramuscular implantation of FP. According to the technique of Wang et al. (*10*), 20–21-day gestational pups were isolated by cesarean section from time-pregnant Lewis rats under anesthesia using isoflurane (AErrane, Anaquest Inc., Liberty Corner, NJ) through a vaporizing system (Omni Medical Equipment, Inc., Pleasanton, CA). FPs were removed by blunt dissection. FPs were minced into 1-mm^3 pieces and washed twice in Hanks' balanced salt solution. Tissues were kept on ice in Hanks' balanced salt solution until transplantation. After sterile preparation of male Lewis recipients (150–250 g), an incision was made over the anterior surface of each hind leg and a 1-cm pocket created in the underlying muscle. Grafts of 8 or 16 FP were divided equally between the two pockets. The grafts were covered by approximation of the muscle, and the skin closed with surgical clips. Growth factors were delivered directly into the transplant bed by osmotic minipumps (Alza Corp., Palo Alto, CA) placed in an anterior abdominal subcutaneous space. A polyethylene catheter (PE 50, Clay Adams, Parsippany, NJ) leading from the pump to the bed was secured in place with suture. ALR (kindly supplied by T.E. Starzl) was delivered in doses of 40 or 400 ng/kg/day for 14 days. IGF-I (recombinant human IGF-I; kind gift of Genentech, San Francisco, CA) was delivered in doses of 69 μg/kg/day for 14 days. Animals receiving FP grafts and treated with vehicle alone served as controls. Pumps were removed on day 15 after transplantation and bisected to confirm complete drug delivery.

Blood glucose determination. After transplantation, blood glucose determinations were made by tail vein bleedings three times a week using an Ames Glucofilm system (Miles, Elkhart, IN). Reversal of diabetes was defined as blood sugars less than 200 mg/dl on three consecutive measurements.

Glucose tolerance testing. Glucose tolerance tests were performed on transplanted rats that had converted to euglycemia. Dextrose was diluted to 500 mg/kg in normal saline and administered by intravenous injection. Blood glucose determinations were made before dextrose injection and at 5-, 15-, 30-, 60-, and 120-min intervals thereafter.

Histology. After fixation in 10% neutralized formalin, sections were stained with hematoxylin and eosin. Insulin immunoreactivity was demonstrated with mouse anti-human insulin monoclonal antibody (E54071 M, Biodesign International, Kennebunkport, ME) on 6-μm paraffin-embedded sections using a commercially available kit (Histostain-DS Broad Spectrum, Zymed Labs, Inc., South San Francisco, CA).

Data analysis. Results are reported only on those animals that reverted to hyperglycemia after graft removal and had no histological evidence of islets remaining in the native pancreas. Categorical differences were analyzed by Fisher's exact probability test, and interval differences by analysis of variance. A *P*-value of less than 0.05 was considered significant.

RESULTS

Treatment of FP isografts with ALR significantly increased the rate of conversion to euglycemia when compared with vehicle alone. This was true of both doses tested. Administration of 40 ng/kg/day increased the conversion rate to 89% (eight of nine) with an interval of 76 ± 54 days from transplant to conversion. Administration of 400 ng/kg/day had similar results with a conversion rate of 88% (seven of eight) and an interval of 61 ± 11 days. None of the animals treated with vehicle alone converted to euglycemia. See Table 1.

Reducing the mass of transplanted FP resulted in a one-third decrease in the efficiency in the rate of conversion. When eight FP isografts were treated with 40 ng of ALR/kg/day for 14 days, 62% (five of eight) of the rats converted with a mean interval of 51 ± 17 days. See Table 2. As reported previously (*14*), IGF-I administered at a rate of 69 μg/kg/day resulted in a conversion rate of 89% (eight of nine) with a mean interval of 57 ± 27 days (*14*). Combination of ALR with IGF-I did not offer an advantage over either agent alone. Animals receiving eight FP grafts and treated with 40 ng of ALR/kg/day plus 69 μg of IGF-I/kg/day converted at a rate of 83% (five of six) with a mean interval of 63 ± 14 days ($P=0.114$).

Figure 1 shows the typical blood glucose trends in a single ALR-treated rat after transplant. Random blood glucose levels in normal rats averaged 111 ± 7 mg/dl by tail vein bleeding. Streptozotocin-treated rats were consistently above 300 mg/dl. All animals receiving FP isografts at day 0 remained hyperglycemic during the 14-day treatment period during which ALR was administered. For 1–2 weeks before conversion to euglycemia, the glucose levels began to fluctuate. Once conversion had occurred, blood glucose levels re-

TABLE 1. Effects of two doses of ALR treatment on 16 fetal pancreatic isografts transplanted into the intramuscular site

Group[b]	FP	% Euglycemic[c]	Interval[d]	P[e]
40	16	89 (8/9)	76 (±54)	0.0003
400	16	88 (7/8)	61 (±11)	0.0007
Vehicle	16	0 (0/6)		

[a] % Euglycemia is listed as percent of animals cured (animals cured/sample size).

[b] Concentration of ALR in ng/kg/day, delivered for 14 days.

[c] Glucose <200 mg/dl × 3 days (# cured/# total).

[d] Mean interval (±SD) transplant to euglycemia in days.

[e] Fishers exact test compared to untreated controls.

TABLE 2. Success rates of engraftment of 8 fetal pancreas isografts in the intramuscular site following treatment with ALR, IGF-I, or ALR plus IGF-I

Group	FP	% Euglycemic	Interval
ALR 40[b]	8	62 (5/8)	51 (±17)
IGF 69[c]	8	89 (7/9)	57 (±27)
IGF/ALR	8	83 (5/6)	63 (±14)

[a] % Euglycemia is listed as percent of animals cured (animals cured/sample size).

[b] Concentration of ALR in ng/kg/day, delivered for 14 days.

[c] Concentration of IGF in μg/kg/day, delivered for 14 days.

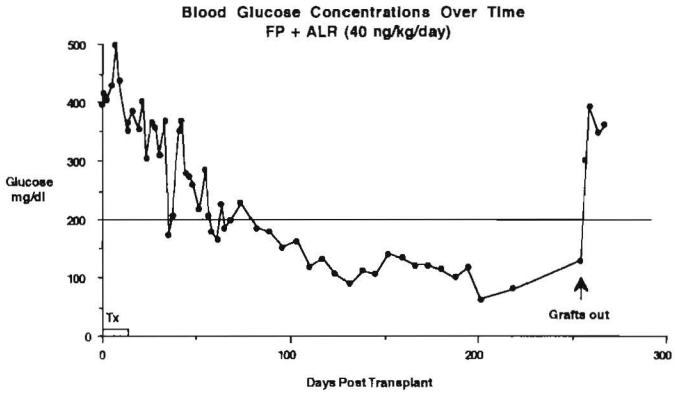

FIGURE 1. Glucose measurements over time in a FP recipient treated with ALR at 40 ng/kg/day.

mained normal until the grafts were removed from the intramuscular site, at which time serum glucose levels returned to the diabetic range.

Intravenous glucose tolerance tests were performed on all euglycemic animals at more than 100 days after transplant. Averaged results of those data are shown in Figure 2. Glucose tolerance tests of the ALR-treated isograft recipients were not significantly different from those from normal rats (P=0.12 by analysis of variance).

Histological analysis of the excised intramuscular grafts from euglycemic animals routinely showed islet tissue imbedded in fat (Fig. 3, top). Immunohistochemical staining showed insulin reactivity in β cell granules (Fig. 3, bottom). Histology of the native pancreas after streptozotocin treatment revealed a paucity of islets and an atrophic appearance to those identified. Those islets present lacked insulin reactivity by immunohistochemistry.

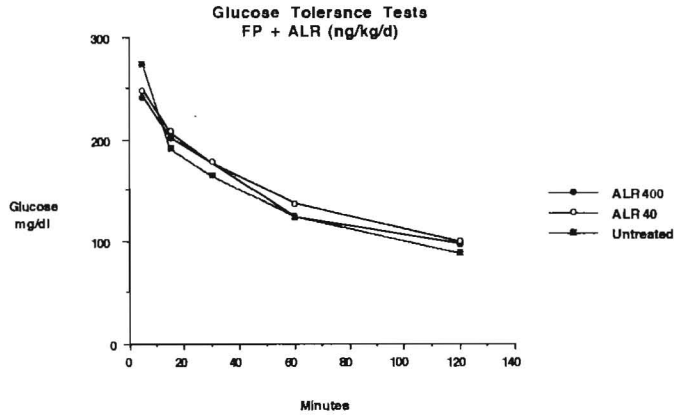

FIGURE 2. Similar mean results of glucose tolerance tests comparing rats treated with ALR at 40 ng/kg/day and 400 ng/kg/day to normal nondiabetic rats. The graph represents clearance of sugar from the blood stream after a glucose challenge.

DISCUSSION

Currently, only vascularized whole pancreas grafts are routinely used clinically. These grafts may achieve near-euglycemia (*11*). Usually, however, the recipients already have end-stage diabetic nephropathy, which requires a simultaneous kidney transplant. In this setting, the one year pancreas graft survival has improved steadily. However, there is still significant morbidity and mortality associated with this procedure (*12*).

Results of the clinical transplantation of isolated adult islets have been disappointing (*13*). Insufficient yield of islets, impure preparations, lack of engraftment, and rejection continue to frustrate efforts.

The goal of FP transplantation is to introduce a functional islet cell mass into patients with type 1 diabetes mellitus. This tissue is expected to achieve the physiological glucose control thought necessary for the prevention, retardation, or reversal of diabetic neurovascular complications. The advantages of FP include availability, proliferative capacity, and viability after preservation. Allografts of human FP have

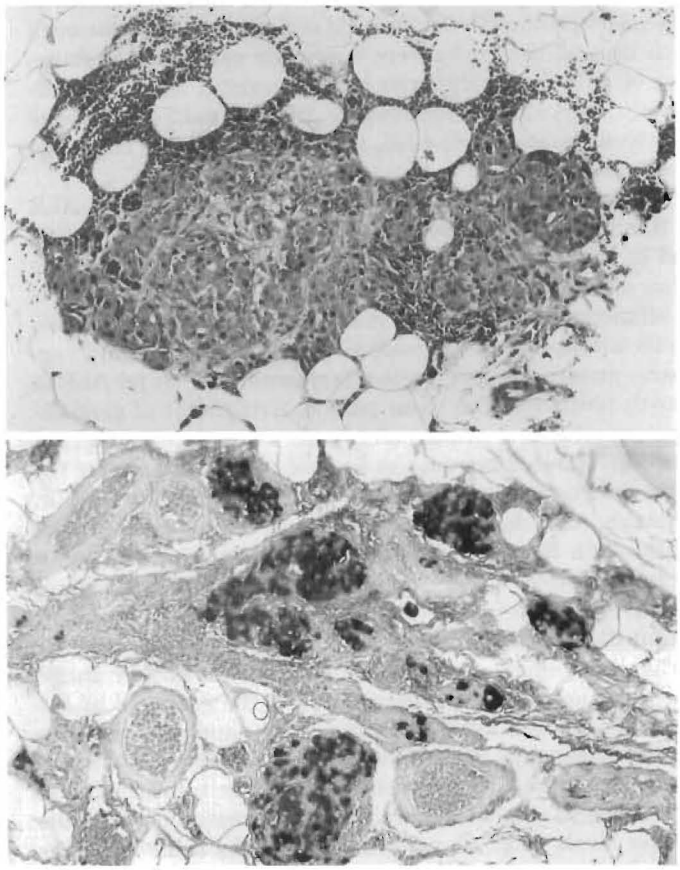

FIGURE 3. (Top) Hematoxylin-eosin stained FP graft tissue excised from the intramuscular site (original magnification, ×50). (Bottom) Insulin immunoreactivity in the FP graft tissue (original magnification, ×50).

been transplanted into various sites including intramuscularly and under the capsule of a kidney, with only transient C-peptide production (*6*). However, a better understanding of well-defined growth factors and the production of these growth factors by recombinant technology offer the opportunity to re-evaluate FP transplantation.

We have reported that diabetes may be reversed in rats receiving FP allografts and treated with IGF-I (*14*). We also found that treatment of grafts with anti-IGF-I receptor antibody increased the interval to conversion in the successful FP/fetal liver co-transplantation model (*15*). Administration of IGF-I to cultured adult rat islets or to neonatal rat pancreatic monolayers has been shown to increase insulin secretion and β cell replication (*16*, *17*). It is likely that other mediators elaborated by liver may have trophic effects on FP. An incomplete list of growth factors that may play a role in this experimental system includes the cytosolic pancreatic factor ilotropin (*18*) and hepatocyte growth factor (*19*), in addition to IGF-I and -II (*20*).

The choice of ALR was based on its trophic effects on liver despite its lack of mitogenic stimulation. Initially, it was believed that ALR may be synergistic with IGF-I with respect to the IGF-I effects on FP.

Our evidence shows that local delivery of ALR or IGF-I to FP isografts transplanted intramuscularly improves the success rate of correction of streptozotocin-induced diabetes in rats. Milligram for milligram, ALR may be roughly 100 times more potent than IGF-I with respect to its effects on FP, based on the concentrations chosen here. Once conversion to euglycemia occurred, the animals remained euglycemic until such time as the grafts were surgically removed. Combination of ALR and IGF-I was not synergistic, nor did it adversely affect the conversion rate. In all cured FP recipients that were treated with ALR, glucose clearance to challenge was normalized.

The precise mechanism(s) of the beneficial effects of ALR in this model is unknown. ALR is found mainly in platelets and in germ cell lines and does not have tight homology to other known peptide growth factors. The closest homology is to bifunctional gene in the yeast *Saccharomyces cerevisiae,* which is responsible for oxidative phosphorylation and vegetative growth. This suggests a very primitive role for ALR in growth functions. The yeast gene is a regulator of gene expression related to growth and is not a direct growth factor. The ALR-induced increase in hepatic regeneration after hepatic resection suggests a role in liver growth or repair, although ALR does not appear to directly increase DNA synthesis in the resting hepatocyte in vitro. This argues for an indirect role, either via stimulation of other mitogenic agents or by reversal of those factors that may restrict or inhibit hepatocyte growth.

The direct effect of ALR on FP is worthy of further study. ALR is no longer being administered to the graft bed by the time the pancreas reaches a critical mass and glucose levels begin to normalize. But the trend toward euglycemia is apparent in Figure 1 by the second week after treatment. It is interesting to note that there was no overcompensation; the glucose levels after conversion were in the same range as normal rats.

To optimize FP transplantation as an approach to the treatment of diabetes, we need to define better the role that growth factors play, in particular, IGF-I and ALR, in the maturation of FP grafts. Our current studies demonstrate that IGF-I increases the frequency of successful transplantation, and decreases the amount of tissue required for successful FP transplantation. ALR also has positive effects in this system. Similar strategies incorporating the paracrine support of engrafting islets may improve the previously poor results of clinical fetal islet transplantation.

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