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Successful pancreatic allografts in combination with bone marrow transplantation in mice

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SUMMARY

We have established a new method for pancreatic allografts in mice by combining pancreatic transplantation with allogeneic bone marrow transplantation. In this approach, we first transplanted bone marrow to induce tolerance to both donor-type and host-type major histocompatibility complex (MHC) determinants. Pancreatic tissue from the same mouse strain as bone marrow donor was then grafted under the renal capsule. Acceptance of the grafts was confirmed by histopathological and immunohistochemical techniques. BALB/c mice reconstituted with C57BL/6J bone marrow cells accepted pancreatic tissue from both bone marrow donor (C57BL/6J)-type and host (BALB/c)-type mice. An immunohistochemical study revealed the presence of functional islets under the renal capsules. Assays for both mixed lymphocyte reaction (MLR) and induction of cytotoxic T lymphocytes indicated that the newly developed T cells are tolerant of both donor (stem cell)-type and host-type MHC determinants. By contrast, the T cells of these chimeras showed a significant responsiveness to third party MHC determinants. These findings suggest that pancreatic allografts combined with bone marrow transplantation may become a viable strategy for the treatment of patients with diabetes or patients who have undergone pancreatectomy.

INTRODUCTION

More than 500 pancreatic transplantations have been reported since 1966, when such transplantations were first performed in humans (Sutherland & Kendall, 1985; Sutherland *et al.*, 1985). However, the success rate is very low and has never been over 30%. In animals as well, pancreatic allografts have been reported to be rejected aggressively, in contrast with liver allografts. Faustman *et al.* (1980, 1981) demonstrated that rejection of islet allografts in mice is prevented by pretreatment of the grafts with anti-Ia antiserum plus complement. More recently, these investigators have also reported that they succeeded in preventing rejection of islet allografts by pretreatment of the grafts with anti-dendritic cell antibody (Faustman *et al.*, 1984).

Recently, conflicting data have come to light as a result of studies of self-tolerance induction in nu/nu mice and radiation bone marrow chimeric mice (Zinkernagel *et al.*, 1980; Kindred & Loor, 1974; Seger, Rogers & Catty, 1974; Miller, Derry & Sarjeant, 1983; Onoe *et al.*, 1985a, b). Zinkernagel *et al.* (1980) reported that cytotoxic T lymphocytes (CTLs) in radiation bone marrow chimeras were tolerant of thymus-type major histocompatibility complex (MHC) determinants. Kindred & Loor (1974) also reported that BALB/c nu/nu mice engrafted with allogeneic thymus sometimes accepted, but sometimes rejected, skin grafts from thymus-donor strains. In addition, when hearts were grafted to nu/nu mice bearing an allogeneic thymus, the hearts from the thymus-donor strain were rejected in one study and accepted in another (Seger *et al.*, 1974). Recently, stable chimerism and specific tolerance have been induced in adult mice and rats by lethal whole body irradiation of recipients followed by the i.v. infusion of allogeneic fetal liver cells (Yunis *et al.*, 1976) or adult bone marrow cells treated *in vitro* to remove thymus-derived (T) cells and/or T-cell precursors (Von Boehmer, Sprent & Nabholz, 1976; Müller-Rucholtz, Wottge & Müller-Hermelink, 1976). In full allogeneic bone marrow chimeras, T cells are restricted to histocompatibility characteristics attributable to recipient thymus predominate, but a small expandable T-cell population self-restricted to donor cells is also found (Onoe *et al.*, 1985a, b).

We have recently reported that fully allogeneic chimeras in nu/nu mice readily accept skin from both thymus-type and bone marrow-type mice (Furukawa *et al.*, 1984). In addition, we have more recently found that mice that have undergone liver

Abbreviation: CTLs, cytotoxic T lymphocytes. FCS, fetal calf serum; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction.

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allografts combined with bone marrow transplantation accept donor (stem cell)-type and host-type liver tissue (Nakamura *et al.*, 1986).

These findings prompted us to examine the fate of pancreatic allografts in radiation bone marrow chimeras in mice. In this report we demonstrate that chimeric mice accept pancreatic tissue from both bone marrow donor-type and host-type mice.

MATERIALS AND METHODS

Animals

Inbred BALB/c (H-2^d), C57BL/6J (H-2^b) and C3H/HeN (H-2^k) mice were obtained from the Japan Clea Co. Ltd, Osaka, Japan and maintained under specific pathogen-free conditions in our animal facilities, Kansai Medical College, Osaka, Japan.

Transplantation of bone marrow cells and pancreas

Two-month-old BALB/c mice were lethally irradiated with 8·5 Gy (1·0 Gy/min) from a ^{60}Co source and subsequently injected with 2×10^7 bone marrow cells from 6-week-old C57BL/6J mice; the bone marrow cells had been treated with anti-Thy-1.2 antibody (clone F7D5; Serotec Ltd, Bicester, Oxon) plus rabbit complement. A few days following bone marrow transplantation, the mice were anesthetized with pentobarbital (0·05 mg/g body weight, Pitman-Moore, Washington Crossing, NJ). Pancreases taken from new-born BALB/c, C57BL/6J or C3H/HeN mice were grafted under the left renal capsules of the BALB/c mice. The mice were killed 2 months later, and the grafted pancreatic tissue was examined histologicaly and immunohistochemicaly. The chimerism was checked using anti-H-2 antibody; H-2 typing revealed that more than 95% of spleen cells were of donor-type cells.

Cell preparation

The spleens were aseptically removed, minced, and gently passed through a fine mesh stainless steel sieve into phosphate-buffered saline.

Mixed lymphocyte reaction (MLR)

MLR was examined by counting [^{3}H]thymidine ([^{3}H]TdR, 0·5 μCi; New England Nuclear, Boston, MA) incorporated into DNA. Triplicate cultures were set up in 96-well round-bottomed microtitre plates (Corning Glass Works 25850, Corning, NY). Each well contained 2×10^5 responder cells and 1×10^5 stimulator cells in a total volume of 0·2 ml RPMI-1640, streptomycin, 100 μg/ml, 5% fetal calf serum (FCS; Gibco, Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (2-ME; Wako Pure Chemical Industries, Tokyo, Japan). Stimulator cells were exposed to 20 Gy from a ^{60}Co source. The cultures were incubated for 96 hr in a humidified atmosphere of 5% CO_2 in air. [^{3}H]TdR was present during the last 4 hr of the culture period. The radioactivity of [^{3}H] incorporated into trichloroacetic acid-insoluble material was determined using a liquid scintillation counter.

Induction of cytotoxic T lymphocytes (CTLs)

Responder cells ($7{\cdot}5 \times 10^6$) and irradiated (20 Gy) stimulator cells ($2{\cdot}5 \times 10^6$) were co-cultured in RPMI-1640 medium containing 10% FCS, supplemented with 5×10^{-5} M 2-ME, 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cultures were incubated for 5 days at 37° in a humidified atmosphere of 5% CO_2 in air. P815 (H-2^d), EL-4 (H-2^b) and X5563 (H-2^k) cell lines were used as targets. The target cells were labelled with 100 μCi Na_2 [^{51}Cr]O_4 (New England Nuclear) by means of incubation for 1 hr at 37°. Labelled cells were washed three times. These cells (5×10^4) were then mixed with effector cells in 200 μl of RPMI-1640 medium in round-bottomed microtitre plates and incubated at 37° in 5% CO_2 for 4 hr. Using the Titertec Supernatant Collection System (Flow Lab., Irvine, Ayrshire), supernatant was harvested, and the released radioactivity was determined. Specific lysis was calculated according to the following formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneus release}} \times 100.$$

Histopathology

The left kidney with grafted pancreatic tissue was obtained at necropsy. A portion of this tissue was stained with haematoxylin and eosin to evaluate graft acceptance. An immunohistochemical technique using peroxidase-labelled antibody was used to assess the presence of functional islets under the renal capsules, as described in our previous paper (Ikehara *et al.*, 1985a).

Table 1. Fate of pancreatic allografts in BALB/c mice reconstituted with bone marrow cells of C57BL/6J mice

Mouse	Donor of pancreas	No. examined	No. accepted
BALB/c	BALB/c	8	7
BALB/c	C57BL/6J	8	0
BALB/c	C3H/HeN	8	0
[C57BL/6J→BALB/c]*	BALB/c	10	10
[C57BL/6J→BALB/c]	C57BL/6J	9	9
[C57BL/6J→BALB/c]	C3H/HeN	5	0

* BALB/c (H-2^d) mice were irradiated (8·5 Gy) and reconstituted with bone marrow cells (2×10^7) of C57BL/6J (H-2^b) mice. A few days after bone marrow transplantation, pancreatic grafts obtained from new-born BALB/c, C57BL/6J, or C3H/HeN mice were grafted under the renal capsules of BALB/c.

RESULTS

BALB/c (H-2^d) mice reconstituted with C57BL/6J (H-2^b) bone marrow cells, [C57BL/6J→BALB/c] chimeras, survived more than 8 weeks without GVHR. H-2 typing of spleen cells in the chimeras revealed that more than 95% of spleen cells are donor derived (H-2^b cells >95% and H-2^d cells <5%). The mice possessed normal numbers of Thy-1$^+$ cells in the spleen, and the spleen cells responded normally to PHA, Con A and LPS (data not shown).

The fate of the engrafted pancreatic tissue is summarized in Table 1. Non-treated BALB/c mice rejected allogeneic pancreatic tissue from C57BL/6J (0/8) and C3H/HeN (0/8) mice. By contrast, [C57BL/6J→BALB/c] chimeras accepted both bone marrow donor (C57BL/6J)-type (9/9) and host (BALB/c)-type (10/10) pancreatic tissue whereas they rejected third-party (C3H/HeN) pancreatic tissue (0/5). As shown Fig. 1, grafted islets and interlobular ducts from C57BL/6J donors were found

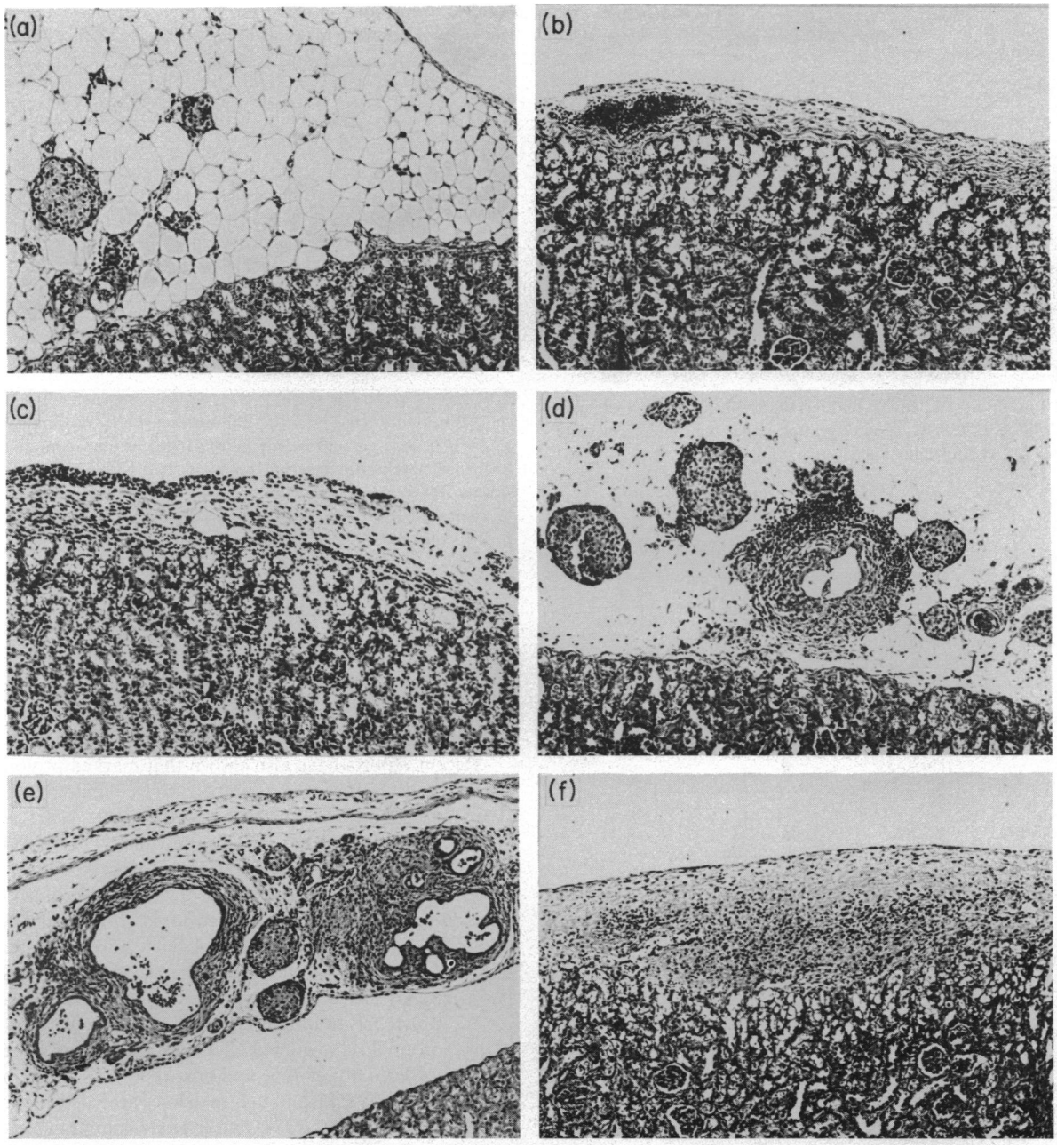

Figure 1. Histological findings of pancreatic tissue grafted under the renal capsules. BALB/c, C57BL/6J, or C3H/HeN pancreatic tissue was grafted under the renal capsules of BALB/c and [C57BL/6J→BALB/c] chimeric mice: (a) BALB/c pancreatic tissue grafted in BALB/c kidney; (b) C57BL/6J pancreatic tissue grafted in BALB/c kidney; (c) C3H/HeN pancreatic tissue grafted in BALB/c kidney; (d) BALB/c pancreatic tissue grafted in [C57BL/6J→BALB/c] chimera; (e) C57BL/6J pancreatic grafted in [C57BL/6J→BALB/c] chimera, and (f) C3H/HeN pancreatic tissue grafted in [C57BL/6J→BALB/c] chimera. As shown in a, d and e, grafted islets and interlobular ducts are found under the renal capsule. As shown in b, c and f, however, all grafted tissue under the renal capsule was replaced by fatty and fibrous tissue.

under the renal capsules of [C57BL/6J→BALB/c] chimeras. However, the exocrine components of the pancreas were replaced with fibrous and fatty tissue following autolysis. An immunohistochemical study revealed the presence of insulin-producing cells (Fig. 2). However, C3H/HeN pancreatic tissue grafted in [C57BL/6J→BALB/c] was found to be rejected and replaced by fibrous and fatty tissue with the infiltration of mononuclear cells. No persistent islets were found. Other combinations with C57BL/6J as a recipient and C3H/HeN as a bone marrow donor were performed, and results similar to those described above were obtained (data not shown).

MLR and CTL induction assays were performed to ascertain that the newly developed T cells of the chimeras are tolerant of both host-type and donor-type MHC determinants. Figure 3 shows that spleen cells of chimeras respond well to third party cells, whereas they do not respond to cells of either bone marrow

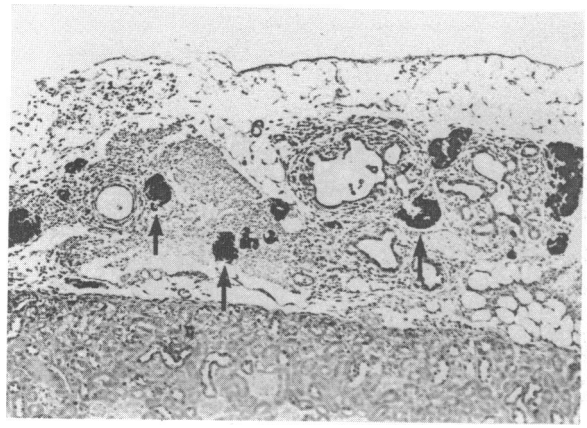

Figure 2. Immunohistochemical findings of insulin-producing beta cells (arrow) in islets of C57BL/6J mice grafted under the renal capsules of [C57BL/6J→BALB/c] chimeras.

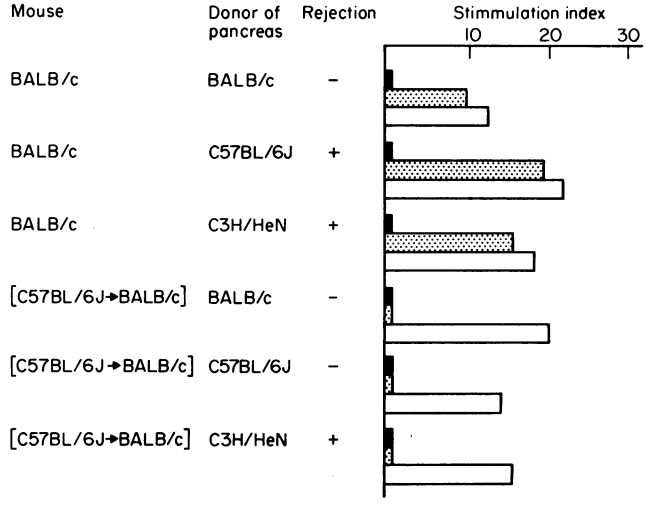

Figure 3. Mixed lymphocyte reaction (MLR) after bone marrow transplantation reveals that [C57BL/6J→BALB/c] chimeras are tolerant of both bone marrow donor (C57BL/6J)-type and host (BALB/c)-type MHC determinants. Stimulator: BALB/c (■), C57BL/6J (■), and C3H/HeN (□).

donor-type or host-type mice. As shown in Table 2, the assay for induction of CTLs also revealed that the T cells are tolerant of both donor-type and host-type MHC determinants, and respond normally to third party MHC determinants.

DISCUSSION

It has been reported that the acceptance rate for pancreatic allografts is lower than that for other organs, and that even if the pancreatic graft is accepted, the patient requires almost continuous administration of immunosuppressive agents, e.g. cyclosporin, azathioprine, and/or steroid hormone (Sutherland *et al.*, 1985; Schulak & Drevyanko, 1985; Groth, 1985). Cyclosporin has recently been shown to be effective in prolonging kidney, heart, liver, and pancreatic allograft survival in man

Table 2. Generation of cytotoxic T lymphocytes from BALB/c mice reconstituted with bone marrow cells of C57BL/6J mice*

Spleen cells from	Donor of pancreas	E:T*† ratio	% specific release from target cells (H-2^d) P815	(H-2^b) EL-4	(H-2^k) X5563
BALB/c	BALB/c	4:1	0	53	63
BALB/c	C57BL/6J	4:1	6	31	50
BALB/c	C3H/HeN	4:1	5	38	35
[C57BL/6J→BALB/c]‡	BALB/c	4:1	7	2	40
[C57BL/6J→BALB/c]	C57BL/6J	4:1	1	0	33
[C57BL/6J→BALB/c]	C3H/HeN	4:1	1	0	42

* Responder cells ($7{\cdot}5 \times 10^6$) and irradiated (20 Gy) stimulator cells ($2{\cdot}5 \times 10^6$) were co-cultured in RPMI-1640 medium containing 10% heat-inactivated human serum, supplemented with 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5×10^{-5} M 2-mercaptoethanol. After 5 days of co-culture, the cells were used for a ^{51}Cr release assay, as described in the Materials and Methods.

† Effector:target cell ratio.

‡ BALB/c (H-2^d) mice were irradiated and reconstituted with bone marrow cells of C57BL/6J mice.

(Calne, 1983) as well as animals (Flye *et al.*, 1983), but reports show that high doses of cyclosporin have toxic effects on the kidney and liver.

Recent reports have also shown that marked prolongation of islet allograft survival can be achieved by eliminating Ia$^+$ cells (Lacy *et al.*, 1979; Faustman *et al.*, 1980, 1981; Farr & Anderson, 1985) or dendritic cells (Faustman *et al.*, 1984) from the islets prior to transplantation. Faustman *et al.* (1984) demonstrated that glucose levels of C57BL/6J diabetic mice induced by streptozotocin were restored to normal after islet allografts for more than 200 days. Such mice exhibited graft rejection and hyperglycemia when injected with donor cells that had been enriched with dendritic cells.

We previously reported that mice that underwent transplantation of T cell-depleted allogeneic bone marrow cells are tolerant of both donor-type and host-type MHC determinants (Ikehara *et al.*, 1985a), and that such mice accepted allografts of skin (Furukawa *et al.*, 1984) and liver (Nakamura *et al.*, 1986) from the same donor as the bone marrow. These mice did not develop GVHR (Ikehara *et al.*, 1985b).

In the present study, we have demonstrated that non-treated BALB/c (H-2^d) mice accept syngeneic pancreatic tissue from BALB/c donors, whereas they reject allogeneic pancreatic tissue from both C57BL/6J (H-2^b) and C3H/HeN (H-2^k) mice. By contrast, when BALB/c mice were irradiated and reconstituted with C57BL/6J bone marrow cells purged of T cells, these BALB/c mice accepted pancreatic tissue from both host (BALB/c)-type and bone marrow donor (C57BL/6J)-type mice, but rejected pancreatic tissue from third party (C3H/HeN) mice (Table 1). Immunohistochemical studies revealed the presence of functional islets in accepted pancreatic grafts (Figs 1 and 2). Although we did not examine serum insulin levels in the present study, we have recently found that insulin levels of non-obese diabetic (NOD) mice with overt type I diabetes treated with this method are restored to normal. Such mice exhibit normal

responses in glucose tolerance tests, and survive more than 1 year after pancreas allografts (Yasumizu *et al.*, 1987).

Using assays for both MLR (Fig. 3) and induction of CTLs (Table 2), we demonstrated that the newly developed T cells in these chimeras are tolerant of both donor-type and host-type MHC determinants. Thus, we have established a new method for pancreatic allografts in which we induce tolerance without using immunosuppressive agents and in which HLA-mismatching can be overcome.

A major clinical problem is to obtain HLA-matched bone marrow and pancreas donors. This problem may, however, be overcome by using aborted fetuses, from which we can obtain not only haematopoetic stem cells from the liver but also pancreatic tissue suitable for pancreas grafts. This is because the fetal pancreas has less exocrine tissue and denser endocrine tissue than adults.

Recently, bone marrow transplantation is often performed as a therapy for aplastic anaemia and severe combined immunodeficiency (SCID) (Storb & Thomas, 1983; Good, Kapoor & Reisner, 1983). Bone marrow transplantation is also becoming common as a therapy for leukaemia and malignant lymphoma (Storb & Thomas, 1983; Mascret *et al.*, 1986). Good *et al.* (1983) have reported that more than 50 otherwise lethal diseases can be treated with bone marrow transplantation from HLA-matched or haplotype-identical mismatched donors. We think that this method of organ transplantation combined with bone marrow transplantation can be developed into a viable strategy for the treatment of patients with diabetes, in whom beta cells of the islets have been destroyed, and also of patients whose pancreases have been removed in the treatment of pancreatic cancer and pancreatic cysts.

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