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Ex vivo perfusion of canine pancreaticoduodenal allografts using class-II-specific monoclonal antibody delays the onset of acute rejection*

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Abstract. In the following study, we investigated whether ex vivo perfusion of canine pancreaticoduodenal allografts prior to transplantation using a class-II-specific monoclonal antibody (MoAb) OKIa1) could prevent acute rejection. Untreated grafts were rejected within 6 days after transplantation, and all of these recipients suffered severe hyperglycemia. In contrast, in recipients who received grafts which underwent ex vivo class-II-specific MoAb perfusion treatment, the mean urinary amylase levels were sustained significantly higher (11733 ± 4493 vs. 3274 ± 2108 U/L on day 7, $P < 0.005$), and mean fasting blood glucose (FBG) levels remained within the normal range (13.4 ± 5.8 vs. 23.4 ± 3.9 m*M* on day 7, $P < 0.0005$). Low doses of cyclosporin A (CsA) were necessary in order to maintain lower FBG levels. Histopathology analysis on day 7 after transplantation showed that endotheliitis and necrosis were much less prominent in the MoAb-treated grafts. In the light of our results, we conclude that ex vivo perfusion of canine pancreaticoduodenal allografts using a class-II-specific MoAb is effective in delaying the onset of acute rejection, and low doses of CsA could extend this effect.

Key words: Pancreas transplantation – Class-II-specific monoclonal antibody – Immunomodulation – Dendritic cell

In pancreas transplantation, the most powerful stimulus for the initiation of acute rejection has been shown to come from interstitial dendritic cells (DCs), expressing large quantities of major histocompatibility complex (MHC) class II antigens. DCs are known to be present in the exocrine pancreas and circulate rapidly through the tissues to carry foreign antigens in a highly immunogenic form to the organized lymphatic tissues [8, 13, 14]. It has been suggested that inactivation of the DCs would result in the removal of the stimulating capacity of the allograft. Manipulation of allografts to modulate their antigenicity might be an attractive and potentially clinically applicable strategy as an alternative to current immunosuppression techniques. Pretreatment of the pancreas or islet allografts using class-II-specific monoclonal antibodies (MoAbs) was shown to result in prolongation of graft survival in rodent models [6, 9] but was ineffective in canine models [2, 15]. The following experiments were designed to determine whether or not ex vivo perfusion of canine pancreaticoduodenal allografts using class-II-specific MoAb could delay the onset of acute rejection. We also examined the effect of the combined use of cyclosporin A (CsA) at levels inadequate for normal grafts, since pretreatment with class-II-specific MoAb seems limited to the inhibition of acute rejection in a large animal model like a dog.

Materials and methods

Animals. Unrelated adult mongrel dogs of both sexes weighing 9–13 kg without infections were used in these experiments as the donors and recipients. All surgical procedures were performed under general anesthesia with ketamine hydrochloride (10 mg/kg i.m., Sankyo, Tokyo, Japan).

MoAb. OKIa1 (Ortho, Raritan, N.J.), a murine IgG2 antibody against human DR antigen complex, was used in this experiment. It has also been shown to crossreact with canine class II antigens [7].

Harvesting procedure for pancreaticoduodenal allografts. As shown in Fig. 1 A, the harvesting of the canine pancreaticoduodenal allografts was performed basically according to the method of Ekberg et al. [5]. Briefly, the whole pancreas and the duodenal segment were harvested together with a vascular pedicle comprising the portal vein and the aortic conduit with the celiac axis and the superior mesenteric artery (SMA). The common bile duct was divided where it approached the pancreas, and two or three hepatic branches originating from the common hepatic artery were divided. The pancreaticoduodenal artery along the duodenum was divided distal to the origin of the pancreaticoduodenal arcade to the SMA, and the arcade was preserved in its entirety. The distal part of the uncinate

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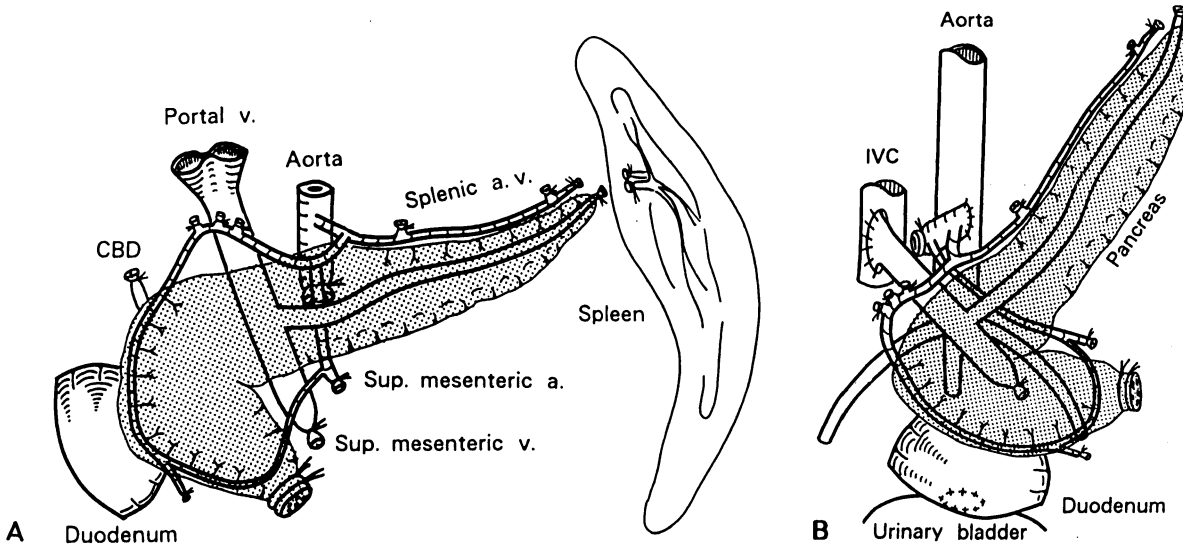

Fig. 1 A, B. Procedure for canine pancreaticoduodenal allotransplantation: harvesting (**A**) and transplantation (**B**). *CBD*, common bile duct; *IVC*, inferior vena cava

process that became ischemic was resected. The splenic artery and vein were divided distal to the pancreas, and the porta hepatis was divided up to its bifurcation at the hepatic hilum. After harvesting and perfusion with 300 ml Euro-Collins' solution (EC) at 4 °C via the aortic conduit, the grafts were maintained in saline-ice slush at 4 °C.

Transplantation procedure. As shown in Fig. 1 B, the portal vein was anastomosed end-to-side to the inferior vena cava, and the aortic conduit was anastomosed end-to-side to the aorta. The arterial supply of the allograft was based on the celiac axis and SMA. After revascularization, the closed duodenal segment was anastomosed side-to-side to the dome of the urinary bladder for exocrine drainage. Finally, the recipient pancreas and spleen was totally removed with preservation of the duodenum and its vasculature.

Experimental groups. Twenty dogs were divided into the following 4 groups. In group 1, 5 dogs received untreated grafts and were given no immunosuppressant. In group 2, 5 dogs received grafts after ex vivo perfusion with OKIa1 and were given no immunosuppressant. Following the perfusion with 250 ml of EC, the grafts were perfused with 50 ml of EC supplemented with 300 μg of OKIa1 and incubated for 90 min at 4 °C. In group 3, 5 dogs received untreated grafts and were treated with subtherapeutic doses of CsA (Sandoz, Basel, Switzerland). CsA was started from the day of transplantation and maintained at a dose of 2.5 $mg \cdot kg^{-1} \cdot day^{-1}$ intramuscularly. This treatment resulted in CsA trough levels of 100–200 ng/ml, which are inadequate to prevent canine islet allograft rejection [1]. In group 4, 5 dogs received grafts after ex vivo perfusion with OKIa1 and were treated with the same doses of CsA as group 3. Mean cold ischemic time was 126 ± 23 min, and there was no significant difference among the 4 groups.

Immunoperoxidase technique. Biopsy specimens were obtained from the grafts before and after ex vivo perfusion and were examined to confirm whether the class-II-specific MoAb in the perfusate had combined with the class II antigens. The specimens were fixed using the AMeX method [11] and embedded in ordinary paraffin. Thin paraffin sections were deparaffinized with xylene and stained with the avidin-biotin-peroxidase technique. The sections were incubated with class-II-specific MoAb at 4 °C overnight. This was followed by incubation with a biotinylated goat antimouse antibody (Biogenex, St Ramon, Calif.) for 60 min at 37 °C and subsequently by incubation with avidin-biotin complex (Biogenex) for 60 min at 37 °C. Diaminobenzidine tetrahydrochloride (0.025% solution, Sigma, St Louis, Mo.) was used as the chromogen.

Postoperative treatment and monitoring of graft function. In addition to the standard postoperative care, the following routines were observed: Some 300 mg gabexate mesilate (courtesy of Ono, Tokyo, Japan) was given intravenously on the day of transplantation, and adequate bicarbonate was given intravenously daily for 7 days. The exocrine pancreatic function was monitored by daily measurement of urinary amylase (UA) levels, and rejection was defined as UA < 5000 U/l in two consecutive samples. Fasting blood glucose (FBG), serum amylase (SA), serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) levels were also monitored daily.

Open biopsies of the pancreas allografts in all recipients were taken on day 7 and examined by histopathology for the assessment of acute rejection.

Histopathology assessment. Biopsy specimens taken on day 7 were fixed in neutral-buffered formalin and embedded in paraffin. Multiple sections from each specimen were stained with H&E. Rejection features were defined according to the criteria of Carpenter et al. [4] as mixed neutrophilic and mononuclear cell infiltrates of pancreatic lobules, endotheliitis, thrombus, and coagulation necrosis. Each finding was graded as absent (−), mild (+), moderate (++), and severe (+++) for each specimen.

Statistical analysis. Differences between the mean values were assessed for significance by Student's unpaired *t*-test, with $P < 0.05$ considered significant.

Results

Immunostaining of the canine pancreas

In the specimens obtained before ex vivo perfusion with class-II-specific MoAb, a few cells expressing class II antigens were stained in the exocrine pancreas and within the parenchyma of the islets, as shown in Fig. 2 A. They were morphologically recognized as DCs. Class-II-specific MoAb did not react with parenchymal cells or with endothelial cells. After ex vivo perfusion with class-II-specific MoAb and incubation with biotinylated goat anti-mouse antibody, an almost similar number of cells was labeled, as shown in Fig. 2 B.

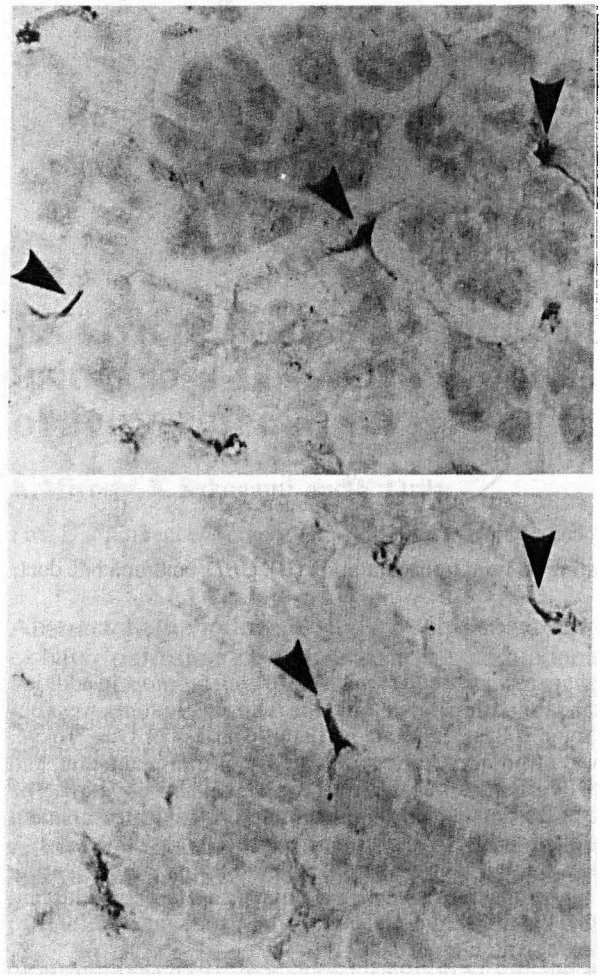

Fig. 2 A, B. Distribution of scattered class-II-positive cells showing dendritic morphology in the canine pancreas. Positive cells are present in the exocrine pancreas and within the parenchyma of the islets *(arrows)* (**A**). After ex vivo perfusion with class-II-specific monoclonal antibodies (MoAb), an almost similar number of cells was labeled *(arrows)* (**B**). Stained with the indirect immunoperoxidase technique and lightly counterstained with hematoxylin. × 800

Postoperative graft functions

Table 1 depicts the summary of graft function in the 4 groups during the 1st week after pancreaticoduodenal allotransplantation. The UA level in all successfully transplanted cases rose to over 10000 U/L immediately after allotransplantation. In group 1, mean UA levels deteriorated progressively and fell below 5000 U/L on day 6. By contrast, in group 2, mean UA levels were sustained significantly higher for 7 days posttransplant (11733 ± 4493 vs. 3247 ± 2108 U/L on day 7, $n = 5$ in each group, $P < 0.005$). This significance was also seen between CsA-treated groups. All transplanted cases were normoglycemic (FBG < 14 m*M*) on the 1st postoperative day. In group 1, hyperglycemia occurred acutely on day 6 posttransplant. By contrast, in group 2, normoglycemia was sustained for at least 7 days after transplantation (13.4 ± 5.8 vs. 23.4 ± 3.9 m*M* on day 7, $n = 5$ in each group, $P < 0.0005$). The effect of CsA treatment in recipients was not significant but was helpful in maintaining the lower FBG levels.

SA levels were usually elevated on day 1, and SGOT and SGPT levels were maximized on day 1 or 2 posttransplant. They did not vary significantly in each study group (data not shown). Our protocol for CsA administration resulted in blood CsA trough levels of 100–200 ng/ml.

Histopathological findings

At the time of open biopsy on day 7, the anastomosis of either the aortic conduit or the porta hepatis was patent, and the duodenocystostomy was intact in all recipients. Table 2 depicts the comparison of histopathological findings in each group. All untreated grafts transplanted in nonimmunosuppressed recipients showed mild to severe mixed, predominantly mononuclear, cellular infiltration which was densest around the vascular tracts in the center of the lobules. Mild to severe endotheliitis and coagulation necrosis (arterial, venous, or both) was detected in all grafts. Thrombus formation was present in large vessels near the anastomosis in 2 of the 5 grafts. After ex vivo perfusion with class-II-specific MoAb, although a few grafts showed mild mixed cellular infiltrate, endotheliitis and thrombi were absent in all grafts. CsA treatment in recipients could, in part, suppress mixed cellular infiltration.

Discussion

It is well-known that MHC class II antigen-bearing cells possess an antigen presenting cell (APC) function, and they can stimulate T lymphocytes reactive to either antigen plus MHC or foreign MHC alone [10].

Several studies indicated that DCs are potentially important components of the rejection reaction and that the presence of DCs alone within the pancreas is sufficient for the initiation of pancreas allograft rejection. The results of our immunohistochemical studies provided evidence that class-II-specific MoAb could label the surface antigens of DCs in canine pancreas (recognized morphologically). This demonstrated that class-II-specific MoAb in the perfusate could permeate into the canine pancreas tissue and combine with MHC class II antigen epitopes on DCs during the incubation period (Fig. 2).

DCs appear to be as much as 10000-fold more potent than resting B cells [10]. DCs expressing large quantities of MHC class II antigen epitopes are known to be specialized to transport various antigens to the T area via blood or lymph. Migration to the lymphoid organ is suggested to be a more efficient means for DCs to select rare antigen-specific T cells from the recirculating pool in the early sensitization phase of an immune response [14]. Such observations on the function of DCs in the initiation of pancreas allograft rejection led several authors to attempt to modulate the antigenicity of DCs by treating the pancreas to be grafted rather than the recipient. The strategies of immunomodulation using class-II-specific MoAb in rodent pancreas transplantation models has succeeded in preventing acute rejection. Murine islets treated with DC-specific MoAb and complement were shown to survive for over 200 days [6]. Normothermic perfusion of rat pancreas allografts with class-II-specific MoAb using a perfusion circuit was demonstrated to pro-

Table 1. Summary of graft function in the 4 groups during 1st week after pancreaticoduodenal allotransplantation ($n = 5$ in each group)

	Before transplantation	Day 2	Day 6	Day 7
Urinary amylase (U/L)				
Group 1	44 ± 43	21 932 ± 7040	3664 ± 3539	3274 ± 2108
Group 2	28 ± 16	36 124 ± 9164	12 586 ± 5673*	11 733 ± 4493*
Group 3	30 ± 25	25 572 ± 5444	5065 ± 2035	2044 ± 1181
Group 4	32 ± 12	23 627 ± 8411	15 451 ± 8716*	11 689 ± 3777*
Fasting blood glucose (m*M*)				
Group 1	5.7 ± 0.4	7.8 ± 4.2	21.4 ± 4.1	23.4 ± 3.9
Group 2	4.8 ± 1.8	9.4 ± 4.8	12.9 ± 7.3*	13.4 ± 5.8*
Group 3	5.9 ± 1.6	6.3 ± 3.6	24.7 ± 7.8	27.3 ± 5.2
Group 4	5.2 ± 1.8	6.0 ± 2.2	7.6 ± 2.9*	9.2 ± 4.4*

* Data points where differences are significant ($P < 0.05$) vs. group 1 (control)

Table 2. Summary of histopathological findings in the 4 groups on day 7 after pancreaticoduodenal allotransplantation

Group	Ex vivo perfusion with OKIa1	Cyclosporin A	Dog no.	Rejection findings			
				Mixed cellular infiltrate[a]	Endotheliitis	Thrombus	Coagulation necrosis
1	No	None	1	+ +	+ +	−	+ +
			2	+	+	−	+
			3	+ + +	+ + +	+	+ +
			4	+	ND[b]	ND[b]	+ +
			5	+ +	ND[b]	+ +	+ + +
2	Yes	None	1	+	−	−	−
			2	+	−	−	−
			3	+	−	−	+
			4	+	−	−	−
			5	−	−	−	−
3	No	2.5 mg·kg^{-1}·day^{-1}	1	+ +	+	+ +	+ +
			2	+	+	+	−
			3	+	−	+	−
			4	+	−	+	+
			5	−	−	−	−
4	Yes	2.5 mg·kg^{-1}·day^{-1}	1	+	−	−	−
			2	+	−	−	−
			3	−	−	−	−
			4	−	−	−	−
			5	−	−	−	−

[a] Defined as mixed neutrophilic and mononuclear cell infiltrate
[b] Not diagnostic because of severe pancreatic necrosis

long graft survival significantly [9]. The mechanism of these beneficial effects with pretreatment using class-II-specific MoAb has been suggested: when DCs combine with MoAb, they would lose their APC function in pancreas allografts, and T-cell sensitization from alloantigens could not be carried out.

It is not yet known whether or not similar manipulations to modify allograft antigenicity are applicable to larger non-inbred mammals like dogs. There are few reports indicating the efficacy of treatment using class-II-specific MoAb in canine pancreas transplantation models. Treatment of freshly isolated canine islets with Ia-specific MoAbs and complement was reported to be inadequate to prevent acute rejection in outbred beagles [2]. Simple infusion of canine whole pancreas allografts with DC-specific MoAbs prior to transplantation was reported not to prolong graft survival [15].

Our results demonstrated that in recipients whose grafts received ex vivo perfusion using class-II-specific MoAb, the UA levels were sustained above 5000 U/L, and the FBG levels were maintained within 14 m*M* for 7 days posttransplant. By contrast, the function of untreated grafts was abrogated due to acute rejection by day 6 posttransplant. On days 6 and 7, the difference in the UA and FBG levels between the treated and untreated grafts was significant (Table 1). Histopathology analysis of the grafts on day 7 agreed with the above shifts in graft function. In the grafts undergoing ex vivo perfusion using class-II-specific MoAb, endotheliitis comprising cell swelling, mononuclear cell infiltrate, and basement membrane disruption [3] was not seen, and the results suggested that the tissue blood supply through the capillaries was sufficient when the open biopsy was performed (Table 2). Since it was reported that canine resting endothelial cells lack MHC class II antigens, we do not yet have a good explanation for the suppression of endotheliitis by our manipulation. Despite the failure to achieve prolongation of canine pancreas graft survival by other authors, our manipulation could delay the onset of acute rejection for a few days. It is known that DCs turn over fairly rapidly in the tissues, with a half-life on the order of 2 or 3 days [13], and that the degree of the expression of class II antigens in allografts in-

creases dramatically after transplantation [12]. These facts explain the ability of our manipulation to inhibit the onset of acute rejection, which is limited in such a short period. Our results must have arisen because class-II-specific MoAb penetrated the pancreatic tissue sufficiently to interact with most of the DCs and its affinity to antigens was enough to reduce the antigenicity of the DCs. On the other hand, that the SA levels were not elevated suggests that the perfused class-II-specific MoAb did not induce parenchymal tissue damage in pancreas grafts. Similar changes in both SGOt and SGPT levels in the control group led to the conclusion that it also would not induce deterioration of liver function.

The relative strength of the rejection responses induced by pancreas allografts inactivated with DCs using class-II-specific MoAb is less than that induced by untreated allografts. This suggests that minimal immunosuppression at levels ineffective for untreated grafts would prevent acute rejection in DC-inactivated allografts. Successful prolongation of untreated canine islet allograft survival was demonstrated when serum CsA levels exceeded 400 ng/ml [1]. In the present study, subtherapeutic doses of CsA (blood CsA levels ranged from 100 to 200 ng/ml) could maintain the lower FBG levels and suppress, in part, mixed cellular infiltration to the grafts, but the difference was not significant.

In conclusion, we have described a new approach to reduce the antigenicity of canine pancreaticoduodenal allografts by ex vivo perfusion using class-II-specific MoAb prior to transplantation. This manipulation significantly delayed the onset of acute rejection. Additionally, subtherapeutic doses of CsA contributed to normoglycemia. Direct clinical application of this therapeutic approach is envisioned as an alternative to current nonspecific immunosuppression therapy, since no apparent side-effects in recipients were recognized during this study.

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