levels of >140 mg/dl and/or 2-h postprandial blood glucose levels of >180 mg/dl in a single week, meriting the reintroduction of exogenous insulin (6).

Assessment of Transplanted Islet Function

Islet functioning was assessed in terms of daily serum glucose levels, the amount of insulin requirement, and HbA_{1C} before and after islet transplantation. The patients were also assessed using the SUIT index (15,42) for the simple evaluation of engraftment, and the EIR index, which shows the percentage of engrafted islets per transplanted islets. Assuming normoglycemic subjects aged <40 years have normal pancreatic β-cell mass, SUIT can be accessed from the fasting blood glucose (F-BG) and fasting C-peptide immunoreactivity (F-CPR) using the formula: $250 \times$ F-CPR (nM) ÷ [F-BG (mM) − 3.43], where the SUIT index of normal subjects is 100.0×11.7 (42). For convenient daily usage the formula was simplified to: $1500 \times$ F-CPR (ng/dl) ÷ [F-BG (mg/dl) − 63]. The SUIT index is based on the correlation of fasting blood C-peptide and glucose levels in patients after islet transplantations with that of normal individuals (42). A SUIT index of 30 means that the patient receiving islet transplantation has 30% of the functional islet mass of normal subjects. Undetectable C-peptide levels (<0.1 ng/ml) were taken as 0. The EIR was assessed using the SUIT index and transplanted islet mass (islet equivalents; IEQ) for transplantation by the formula: [SUIT after transplant − SUIT before transplant (ΔSUIT)] ÷ transplanted islet mass (IEQ) $\times$ 1,000,000. Because the SUIT index shows the percentage of functional (engrafted) islet mass compared with normal subjects, the ratio of ΔSUIT/transplanted islet mass might correlate with the rate of engrafted islets; therefore, the EIR shows the rate of functional (engrafted) islets from transplanted islets.

Assuming that a normal subject has 1 million IEQ, a SUIT index of 100 also means 1 million IEQ for that person and SUIT $\times$ 10,000 shows the number of engrafted islet equivalents. Therefore, EIR also shows the percentage of engrafted islets from transplanted islets, assuming that a normal subject has 1 million IEQ.

Statistics

The values for the collected data are represented by the mean ± SE. Two groups were compared using Student's *t*-test. A multiple regression analysis was performed with the data of the variables with a value of $p < 0.05$ in the univariate analysis. Values of $p < 0.05$ were considered to be significant.

RESULTS

Human Islet Characteristics

Islet characteristics based on the islet isolation protocol are shown in Table 1. The current criteria for the approval of clinical transplantation are an islet yield of more than 5,000 IEQ/kg body weight, purity more than 30%, viability more than 70%, tissue volume less than 10 ml, endotoxin level less than 5 EU/kg body weight, and a negative gram stain based on the Edmonton protocol (39). According to these criteria, all cases met the transplant criteria except for the islet yields. Sixteen of the 19 islet preparations were used to perform islet transplantations for seven type 1 diabetic patients. One patient was excluded from this study because her creatinine clearance was less than 80 ml/min, whereas the other six recipients were over 80 ml/min. The recipient characteristics and islet infusion characteristics are shown in Table 1.

Postoperative Course of all Cases

All transplanted islets started to secrete insulin-based on C-peptide measurements and all patients demonstrated improved blood glucose control without experiencing any hypoglycemic loss of consciousness. Three patients (#1, #4, #5) became insulin independent as shown previously (20), and the other three patients reduced their insulin amount. Patient #1 had a single positive autoantibody and patients #4 and #5 had negative autoantibodies, whereas patients #2 and #3 had double-positive autoantibodies and patient #6 had triple-positive autoantibodies (autoantibodies: anti-GAD antibody, anti-insulin antibody, anti-IA-2 antibody). The HbA_{1C} levels of all six patients gradually decreased and reach less than 6% within 3 months irrespective of their receiving single or multiple islet transplantation. C-peptide in patients #2 and #3 dramatically decreased from 3 months after the first transplantation to the next transplantation and became undetectable (C-peptide <0.1 ng/ml) before the next transplantation, as reported previously (20).

SUIT Index

To evaluate islet engraftment simply, the SUIT index was used (15,42). Table 2 shows the average SUIT index from day 7 to 3 months after transplantation or the next transplantation. The SUIT index of all patients before transplantation was 0, but increased in all patients after transplantation. The increase of the SUIT index in patients who received cultured islets was significantly lower than in patients who received fresh islets (fresh: 12.1 ± 1.7, $n = 11$; cultured: 3.8 ± 2.1, $n = 3$) (Table 4). The islet equivalents decreased by about 30% after overnight culture (before culture: $665{,}378 \pm 101{,}075$ IEQ; after culture: $481{,}469 \pm 76{,}708$ IEQ, $n = 3$). These data suggest that noncultured islets are more effective than cultured islets for islet transplantation from NHBDs. With the transplantation of fresh islets, the increase of the SUIT index in patients with double autoantibodies was significantly lower than in patients with a single or

Table 1. Recipient Characteristics and Islet Infusion Characteristics

	Patient No.					
	1	2	3	4	5	6
Age (years), male/female	36F	39M	39F	47F	58M	36F
Body weight (kg)	60	71	58	51	72	51
BMI	20.3	21.9	21.2	20.2	22.0	20.1
Autoantibodies*	+	++	++	−	−	+++
Daily insulin (U/kg)	0.67	0.70	0.67	0.88	0.49	0.58
Islet transplantation						
IEQ of first Tx	354,384	406,082	621,551	465,598	479,656	262,319
IEQ of second Tx	474,234	380,726	498,928	357,259	869,826	—
IEQ of third Tx	—	393,588	—	447,931	297,720	—
Total IEQ	828,618	1,180,396	1,120,479	1,270,788	1,647,202	261,329
IEQ/kg	13,810	16,626	19,319	24,917	23,832	5,103
Daily insulin (U/kg)	0	0.28	0.38	0	0	0.39

−: negative, +: single positive, ++: double positive, +++: triple positive.
*Autoantibodies: anti-GAD antibody, anti-insulin antibody, anti-IA-2 antibody.

no autoantibody (no or single autoantibody: 15.2 ± 2.4, $n = 6$; double or triple autoantibodies: 8.4 ± 0.8, $n = 5$) (Table 4). The increase of the SUIT index in patients with 5 or 6 (full) mismatched HLA was significantly lower than in patients with 2–4 mismatched HLA (mismatch 2–5: 15.4 ± 2.3, $n = 6$; mismatch 5 or 6: 8.2 ± 0.7, $n = 5$) (Tables 4 and 5). Multivariate analysis including the variables of culture, autoantibody, and HLA mismatch identified only culture as significantly contributing to the variations in the SUIT index ($p = 0.03$).

Patients #1, #4, and #5 became insulin independent and the mean of their final SUIT was 33.8 ± 3.0. The other patients had positive C-peptide with reduced insulin 1 month after each transplantation but did not achieve insulin independence and their mean SUIT was 8.4 ± 2.3, significantly lower than the independent group ($p < 0.01$). Data from patient #4 showed the lowest SUIT value among insulin-independent patients, 28 of SUIT index, thus suggesting that 28% β-cell mass is required to become insulin independent.

EIR Index

The EIR index was used to evaluate the rate of islet engraftment. EIR shows the percentage of engrafted islets per transplanted islets, assuming that a normal subject has 1 million IEQ. As shown in Table 3, the percentage of engrafted islets for one transplantation is about 30%. The EIR index in patients who received cultured islets was significantly lower than in patients who received fresh islets (fresh: 28.9 ± 4.3, $n = 11$; cultured: 9.7 ± 6.3, $n = 3$) (Table 4). Because IEQ decreased after overnight culture by about 30% and the EIR index in patients who received cultured islets was significantly lower, cultured islets were less effective than fresh islets in islet transplantation from NHBDs. With the transplantation of fresh islets, the EIR index in patients with dou-

Table 2. Increase of SUIT Index in Each Islet Transplantation

	Patient No.					
	1	2	3	4	5	6
SUIT index before Tx	0	0	0	0	0	0
ΔSUIT after first Tx	16.4	8.2*	1.3*†	2.2†	18.4	8.9
ΔSUIT after second Tx	22.1	9.0*	5.6	8.0†	7.9	—
ΔSUIT after third Tx	—	10.1	—	18.0	8.4	—
SUIT index after final Tx	38.5	10.1	5.6	28.2	34.7	8.9

ΔSUIT: increase of SUIT index, Tx: Transplantation.
*SUIT index decreased and become 0 before additional transplantation.
†Cultured islets.

Table 3. EIR Index in Each Islet Transplantation

	Patient No.					
	1	2	3	4	5	6
First transplant	46.3	20.2	2.1*	4.7*	38.3	34.0
Second transplant	46.6	23.6	11.2	22.4*	9.1	—
Third transplant	—	25.6	—	40.2	28.2	—

*Cultured islets.

ble or triple autoantibodies was lower, but not significantly, than in patients with a single or no autoantibody (no or single autoantibody: 34.8 ± 5.9, $n = 6$; double autoantibodies: 22.9 ± 3.7, $n = 5$) (Table 4). The EIR in patients with 5 or 6 (full) mismatched HLA was significantly lower than in patients with 2–4 mismatched HLA (mismatched 2–5: 38.9 ± 2.9, $n = 6$; mismatched 5 or 6: 18.0 ± 3.3, $n = 5$ (Tables 4 and 5). Multivariate analysis including the variables of culture, autoantibody, and HLA mismatch identified only culture as barely significantly contributing to the variation of the EIR index ($p = 0.05$). There was no significance in other factors such as WIT, CIT, donor age, amylase level (Table 4), AST/ALT level, BUN/Cr level, or BMI (data not shown).

In summary, a statistically significant contribution of SUIT and EIR indexes in multivariate analysis was found in culture. However, the number of subjects evaluated was not sufficient to conclude that fresh islets are better than cultured islets.

DISCUSSION

Allogenic islet transplantation was effectively performed from NHBDs using the Kyoto Islet Isolation Method for the treatment of type 1 diabetes. Fasting blood glucose and C-peptide levels was examined in seven posttransplantation stages of four patients and found a linear relationship between F-BG and F-CPR in each stage (15,42). The Miami group also showed that the C-peptide to glucose ratio was constant from 1 week to 30 days and the ratio after the second islet transplantation doubled compared to the first islet transplantation (5). The SUIT index is correlated with the acute insulin response to glucagon after islet transplantation (42). The Edmonton group showed that the functional capacity of transplanted islets in insulin-independent patients was estimated to correspond to only about 20–40% of that in nondiabetic subjects (34,35), which corresponded with the SUIT index of our insulin-independent patients (SUIT index 28.2–38.5). Also, in general, diabetes after

Table 4. Evaluation of Islet Transplantation From NHBDs Using SUIT and EIR Indexes

	Increase of SUIT Index	*p*-Value	EIR Index	*p*-Value
Culture				
(−) ($n = 11$)	12.1 ± 1.7		28.9 ± 4.3	
(+) ($n = 3$)	3.8 ± 2.1	$p = 0.03$	9.7 ± 6.3	$p = 0.03$
Fresh islet transplantation				
HLA				
Mismatch <5 ($n = 6$)	15.4 ± 2.3		38.9 ± 2.9	
Mismatch ≥5 ($n = 5$)	8.2 ± 0.7	$p = 0.02$	18.0 ± 3.3	$p = 0.001$
Autoantibody				
(−) or (+) ($n = 6$)	15.2 ± 2.4		34.8 ± 5.9	
(++) or (+++) ($n = 5$)	8.4 ± 0.8	$p = 0.03$	22.9 ± 3.7	$p = 0.14$
WIT				
≥5 min ($n = 5$)	12.2 ± 2.2		29.5 ± 6.3	
<5 min ($n = 6$)	12.0 ± 2.7	$p = 0.94$	29.3 ± 5.4	$p = 0.98$
CIT				
≥240 min ($n = 6$)	9.5 ± 1.5		24.4 ± 5.8	
<240 min ($n = 5$)	15.2 ± 2.7	$p = 0.09$	35.4 ± 4.2	$p = 0.17$
Donor age				
≥50 years ($n = 7$)	12.2 ± 2.2		27.8 ± 6.0	
≤50 years ($n = 4$)	11.9 ± 3.4	$p = 0.93$	31.7 ± 7.8	$p = 0.70$
Amylase				
≥150 ($n = 5$)	11.1 ± 2.8		25.5 ± 6.1	
<150 ($n = 6$)	12.9 ± 2.2	$p = 0.62$	32.6 ± 5.1	$p = 0.40$

Table 5. HLA Typings of the Patients and Islet Donors

	HLA			
	A	B	DR	Mismatch
Patient #1	**2, 11**	**34, 54**	**4, -**	
Donor 1	1, 24	37, **54**	**4**, 10	4
Donor 2	**2**, -	51, 61	**4**, 9	3
Patient #2	**2, 24**	**7, 54**	**1, 4**	
Donor 1	11, 26	61, 67	9, 16	6
Donor 2	26, 31	51, 61	8, 9	6
Donor 3	33, -	44, -	13, -	6
Patient #3	**2, 33**	**44, 61**	**9, 13**	
Donor 1*	26, -	54, 62	8, **9**	5
Donor 1'*	**2**, 24	48, 49	8, **9**	4
Donor 2	**2**, 26	35, 51	1, 15	5
Patient #4	**2, 33**	**44, 54**	**4, 13**	
Donor 1*	**24, -**	**52, 54**	14, 15	5
Donor 2*	**2**, 24	52, -	5, -	5
Donor 3	11, **33**	**44, 54**	9, **13**	2
Patient #5	**11, 33**	**44, 54**	**4, 11**	
Donor 1	2, 31	48, 55	**4, 11**	4
Donor 2	24, 26	51, 61	**4**, 9	5
Donor 3	24, **33**	**44**, 60	**11**, 13	3
Patient #6	**26, -**	**61, -**	**9, -**	
Donor 1	**26**, 31	51, **61**	4, 9	3

*Cultured islets.

a pancreatectomy usually occurs when the remnant pancreas is less than 30%, which corresponds with the lowest SUIT value among insulin-independent patients, 28, which might indicate that 28% β-cell mass is required to become insulin independent. Therefore, the SUIT index could predict the efficacy of islet transplantation.

The EIR shows the ratio of ΔSUIT/transplanted islet mass × 1,000,000, which might correlate with the rate of engrafted islets from transplanted islets. It was shown that a normal person has about 0.5–1 million IEQ (9,37). Assuming that a normal subject has 1 million IEQ, a SUIT index of 100 also means 1 million IEQ for that person and SUIT × 10,000 shows the number of engrafted IEQ. Therefore, the EIR also shows the percentage of engrafted islets per transplanted islets, assuming that a normal subject has 1 million IEQ. These data show that the percentage of engrafted islets from one transplantation is about 30%. In experimental islet transplantation, it is generally accepted that 50–70% of transplanted islets will be lost in the immediate posttransplantation period (3,4), although these experiments were based on multi-islet grafts implanted beneath the kidney capsule of immunocompromised recipients. These data also show that only a small fraction of transplanted islets are successfully engrafted.

It is known that the results of human islet isolation depend on the quality of the donor pancreata (10,16), so it is not easy to isolate suitable human islets successfully from NHBDs. This study suggests that it should be possible to cure type 1 diabetes by means of islet transplantation using the current islet isolation method with NHBDs. However, patients #2 and #3, who received over 15,000 IEQ/kg patient body weight of islets, did not become insulin independent and patients #4 and #5 needed over 20,000 IEQ/kg of islets to become insulin independent, although patients who received over 10,000 IEQ/kg of islets from brain-dead donors usually became insulin independent (39). This may be due to the quality of islets. Indeed, the SUIT index in a patient who received living-donor islet transplantation was 38.5 and the EIR was over 90, while the average SUIT and EIR in islet transplantation from NHBDs were about 10 and less than 30, respectively. Therefore, the islet quality strongly affects the engrafted islet rate.

SUIT indexes in patients #2 and #3 from 3 months after the first transplantation to the next transplantation dramatically decreased and the indexes became 0 (C-peptide <0.1 ng/ml) before the next transplantation, although their EIR within 3 months after the first transplantation was not significantly different, in comparison to the EIR of patients #1, #4, and #5. This may be due to autoimmunity and/or alloimmunity, and glucotoxicity. The interval between transplantations in patients #2 and #3 was relatively long (20); therefore, these patients may have suffered autoimmunity and/or alloimmunity and glucotoxicity over a long period. The SUIT index could also be used to evaluate long-term graft survival.

Recently, it has been shown that fresh islets are more effective than cultured islets at reversing hyperglycemia in a mouse model (8,31). Moreover, the Edmonton group showed that the clinical outcome of cultured islet transplantation is worse than that of fresh (noncultured) islet transplantation (presented at IPITA meeting, 2005). The present data show that increases in the SUIT and EIR indexes in patients who received fresh islets are significantly higher than in patients who received cultured islets, although the number of cultured islet transplants performed was low. Although some groups have shown the effectiveness of using cultured islets (6,7), the effect seems due to the pretreatment of patients and improvement of islet purity (and therefore decreased tissue volume) rather than improved islet quality. In addition, some groups have transplanted cultured islets from brain-dead donors but the present study transplanted islets from NHBDs, which received greater stress than those from brain-dead donors. Based on these data, islets without culture have been used for transplantation. However, the number of subjects evaluated was insufficient to

conclude that fresh islets are better than cultured islets and this study yielded no data concerning cultured islets from brain-dead donors.

The SUIT index resembles that in the computer-solved model of pancreatic β-cell function, HOMA-β, which is 20 × insulin (mU/L)/(F-BG − 3.5) (17). However, HOMA-β cannot be used to assess βcell function in those taking exogenous insulin (41), due to the inability of insulin assays to differentiate endogenous insulin from exogenous insulin. The SUIT index is independent of the amount of exogenous insulin. Therefore, it is possible to determine the β-cell function after the injection of long-acting insulin.

In conclusion, this method makes it feasible to use tissue harvested from NHBDs efficiently for islet transplant into type 1 diabetic patients. The SUIT and EIR indexes are useful to evaluate single islet transplantation because two or three islet transplantations are required to achieve insulin independence.

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Analysis of Donor- and Isolation-Related Variables From Non-Heart-Beating Donors (NHBDs) Using the Kyoto Islet Isolation Method

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Recently, we demonstrated that islet transplantation from non-heart-beating donors (NHBDs) using the Kyoto islet isolation method (KIIM) successfully reversed patients' diabetes state. In this study, we evaluated the effects of donor- and isolation-related variables on islet isolation results from NHBDs by KIIM. Twenty-one islet preparations from the pancreata of NHBDs were isolated by KIIM. Islet preparations that met transplantation criteria and achieved improved patient diabetes control after transplantation were defined as successful isolations. Potential risk factors deemed to affect islet isolation results, such as age, gender, body mass index, hospital stay, donors' blood biochemical tests, a modified pancreata procurement method, and isolation and purification procedure-related variables, were analyzed. Seventeen out of 21 islet isolations (81%) were successful isolations. Postpurification islet yield was 447,639 ± 39,902 islet equivalents (IE) in the successful isolation group and 108,007 ± 31,532 IE in the failure group. Donor age was significantly younger in the success group (41.9 ± 4.0 years old in the success group vs. 57.5 ± 2.2 years old in the failure group, $p = 0.003$). Chronic pancreatitis significantly decreased islet yields ($p = 0.006$). Phase I time was significantly shorter ($p = 0.010$) and undigested tissue volume was significantly smaller ($p = 0.020$) in the success group. Purity was in positive correlation to postpurification islet yield, while donor age was in reverse correlation to postpurification islet yield. KIIM enables us to perform islet transplantation from NHBDs; however, the decision to use pancreata from older donors or those with chronic pancreatitis requires careful consideration.

Key words: Non-heart-beating donor; Kyoto islet isolation method; Pancreatic islet transplantation; Type 1 diabetes

INTRODUCTION

After the Edmonton group showed that transplantation of human islets of Langerhans could be a novel treatment for patients with type 1 diabetes mellitus (22), the problem of donor shortage became prominent in islet transplantation. A non-heart-beating donor (NHBD) pool might be a beneficial supplement to alleviate donor shortage in the islet transplantation field (5); however, few islet teams prefer manipulating such a pancreas because of its notorious difficulty in islet isolation and purification (9). In Japan, pancreatic islet transplantation is categorized as tissue transplantation and heart-beating brain-dead donors are not allowed. This leaves the potential donor pool to come from only non-heart-beating donors (NHBDs) (10,11,14) and living donors (12,15). Previously, we initiated the Kyoto Islet Isolation Method (KIIM), which was modified for NHBDs (11,19) and manipulated 21 islet preparations from the pancreata of NHBDs. It was encouraging that 17 out of 21 (81%) islet preparations from NHBDs met the transplantation criteria, and were transplanted into eight patients. All patients achieved improved blood glucose levels after transplantation with no hypoglycemia episodes, reduced

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insulin requirement, and three instances of insulin independence (11).

We were curious to identify the risk factors from NHBDs using the KIIM in order to gain a higher success rate. In this study, we review our experience of islet isolation and purification with KIIM from NHBDs, and analyze possible donor- and procedure-related variables affecting islet yield.

MATERIALS AND METHODS

Donor Background

Twenty-one pancreata from NHBDs were procured through the Japan Organ Transplantation Network between April 7, 2004 and January 21, 2006. The study was approved by the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine. The average donor age was 44.9 ± 3.5 years old, body weight was 59.4 ± 2.7 kg, body mass index (BMI, weight in kg/height in m^2) was 21.9 ± 0.7 kg/m^2, and pancreas weight was 87.9 ± 5.8 g. Average warm ischemic time (WIT, time from donor death to chilling solution perfusion) was 7.9 ± 2.2 min and cold ischemic time (CIT, time from chilling solution perfusion to initiation of isolation) was 253.1 ± 16.9 min.

Pancreata Procurement, Islet Isolation, and Purification

All 21 islet preparations were manipulated according to GMP (Good Manufacturing Practice) at the Center for Cell and Molecular Therapy of Kyoto University Hospital. Islet isolation was performed according to KIIM, which is based on the Ricordi method with some modifications (11). The key procedures are described as follows. We procure pancreata in conjunction with kidneys taken by the kidney procurement team. This is done by inserting double-balloon catheters into the iliac vessels before cardiac arrest and after confirmation of brain death and informed consent. We start regional organ chilling after cardiac arrest using an in situ regional organ cooling (ISRC) system (17). The pancreas is protected during kidney procurement and is removed after the kidney. We remove the duodenum and spleen from the pancreas at the procurement site. A cannula is immediately inserted into the procured pancreas through the main pancreatic duct from the direction of the pancreatic head and M-Kyoto solution is administered intraductally (11,18). M-Kyoto solution is 100,000 U/L of Ulinastatin (Mochida Pharmaceutical Co Ltd, Tokyo, Japan) in ET-Kyoto solution (Kyoto Biomedical Science, Kyoto, Japan). For pancreas preservation during transportation, we use an oxygenated perfluorocarbon/M-Kyoto solution-based two-layer method (11,18) instead of UW solution. We check the density of the isolation aggregates with a test tube density adjustment method as follows. Six density test tubes were prepared with 5 ml of purification solution of different densities (1.085, 1.090, 1.095, 1.100, 1.105, and 1.110 g/cm^3) and covered by 1 ml cap solution (dithizone, Sigma Chemical Co., St. Louis, MO) (0.5 mg/ml, in Hanks' balanced salt solution). The different densities were achieved by adjusting the ratio of iodixanol (OptiPrep, Axis-Shield PoC AS, Norway) to M-Kyoto solution. Samples were taken from the isolation aggregates and added to the discontinuous density test tubes. The tubes were spun at 1000 rpm for 5 min and the densities of aggregates were determined by whether they pelleted or floated in the different density solutions. Islets were purified with a continuous density gradient of iodixanol/M-Kyoto solution (Ulinastatin 50,000 U/L in ET-Kyoto solution) in an apheresis system (COBE 2991 cell processor, Gambro Laboratories, Denver, CO) (11). The heavy density solution was chosen according to the test tube density adjustment method described above and the gradient was achieved by varying the ratio of iodixanol to M-Kyoto solution.

Islet Evaluation

Islet evaluation was independently judged by two investigators. Islet yield was determined with dithizone staining (2 mg/ml, Sigma Chemical Co.) under optical graticule and converted into a standard number of islet equivalents (IE, diameter standardizing to 150 μm) (13,21). Purity was assessed by comparing the relative quantity of dithizone-stained tissue to unstained exocrine tissue. Mantled islets were defined as more than 50% of the islet surface being covered by exocrine tissue (13). Islet viability was evaluated using acridine orange (10 μmol/L) and propidium iodide (15 μmol/L) (AO/PI) staining to visualize living and dead cells simultaneously (13,21). Islet function was evaluated by the insulin secretory response of the purified islets during a glucose stimulation test (13,21).

Grouping

Islet preparations that met transplantation criteria (11,22) and improved patient diabetes control after transplantation were included in the success group, and those that did not, in the failure group. Features of improved patients' diabetes include stable blood glucose control without hypoglycemic unawareness, improved hemoglobin A_{1C} (HbA_{1C}) levels with positive C-peptide, and reduced exogenous insulin requirement.

Donor- and isolation-related factors were analyzed, including: age, gender, BMI, pancreatic status (healthy vs. chronic pancreatitis), donors' hospital stays, donors' blood biochemistry tests [peak transaminase levels, peak creatinine levels, peak amylase, peak blood urea nitrogen (BUN) levels, and peak blood glucose levels], car-

diac arrest or norepinephrine administration, ISRC system, perfusion time and volume, WIT, CIT, phase I time (time from placement in the Ricordi chamber to the start of collection), phase II time (time of collection), undigested tissue volume, and prepurification islet yield (IE, and IE/g).

Statistical Analysis

Differences between means were compared with the independent *t*-test. Differences between ratios were analyzed with Fisher's exact test. Logistic regression analysis was performed to determine factors affecting islet yield. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 10.0, SPSS, Chicago, IL). Data were expressed as the average ± SEM and $p < 0.05$ was considered significant.

RESULTS

Overall Isolation Results

In all cases after islet transplantation, HbA_{1C} levels were improved (Fig. 1) and there was no hypoglycemic unawareness. Therefore, all preparations that met transplantation criteria were included in the success group. Thus, 17 out of 21 islet preparations achieved success (a success rate of 81%). Average islet yield (from both the success and failure groups) before purification was 508,622 ± 59,473 IE (6,010 ± 665 IE/g), and after purification was 382,945 ± 44,146 IE (4,589 ± 504 IE/g). Purity after purification was 46.8 ± 3.3%. Viability assessed by AO/PI after purification was 96.2 ± 0.7%, and all samples were above 85%. The average percentage of mantled islets was 30.9 ± 5.2%. The average insulin stimulation index was 4.2 ± 1.8.

Donor Characteristics

Donor characteristics were analyzed based on success and failure groups (Table 1). The average postpurification islet yield was 447,639 ± 39,902 IE in the success group and 108,007 ± 31,532 IE in the failure group. There were no significant differences between the two groups in gender, BMI, hospital stay, peak creatinine level, peak BUN level, peak blood glucose level, peak amylase level, peak transaminase level, or norepinephrine administration.

Significant differences were found in donors' ages and pancreata status between the two groups. The donor age in the success group was 41.9 ± 4.0 years old and in the failure group 57.5 ± 2.2 years old ($p = 0.003$). All of the 21 pancreata underwent histological examination with hematoxylin and eosin staining (H&E stain). Pancreata with diffuse fibrosis were defined as chronic pancreatitis ($n = 7$) and those without evidence of inflammation were defined as healthy pancreata ($n = 14$). In the success group, 3 out of 17 pancreata had chronic pancreatitis (CP); while in the failure group, all four cases had CP ($p = 0.006$) (Table 1).

Isolation Characteristics

Isolation characteristics were also analyzed based on success and failure groups (Table 2). The success group had a significantly shorter phase I time ($p = 0.010$) and significantly smaller undigested tissue volume ($p = 0.020$). Although there was no significant difference, the suc-

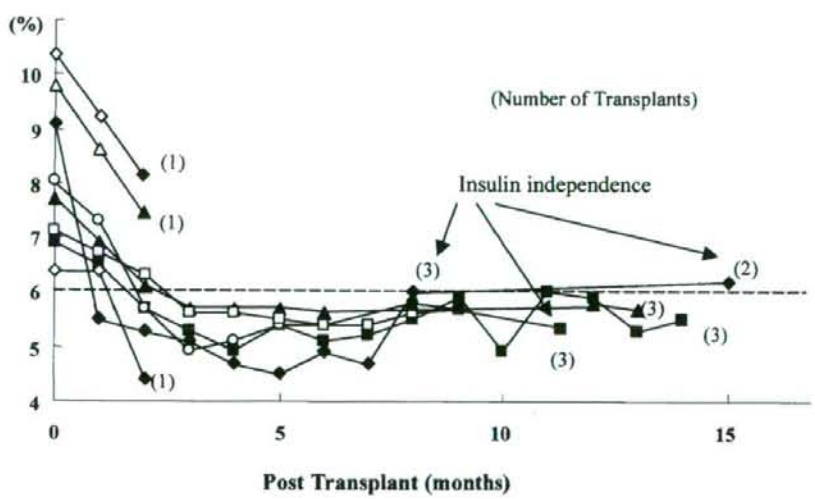

Figure 1. Eight patients' HbA_{1C} levels before and after transplantation of islets from NHBDs.

Table 1. Donor Characteristics

Variables	Success Group ($n = 17$)	Failure Group ($n = 4$)	p-Value
Sex (male/female)	12/5	3/1	0.684
Age (years)	41.9 ± 4.0	57.5 ± 2.2	0.003*
Body mass index (kg/m^2)	21.9 ± 0.8	21.5 ± 2.2	0.845
Pancreas weight (g)	84.1 ± 5.5	104.0 ± 19.5	0.183
Hospital stay (days)	10.2 ± 8.9	5.5 ± 1.6	0.323
Peak transaminase (U/L)	248.2 ± 105.7	56.5 ± 11.6	0.399
Peak creatinine (mg/dl)	3.2 ± 0.6	4.5 ± 2.0	0.372
Peak amylase (IU/L)	343.1 ± 118.9	54.0 ± 28.0	0.331
Peak blood urea nitrogen (mg/dl)	57.9 ± 12.8	52.3 ± 10.6	0.840
Norepinephrine administration or cardiac arrest (No./total)	13/17	2/4	0.081
In situ regional organ cooling (No./total)	16/17	2/3	0.284
Pancreata status (healthy/chronic pancreatitis)	14/3	0/4	0.006*

The two groups were statistically compared using the independent t-test and Fisher's exact test.
*$p < 0.05$ was considered significant.

cess group had about a twofold higher percentage of mantled islets. This was due to our modified purification method using the density adjustment method before purification, which enhanced the recovery of mantled islets in all cases. Obviously, the prepurification islet yield was the most striking factor for success.

Effect of Pancreata Status

Because CP adversely affected the isolation success rate (Table 1), we further analyzed the effect of pancreata status on islet isolation (Table 3).

The donor age was significantly older in the CP group ($p = 0.002$), suggesting that aging is correlated with CP onset. The phase I time was significantly longer ($p = 0.001$) and the undigested tissue volume was significantly larger ($p = 0.016$) in the CP group, which suggests that pancreatitis inhibits pancreas digestion and leads to low islet yield and low purity.

Relationship Between Postpurification Islet Yields and Other Factors

Purity was positively correlated with postpurification islet yield ($R = 0.619$, $p = 0.003$) (Fig. 2A). Donor age was inversely correlated with the postpurification yield ($R = -0.616$, $p = 0.003$) (Fig. 2B). The insulin stimulation index and islet viability showed no correlation with postpurification islet yield (IE/g) ($R = 0.014$, $p = 0.953$ and $R = 0.318$, $p = 0.161$, respectively) (Fig. 2C, D).

DISCUSSION

Previous studies reported several factors that had significant impact on islet isolation using brain-dead heart-

Table 2. Isolation Characteristics

Variables	Success Group ($n = 17$)	Failure Group ($n = 4$)	p-Value
Warm ischemic time (min)	6.4 ± 2.0	14.3 ± 7.6	0.158
Perfusion time (min)	67.1 ± 16.2	84.3 ± 26.7	0.587
Perfusion volume (L)	24.2 ± 6.1	27.3 ± 6.0	0.725
Cold ischemic time (min)	241.1 ± 16.0	304.25 ± 56.1	0.147
Phase I time (min)	19.2 ± 1.3	27.8 ± 2.2	0.010*
Phase II time (min)	53.7 ± 3.0	55.5 ± 8.5	0.810
Undigested tissue volume (g)	11.2 ± 1.1	18.3 ± 3.8	0.020*
Percentage of mantled islets (%)	33.9 ± 6.2	18.5 ± 3.8	0.258
Prepurification islet yield (IE)	590,364 ± 55,238	154,466 ± 51,807	0.002*
Prepurification islet yield (IE/g)	7,099 ± 540	1,382 ± 204	<0.001*

The two groups were statistically compared using the independent t-test.
*$p < 0.05$ was considered significant.

Table 3. Effect of Pancreas State on Islet Yield

Variable	Healthy Pancreata ($n = 14$)	Chronic Pancreatitis ($n = 7$)	p-Value
Age (years)	38.9 ± 4.4	56.7 ± 1.7	0.002*
Phase I time (min)	17.9 ± 1.3	26.7 ± 1.8	0.001*
Phase II time (min)	52.4 ± 3.4	57.3 ± 5.1	0.431
Undigested tissue volume (g)	10.6 ± 1.2	16.6 ± 2.3	0.016*
Prepurification islet yield (IE/g)	7,375 ± 597	3,282 ± 1,007	0.001*
Postpurification islet yield (IE/g)	5,470 ± 458	2,825 ± 922	0.009*
Purity (%)	53.6 ± 3.0	33.3 ± 4.7	0.001*

The two groups were statistically compared using the independent t-test.
*$p < 0.05$ was considered significant.

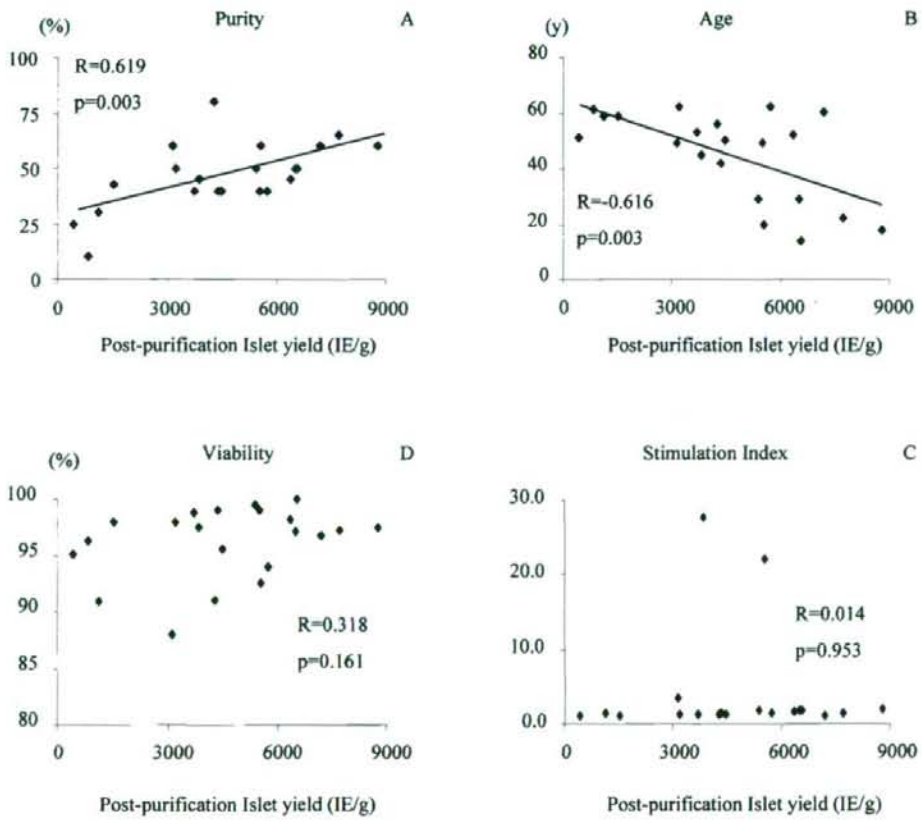

Figure 2. Relationships between postpurification islet yield (IE/g) and purity (A), age (B), insulin stimulation index (C), and viability (D). Postpurification islet yield (IE/g) was strongly correlated with purity (A). Postpurification islet yield (IE/g) was inversely correlated with donor age (B). Islet viability and insulin stimulation index showed no correlation with postpurification islet yield (IE/g) (C, D).

beating donors (2,4,8,16,25); however, this is the first time that factors relating to the use of NHBDs for actual clinical islet transplantation have been reported. Although our data set is small, the results are interesting and should be helpful for assessing pancreata from NHBDs before islet processing.

In terms of donor factors, although overweight older donors have been considered more suitable for islet isolation than younger donors (4,8), our study highlighted the benefit of using pancreata from young donors for islet preparations. In our study, the donor age in the success group was quite young. Additionally, donor age showed an inverse correlation with postpurification islet yield. The main obstacle to gaining high postpurification islet yields from young donors lies in the higher percentage of mantled islets, which are embedded in acinar tissue (20). Due to anatomical and physiological differences of the pancreata from young donors, it is difficult for islets to be separated from acinar tissue without overdigestion and islet fragmentation (8). To avoid overdigestion, mantled islets were accepted and collected after isolation. However, because they were surrounded by exocrine tissue, the density of mantled islets differed from that of isolated islets and resulted in difficulty in extraction from the exocrine tissues during purification. In the case of mantled islets from young donors, the standard islet isolation method with fixed purification density can achieve a large quantity of IE before purification but cannot recover mantled islets after purification.

Socci et al. (23) discovered that the ducts in young pancreata collapse, which hampers the enzyme solution from thoroughly infusing and uniformly distributing throughout the parenchyma, leading to mantled islets. In KIIM, we immediately insert a cannula into the main pancreatic duct on the duodenal terminus after procurement and ductally administer M-Kyoto solution (11,18). Two key features of this approach are: 1) Ulinastatin, a trypsin inhibitor in M-Kyoto solution, helps protect the main duct wall from trypsin injury, and 2) we fill the pancreatic duct system with M-Kyoto solution to maintain the three-dimensional duct system and prevent its collapse. Also, M-Kyoto solution shows a lower Liberase inhibition than UW solution (18), which contributes to sufficient digestion of the pancreas and decreases the amount of mantled islets. Efforts were taken to improve the infusion efficiency of the enzyme and thus increase the level of whole pancreas digestion (to lower the number of mantled islets). Even with this effort, we still collected some mantled islets after isolation. In order to recover this fraction of islets, we individualized the density of the high-density purification solution for each islet preparation before purification based on the results of the test tube density adjustment method. By using an optimal density gradient range, we successfully collected mantled islets. In fact, in our success group, more than 30% of all mantled islets might have been lost if we had not utilized this technique. Although exocrine tissue may hinder the ability of nutrition to diffuse to the islets (6), the detachment of islets from exocrine tissue triggered an apoptotic signal and resulted in islet death (24). Investigators have confirmed the advantages of islets from young donors, both in vitro in terms of insulin secretory function (7) and in vivo after transplanting to diabetic mice (1). We believe that if we can only recover mantled islets from young donors, the islet preparations should be of high quality both in function and in islet number.

The pancreatic status of donors, especially those with CP, could severely affect the isolation results. To analyze this, we further investigated the effect of the pancreata status on islet isolation and transplantation (Table 3). In our study population, CP is defined as diffuse fibrosis after H&E histological staining. Phase I time (digestion time) was significantly longer and undigested tissue volume was significantly larger in the CP group. These were due to the difficulty in digesting a pancreas with CP and led to failed islet isolation. In addition, purity was significantly lower in the CP group, suggesting that, even by sacrificing purity, it was still hard to obtain a high islet yield from pancreata with CP. Because donor age was significantly older in the CP group (Table 3) and older pancreata are more prone to CP, this is another reason to seek younger donors. Based on our data, older NHBDs with chronic pancreatitis are an apparent risk factor for islet isolation. A multicenter, large-scale, randomized trial will be necessary to verify this. Importantly, all cases within the healthy pancreata group (14/14) resulted in successful islet isolation and transplantation. This was especially important for confirming that KIIM was a reliable islet isolation method with normal pancreata. It allowed us to perform living donor islet transplantation successfully with KIIM (12). A reliable islet isolation method is of crucial importance when performing living donor islet transplantation.

Other donor-related factors such as BMI, pancreas weight, length of hospital stay, peak transaminase level, peak creatinine level, peak amylase level, peak BUN level, norepinephrine administration, or cardiac arrest showed no significant effects on islet preparation results. This might be due to our modified procedures in KIIM, which could overcome these obstacles. Another possible reason is the limited size of our study population. For instance, BMI is a decisive indicator of a subject's nutritional status and lean donors (with BMI <24 kg/m^2) had significantly lower islet yields (3,8). However, in our study population, most of the donors were of low BMI (range 14.8–27.2 kg/m^2). It is hard to tell based on our

data whether BMI affects success, but our study justified the utilization of donors with lower BMI for clinical islet transplantation.

In terms of isolation-related factors, isolation characteristics revealed that the phase I time was significantly shorter and the undigested tissue volume was significantly smaller in the success group, suggesting that quick and complete digestion of the donor pancreas was important for the success of islet isolation with NHBDs. Both WIT and CIT were shorter in the success group; however, there were no significant differences because we minimized WIT and CIT in all cases.

Finally, we analyzed the relationship between islet yield and other factors. Unexpectedly, purity was positively correlated with islet yield. We obtained maximum islet yield, including mantled islets, using the density adjusted purification method. This technique enhanced islet yield but it sacrificed purity. Perfect islet isolation should result in high islet yield with a minimum of mantled islets, which is usually shown as high islet yield with high purity. This might be the reason that purity was still positively correlated with islet yields even when attempting to sacrifice some purity to attain higher islet yields.

To summarize, KIIM enables us to perform islet transplantation using NHBDs with a high success rate; however, the use of older donors and those with chronic pancreatitis requires prudent judgment.

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Prominent Lectin-Like Oxidized Low Density Lipoprotein (LDL) Receptor-1 (LOX-1) Expression in Atherosclerotic Lesions Is Associated with Tissue Factor Expression and Apoptosis in Hypercholesterolemic Rabbits

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Background: Despite increasing *in vitro* evidence that lectin-like oxidized low density lipoprotein (LDL) receptor-1 (LOX-1), a cell-surface receptor for oxidized LDL, is implicated in the atherogenesis and thrombus formation, its *in vivo* participation to the atherosclerotic plaque destabilization, rupture and thrombus formation remains unclear. Here, we compared the *in vivo* expression of LOX-1, with tissue factor (TF) expression and cell apoptosis, in atherosclerotic lesions of myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits. Methods and Results: We prepared sixty series of cross sections in the aortic arch and the thoracic aorta from four WHHLMI rabbits. LOX-1 and TF expression, as well as apoptotic events were determined by immunohistochemical staining and TUNEL methods, respectively. LOX-1 expression was mainly observed in the macrophage-rich lipid areas of vulnerable plaque-like atheromatous lesions where TF expression and apoptotic events were prominent. LOX-1 expression was positively correlated with TF expression ($r=0.53$, $p<0.0001$), apoptotic events ($r=0.52$, $p<0.0001$) and morphological vulnerability ($r=0.63$, $p<0.0001$). Conclusions: LOX-1 expression appears to be closely associated with TF expression, apoptotic events and the morphological vulnerability, suggesting the *in vivo* involvement of LOX-1 in the destabilization and rupture of atherosclerotic lesions and the subsequent thrombus formation. The present findings in hypercholesterolemic rabbits should help advance our understanding of the pathophysiology of atherosclerosis.

Key words lectin-like oxidized low density lipoprotein receptor-1; tissue factor; apoptosis; atherosclerosis

Rupture or erosion of vulnerable atherosclerotic plaques and the subsequent formation of occlusive thrombi are currently recognized as the primary causes of acute coronary syndrome and stroke.[1] Vulnerability of atherosclerotic plaques, rather than severity of luminal stenosis, has been suggested to be the most important determinant for the onset of acute coronary syndrome.[2] Accordingly, it is of great importance to determine causative factors in the destabilization of atherosclerotic plaques and in the thrombus formation, which should help develop new therapeutic and diagnostic (imaging) agents of atherosclerosis, leading to the establishment of novel therapeutic strategies for preventing acute coronary syndromes and stroke.

To date, enhanced proinflammatory responses and the expression of matrix metalloproteinases (MMPs) have been suggested to play important roles in the destabilization of atherosclerotic plaques. Apoptosis and prothrombotic factors in atherosclerotic plaques have also been reported to be crucial factors for the plaque vulnerability and thrombus formation.[3] Apoptosis of foam cells and macrophages contributes to the formation of an acellular (cell-poor) lipid core,[4] and the apoptosis of smooth muscle cells further weaken the fibrous cap by decreasing the synthesis of extracellular matrix proteins.[5] Tissue factor (TF), a cofactor for plasma coagulation factor VIIa, which is localized in vascular cells and the lipid core within the atherosclerotic lesions, is an initiator of the coagulation cascade leading to thrombus formation after the plaque rupture *in vivo*.[3]

Lectin-like oxidized low density lipoprotein (LDL) receptor-1 (LOX-1), a type II membrane glycoprotein belonging to the C-type lectin family, acts as a cell-surface receptor for oxidized LDL (Ox-LDL) and mediates several biological effects of Ox-LDL.[6] Recent studies with cultured cells suggest that LOX-1 may play several important roles in destabilization of atherosclerotic plaques, inducing expression of adhesion molecules and chemokines for monocytes,[7] transformation of macrophages into foam cells,[8,9] apoptosis of smooth muscle cells[10,11] and the degradation of extracellular matrix proteins by induction of matrix metalloproteinases.[12] Several studies with cultured cells also showed that Ox-LDL, through LOX-1, also triggers the CD40/CD40L signaling pathway,[13,14] which then induce TF expression.[15] These biological effects mediated by Ox-LDL-LOX-1 interactions may enlarge the lipid core, weaken the fibromuscular cap, and induce proinflammatory responses, resulting in destabilization of the atherosclerotic plaques and thrombus formation.

The actual contribution of LOX-1 to the plaque vulnerability and thrombus formation *in vivo*, however, remains unclear. In this context, we recently demonstrated that LOX-1 expression is related to MMP-9 expression and morphological plaque vulnerability using a rabbit model of spontaneous atherosclerosis,[16] myocardial infarction-prone Watanabe her-

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itable hyperlipidemic (WHHLMI) rabbits (previous strain: Watanabe heritable hyperlipidemic (WHHL) rabbits). The use of WHHLMI rabbits could be effective for investigating causative factors in the destabilization of atherosclerotic plaques, as the histological characteristics of their atherosclerotic lesions have been reported to be similar to those of humans.[17,18] Thus, in the present study, we compared LOX-1 expression with TF expression and apoptosis in atherosclerotic lesions of WHHLMI rabbits, in order to further characterize the roles of LOX-1 to the plaque vulnerability and thrombus formation *in vivo*.

MATERIALS AND METHODS

Animals Four WHHLMI rabbits (female, 12.6±0.8 months old: 3.7±0.3 kg body weight) bred at Kobe University were used in the present study. The rabbits were fed standard rabbit chow (type CR-3; Clea Japan Inc., Tokyo, Japan: 120 g/d) and were given water *ad libitum*. All experimental procedures were approved by the Kyoto University Animal Care Committee.

Preparation of Histological Sections Rabbits were sacrificed with an overdose of sodium pentobarbital (Nembutal, intravenously, Dainippon Sumitomo Pharma, Osaka, Japan). The aortic arch and thoracic aorta were cut into 6 and 9 segments, respectively. Each segment was immediately fixed in a solution containing L-(+)-lysine hydrochloride (75 mmol/l) and 4% paraformaldehyde in phosphate buffer (37.5 mmol/l; pH 7.4), and embedded in paraffin. Consecutive 5-μm thick slices were prepared at the center of each segment.

Histological Analysis Serial sections were subjected to immunostaininig for LOX-1, TF and cell type marker antigens, as well as HE and Azan–Mallory staining. A monoclonal antibody for rabbit LOX-1 (mouse IgG) was established by a standard hybridoma technique.[16] Its specificity was confirmed in CHO cells stably expressing rabbit LOX-1 (data not shown). A monoclonal antibody for rabbit TF (mouse IgG) was obtained from American Diagnostica (Stanford, CA, U.S.A.). Monoclonal antibodies for a rabbit macrophage-specific antigen (RAM-11, mouse IgG) and smooth muscle actin (1A4, mouse IgG) were obtained from Dako Corp., Santa Barbara, CA, U.S.A. Immunohistochemical staining was carried out using a Dako Envision+kit (Dako) with hematoxylin counterstaining. Immunostaining with subclass-matched irrelevant IgG served as a negative control. Apoptotic nuclei were determined by terminal deoxyribonucleotide transferase (TdT)-mediated nick-end labeling (TUNEL) using a commercially available kit (*in situ* apoptosis detection kit, TACS, Trevigen, Gaithersburg, MD, U.S.A.).[19] Specimens stained without TdT enzyme served as negative controls. Counterstaining with hematoxylin was also performed. Tissue fixation, paraffin embedding, and LOX-1 and TF staining procedure were performed in the same condition, respectively.

Classification of Atherosclerotic Lesions The atherosclerotic lesions in WHHLMI rabbits were divided into 4 categories using a classification scheme based on the recommendations of the American Heart Association (AHA)[20,21] by HE and Azan–Mallory staining, as previously described[19]: (**1**) neointimal lesion (Type I—III), (**2**) atheromatous lesion (Type IV), (**3**) fibroatheromatous lesion (Type Va, Vb), (**4**) collagen-rich lesion (Type Vc), as shown in Fig. 1. Neointimal lesions were defined as having adaptive thickening of the intima consisting mainly of smooth muscle cells and few macrophages. Atheromatous lesions consisted of macrophages (foam cells) rich and lipid-rich areas covered with thin fibrous connective tissue, and were considered to be similar to vulnerable lesions in human atherosclerotic plaques. Fibroatheromatous lesions were composed of several macrophages (foam cells) and lipid-rich areas separated by thick layers of fibromuscular connective tissue, which were relatively stable to rupture.[22] Collagen-rich lesions contained predominant collagen components and smooth muscle cells.

Semi-quantitative Analyses Areas (μm^2) occupied by each lesion component were evaluated with a VHX Digital Microscope (Keyence Corp., Osaka, Japan). The vulnerability index, an index of the morphological destabilized characteristics of atherosclerotic lesions in WHHLMI rabbits, was calculated for each atherosclerotic lesion as previously described.[23] The vulnerability index was defined as the ratio of the lipid component area (macrophages+extracellular lipid deposits)/fibromuscular component area (smooth muscle cells+collagen fibers). Collagen-rich fibers and extracellular lipid deposits (extracellular vacuoles and lacunae) were determined with Azan–Mallory staining.[19] The macrophage and smooth muscle cell areas were determined with immunohistochemical staining by use of antibodies RAM11 and 1A4, respectively. LOX-1 and TF expression were assessed as percentages of positively stained areas (% positive). The number of TUNEL-positive cell (apoptotic cell) was counted under the microscope and the TUNEL-positive cell density (number/mm^2) was calculated.

In order to evaluate the detailed expression of LOX-1 and TF in atheromatous and fibroatheromatous lesions, these lesions were subdivided into three subregions; fibromuscular cap, lipid area and boundary regions (Fig. 5I).[21] The intensity of immunohistochemical staining for LOX-1 and TF was examined in each subregion and scored from 0 to 2 (0, negative; 1, equivocal; 2, intense). The evaluation was performed in a blinded fashion four times by two independent observers (S.I. and N.T.) for each specimen.

Statistical Analyses Data are presented as the mean± S.D. Comparisons among lesion types were performed using the Kruskal–Wallis test with *post hoc* analysis by the Scheffe test. Correlation coefficients were assessed with Spearman rank correlation coefficients. Statistical significance was defined as $p<0.05$.

RESULTS

Composition of Atherosclerotic Lesions In the aortic arch and thoracic aorta of 4 WHHLMI rabbits, 191 histopathological features which correspond to the classification criteria (neointimal, atheromatous, fibroatheromatous or collagen-rich lesions) were observed. Thus, the 191 regions were divided into the 4 lesion-categories. Figure 1 shows typical images of four categorized lesion types with HE, Azan–Mallory and immunohistochemical staining (Figs. 1A—H). Fourteen lesions were classified as neointimal lesions showing a thin intimal layer consisting of smooth muscle cells and few macrophages (Fig. 1, left column). Forty-four lesions were classified as atheromatous lesions, consisting of a large

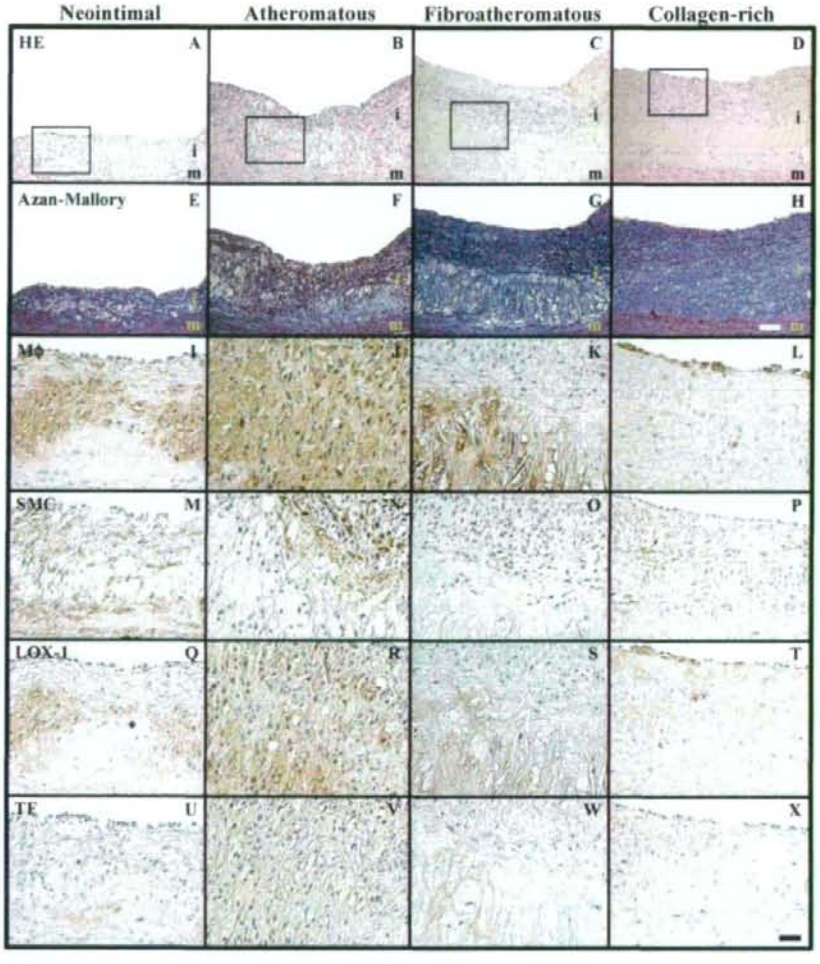

Fig. 1. LOX-1 and TF Expression in 4 Categorized Atherosclerotic Lesions of WHHLMI Rabbits

Atherosclerotic lesions were microscopically classified into four categories as described in Materials and Methods: neointimal lesion (left column), atheromatous lesion (middle-left column), fibroatheromatous lesion (middle-right column) and collagen-rich lesion (right column). HE staining (A—D), Azan–Mallory staining (E—H) and immunohistochemical staining for macrophage staining (Mφ) (I—L), smooth muscle cell staining (SMC) (M—P), LOX-1 (Q—T) and TF (U—X) are shown. The panels of immunohistochemical staining showed higher magnification images of the area indicated in A—D. Bar=100 μm, magnification ×40 (A—H), Bar=300 μm, magnification ×120 (I—X), m: media, i: intima.

lipid area with abundant macrophages and extracellular lipid deposits, accompanied by a thin fibromuscular cap with relatively few smooth muscle cells scattered in the superficial region (Fig. 1, middle-left column). Fifty-six lesions showed typical characteristics of fibroatheromatous lesions, consisting of few macrophages, less extracellular lipid deposits and a thicker fibromuscular cap (Fig. 1, middle-right column). Seventy-seven lesions were categorized as collagen-rich lesions, consisting of a thick neointimal layer with abundant fibrous connective tissue and smooth muscle cells, while the lipid area was minimal or even absent; collagen-rich lesions (Fig. 1, right column). The atheromatous, fibroatheromatous and collagen-rich lesions were similarly observed in both of the aortic arch and the descending thoracic aorta; however, neointimal lesions were found only in the aortic arch, but not in the descending thoracic aorta. Neither plaque rupture nor thrombi (type VI) were detectable in the aortic arch or the descending thoracic aorta of the rabbits used in the present study.

LOX-1/TF Expression and Apoptotic Events in Atherosclerotic Lesions Prominent LOX-1 expression was found in the macrophage-rich lipid areas of atheromatous and fibroatheromatous lesions (Figs. 1Q—T). LOX-1 expression was also observed in the superficial part of neointimal lesions and collagen-rich lesions. In addition, scattered positive staining for LOX-1 was observed in the neointima of collagen-rich lesions. LOX-1 expression was mainly detected in macrophages and endothelial cells, as well as slight to moderate expression in the smooth muscle cells (Figs. 1I—T). TF expression was prominent in the macrophage-rich lipid areas of atheromatous lesions, and the expression was mainly observed in macrophage foam cells (Figs. 1I—L, U—X). TUNEL-positive cells were extensively observed in the macrophage-rich lipid areas of the atheromatous and fibroatheromatous lesions where a large number of foam cells were accumulated (Figs. 2B, C). Scattered TUNEL-positive

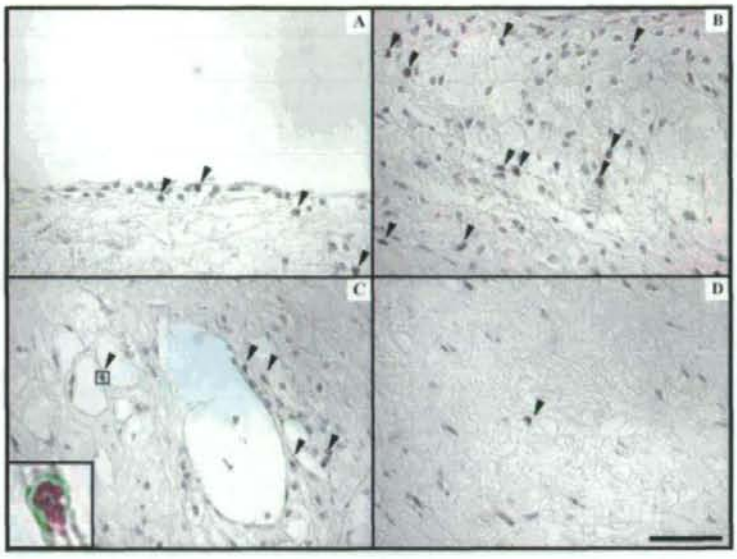

Fig. 2. Detection of TUNEL-Positive Nuclei in 4 Categorized Atherosclerotic Lesions of WHHLMI Rabbits

DNA fragmentation staining by *in situ* end labeling (TUNEL) are shown. Scattered TUNEL-positive nuclei (arrow head) were observed in the intimal region (A), the macrophage-rich lipid area of fibroatheromatous lesion (C) and the collagen layer (D). TUNEL-positive nuclei were mainly observed in the macrophage-rich lipid area of the atheromatous lesion (B). Bar = 50 μm, magnification ×300. In panel (C), a higher magnification image of a TUNEL-positive nucleus was indicated (inlet).

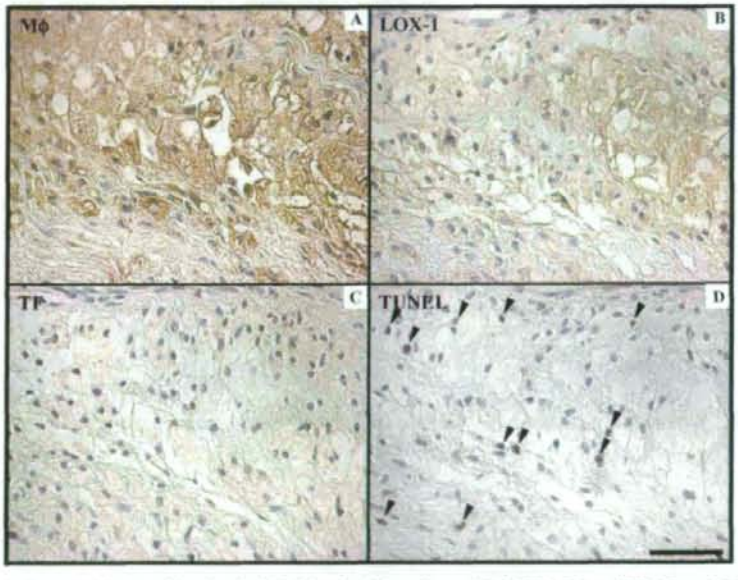

Fig. 3. Typical Microscopic Images of Immunohistochemical Staining for Macrophages (A), LOX-1 (B) and TF (C), and TUNEL Staining (D) for the Macrophage-Rich Area of the Atheromatous Lesion

LOX-1 expression was mainly observed in the macrophage-rich lipid area where TF expression and TUNEL-positive nuclei were prominent. Bar = 50 μm, magnifications ×300.

cells were found in the fibromuscular areas of the collagen-rich lesions (Fig. 2D). TUNEL-positive cells in the superficial region were much less frequently seen among all atherosclerotic lesions (Fig. 2A).

Figure 3 shows typical microscopic images of immunostaining and TUNEL for the macrophage-rich lipid area of atheromatous lesions at higher magnifications. LOX-1 expression, TF expression and TUNEL-positive cells were prominently observed in the macrophage-rich lipid areas (Fig. 3).

There was no notable difference in the staining tendency of LOX-1, TF and TUNEL-positive cells between the aortic arch and the descending thoracic aorta. Neither LOX-1, TF, nor TUNEL-positive cells were detectable by immunostain-

ing in negative control sections (data not shown).

Semi-quantitative Analyses of LOX-1/TF Expression and Apoptotic Events in Relation to Plaque Vulnerability Figure 4a shows the vulnerability index calculated for each lesion category classified as described in Materials and Methods. This index was the highest in the atheromatous lesions ($p<0.0001$ *vs.* other lesions), followed in decreasing order by the fibroatheromatous lesions ($p<0.005$ *vs.* neointimal lesions; $p<0.0001$ *vs.* collagen-rich lesions), the neointimal lesions, and the collagen-rich lesions.

LOX-1 expression (% positive) was the highest in the atheromatous lesions ($p<0.05$ *vs.* neointimal lesions; $p<0.0001$ *vs.* fibroatheromatous and collagen-rich lesions, Fig. 4b), followed in decreasing order by the neointimal lesions, the fibroatheromatous lesions, and the collagen-rich lesions. TF expression (% positive) in the atheromatous lesions was also the highest among the four lesion types ($p<0.0001$ *vs.* other lesions, Fig. 4c). DNA nick end-labeling of tissue sections showed that the TUNEL-positive cell density (number/mm^2) was higher in the atheromatous lesions than those in the fibroatheromatous and collagen-rich lesions ($p<0.0001$, Fig. 4d). In the neointimal lesions, the TUNEL-positive cell density was higher than collagen-rich lesions ($p<0.05$).

Localization of LOX-1 and TF Expression within Atherosclerotic Lesions The atheromatous and fibroatheromatous lesions were subdivided into three subregions: fibromuscular cap (C), lipid area (foam cells+extracellular lipid deposits) (L) and boundary regions (B) as illustrated in Fig. 5I. Staining intensity scores for LOX-1 and TF in each subregion of atheromatous and fibroatheromatous lesions were compared. The expression levels of LOX-1 were significantly higher in the lipid area than in the fibromuscular cap and

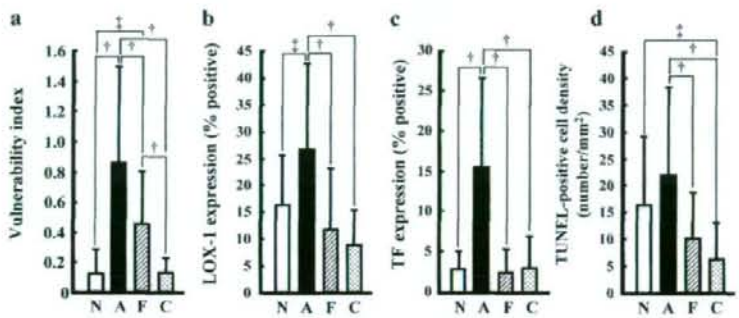

Fig. 4. Vulnerability Index (a), Percentages of Positively Stained Areas (% Positive) for LOX-1 (b) and TF (c), and TUNEL-Positive Cell Density (Number/mm^2) (d) in the Classified Atherosclerotic Lesions

N, A, F and C indicate neointimal, atheromatous, fibroatheromatous and collagen-rich lesions, respectively. Data are represented as the mean±S.D. †$p<0.0001$, ‡$p<0.05$.

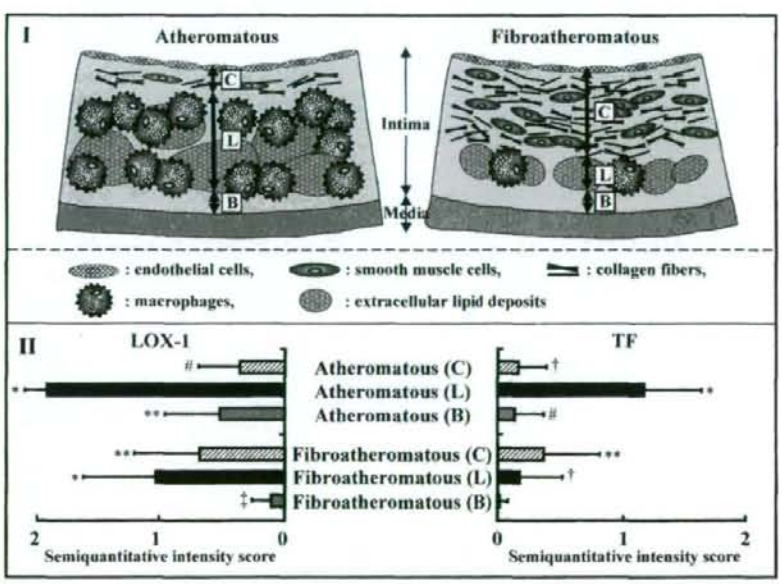

Fig. 5. LOX-1 and TF Expression Scores in Fibromuscular Cap (C), Lipid Area (Foam Cells+Extracellular Lipid) (L), and Boundary (B) Subregions of Atheromatous and Fibroatheromatous Lesions

(I) Schematic illustration of fibromuscular cap (C), lipid area (L) and boundary (B) subregions, which compose atheromatous (left) and fibroatheromatous (right) lesions. (II) Expression scores of LOX-1 (left) and TF (right) in fibromuscular cap (C), lipid area (L) and boundary (B) subregions of 44 atheromatous (upper) and 56 fibroatheromatous (lower) lesions. Data are indicated as the mean±S.D. in each subregion. *$p<0.0001$ *vs.* all other regions. **$p<0.0001$ *vs.* fibroatheromatous (B). #$p<0.0001$ *vs.* fibroatheromatous (C). †$p<0.05$ *vs.* fibroatheromatous (C). ‡$p<0.05$ *vs.* atheromatous (C).

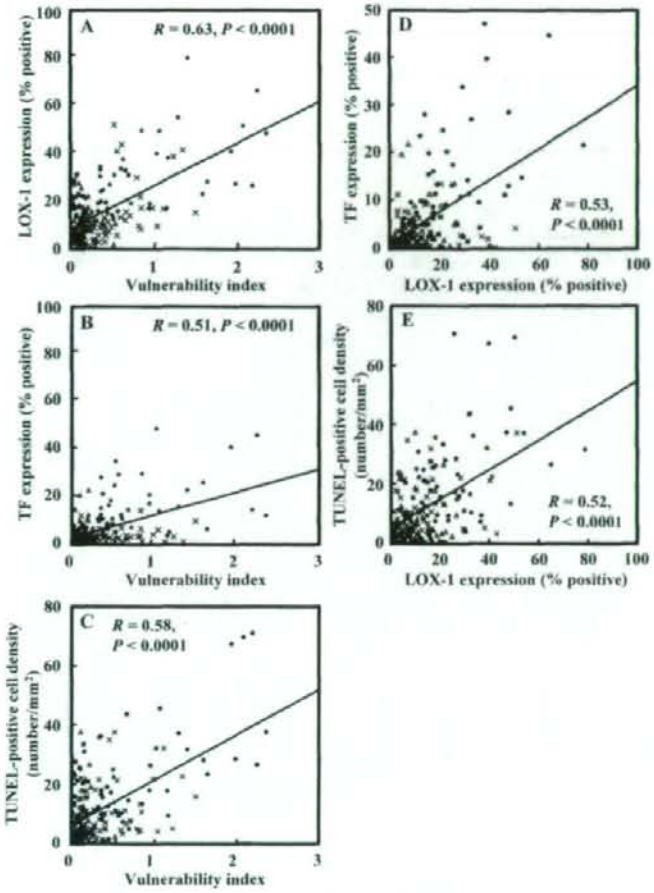

Fig. 6. Analyses of Correlations among LOX-1 and TF Expression (% Positive), and TUNEL-Positive Cell Density (Number/mm^2) and Histological Vulnerability

Regression analyses demonstrated a positive correlation between vulnerability index and LOX-1 expression (r=0.63, p<0.0001) (A), between vulnerability index and TF expression (r=0.51, p<0.0001) (B), as well as between vulnerability index and TUNEL-positive cell density (r=0.58, p<0.0001) (C). In the same manner, a positive correlation between LOX-1 expression and TF expression (r=0.53, p<0.0001), as well as between LOX-1 expression and TUNEL-positive cell density (r=0.52, p<0.0001) are shown in panel (D) and (E), respectively. ■; neointimal, ●; atheromatous, ×; fibroatheromatous, △; collagen-rich lesions.

boundary regions (Fig. 5II). The LOX-1 expression levels were the highest in the lipid area of atheromatous lesions among all subregions of any lesion types (p<0.0001), followed by the lipid area of fibroatheromatous lesions. The TF expression levels were also the highest in the lipid area of atheromatous lesions among all subregions of any lesion types.

Correlation Analyses among LOX-1, TF, Apoptosis and Histological Vulnerability Correlation of the vulnerability index with LOX-1 and TF expression (% positive), and TUNEL-positive cell density (number/mm^2) is shown in Figs. 6A—C. LOX-1 expression was positively correlated with the vulnerability index (r=0.63, p<0.0001, Fig. 6A). Similarly, TF expression and TUNEL-positive cell density showed positive correlation with the vulnerability index (r=0.51, p<0.0001 and r=0.58, p<0.0001, respectively, Figs. 6B, C).

Figures 6D and E show the correlation of LOX-1 expression with TF expression (D) and TUNEL-positive cell density (E). Regression analyses demonstrated positive correlation between LOX-1 and TF expression (r=0.53, p<0.0001, Fig. 6D), as well as between LOX-1 expression and TUNEL-positive cell densities (r=0.52, p<0.0001, Fig. 6E).

DISCUSSION

Our major findings in this study can be summarized as follows: (1) significantly enhanced LOX-1, TF expression and apoptotic events were observed in the atheromatous lesions characteristic of the vulnerable plaque. (2) LOX-1 expression was mainly observed in the macrophage-rich lipid area where TF expression and apoptotic events were prominent. (3) LOX-1 expression levels were positively correlated with morphological vulnerability (vulnerability index), TF expression and apoptotic events. These findings are the first *in vivo* data suggesting the roles of LOX-1 in the processes of plaque destabilization and subsequent thrombus formation.

In the present study, we investigated the LOX-1 expression