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Figure 1 Effects of GIP on endochondral ossification

(A) Growth of *GIPR*$^{+/+}$ and *GIP* $^{-/-}$ mice at 10 weeks of age.

(B) Soft X-ray analyses of *GIPR*$^{+/+}$ and *GIPR*$^{-/-}$ mice

Figure 2 Effects of GIP on bone trabeculae

Histological analyses of tibiae from *GIPR*$^{+/+}$ mice and *GIPR*$^{-/-}$ mice. The tibiae of 8-week-old male mice were examined. Original Magnification: A, ×40; B, ×100.

Figure 3 Dysregulation of bone remodeling in *GIPR*$^{-/-}$ mice

Immunostaining of tissue nonspecific alkaline phosphatase (TNAPase) (A) and tartrate-resistant acid phosphatase (TRAPase) staining (B) of tibiae from *GIPR*$^{+/+}$ and *GIPR*$^{-/-}$ mice are shown.

Figure 4 Effects of GIP on calcium homeostasis *in vivo*

(A) Urinary elimination of deoxypyridinoline crosslinks from *GIPR*$^{+/+}$ (open column) and *GIPR*$^{-/-}$ (filled column) mice. Values are means ± SE. *, $P < 0.05$ for *GIPR*$^{+/+}$ mice vs. *GIPR*$^{-/-}$ mice.

(B) Plasma calcium concentration of *GIPR*$^{+/+}$ and *GIPR*$^{-/-}$ mice before (open) and after (filled) meal was shown. Values are means ± SE. *, $P < 0.05$ vs. fasting.

Figure 5 Effects of GIP on pit-forming activity of osteoclasts *in vitro*

The crude osteoclast preparation was cultured on dentine slices in the presence or absence of GIP at 100 μM, using calcitonin as the positive control. (A) The resorption pits formed on the slices were stained with Mayer's hematoxylin solution for observation by light microscope (upper), or scanned by electron microscope (lower). (B) The number of resorption pits' was calculated.

Figure 6 Effects of GIP on preventing apoptosis of osteoblasts *in vitro*

(A) Saos-2 cells, a human osteoblastic cell line, were stimulated with the indicated concentrations of human GIP and human PTH for 30 minutes, and cAMP levels were measured. **, $P < 0.01$ vs. control.

(B) Saos-2 cells were cultured for 6 hours with 50 μM etoposide in the absence or presence of preincubation for 1 hour with 100 nM GIP. The pyknotic fragmented nuclei typical of apoptotic cells viewed using Hoechst 33342 fluorescent dye. Original Magnification: ×100. Insets: percentage of cells undergoing apoptosis.

(C) Saos-2 cells were cultured for 1 hour in the indicated concentration of GIP or PTH and then incubated for an additional 6 hours in the absence (open) or presence of 50 μM etoposide (filled). Apoptotic cells were enumerated by trypan blue staining. Values are indicated as means ± SE. *, $P < 0.05$; **, $P < 0.01$ vs. etoposide alone.

(D) Primary mouse osteobalsts were cultured for 1 hour with or without GIP and then incubated for an additional 6 hours in the absence (open) or presence of 50 μM etoposide (filled). Apoptotic cells were enumerated by trypan blue staining. Values are indicated as means ± SE. **, $P < 0.01$ vs. etoposide alone.

Figure 7 Schematic model of cyclic (GIP) and persistent (PTH) activation of osteoblasts in bone remodeling.

GIP induces a cyclic increase of the intracellular cAMP concentration ([cAMP]i) in osteoblasts that induces bone formation, calcium from the blood calcium pool depositing on bone. Endogeneous PTH induces a persistent increase of [cAMP]i in osteoblasts that induces bone resorption, calcium from bone releasing into the blood calcium pool.

Table 1. Bone histomorphometry of tibia of *GIPR* $^{+/+}$ and *GIPR* $^{-/-}$ mice.

		GIPR $^{+/+}$	*GIPR* $^{-/-}$
Bone Volume / Tissue Volume	BV/TV (%)	11.0 ± 1.7	9.5 ± 1.0
Trabecular Thickness	Tb. Th (μm)	31.7 ± 2.1	30.2 ± 1.5
Trabecular Separation	Tb. Sp (μm)	287.3 ± 36.6	299.0 ± 20.2
Eroded Surface / Bone Surface	ES/BS (%)	31.6 ± 2.9	34.7 ± 2.0
Osteoclast Surface	Oc. S/BS (%)	12.8 ± 2.0	13.1 ± 1.2
Osteoclast Number	N. Oc/TV (/mm^2)	19.3 ± 2.5	25.0 ± 1.8*
Osteoclast Number	N. Oc/BS (/mm)	3.2 ± 0.6	4.1 ± 0.3
Multinuclear Osteoclast Number	N. Mu. Oc	8.1 ± 1.2	11.6 ± 0.9*
Multinuclear Osteoclast Number	N. Mu. Oc/TV (/mm^2)	12.2 ± 1.8	18.1 ± 1.2**
Multinuclear Osteoclast Number	N. Mu. Oc/BS (/mm)	2.1 ± 0.4	2.9 ± 0.1*
Osteoblast Surface	Ob. S/BS (%)	39.2 ± 4.1	38.4 ± 2.7
Mineral Apposition Rate	MAR (μm/day)	2.8 ± 0.2	2.3 ± 0.1*
Ajusted Apposition Rate	Aj. AR (μm/day)	2.5 ± 0.2	1.8 ± 0.1*
Osteoid Maturation Rate	Omt (day)	1.4 ± 0.1	1.8 ± 0.1*
Mineralizing Lag Time	Mlt (day)	1.5 ± 0.2	2.2 ± 0.2*
Bone Formation Rate	BFR/BV (%/year)	2914.7 ± 261.0	2311.3 ± 120.1*
Bone Formation Rate	BFR/TV (%/year)	273.0 ± 31.3	206.9 ± 20.6
Bone Formation Rate	BFR/BS (mm^3/mm^2/year)	0.43 ± 0.02	0.34 ± 0.02**

* Significantly different from *GIPR* $^{+/+}$, P<0.05.

** Significantly different from *GIPR* $^{+/+}$, P<0.01.

Figure 1

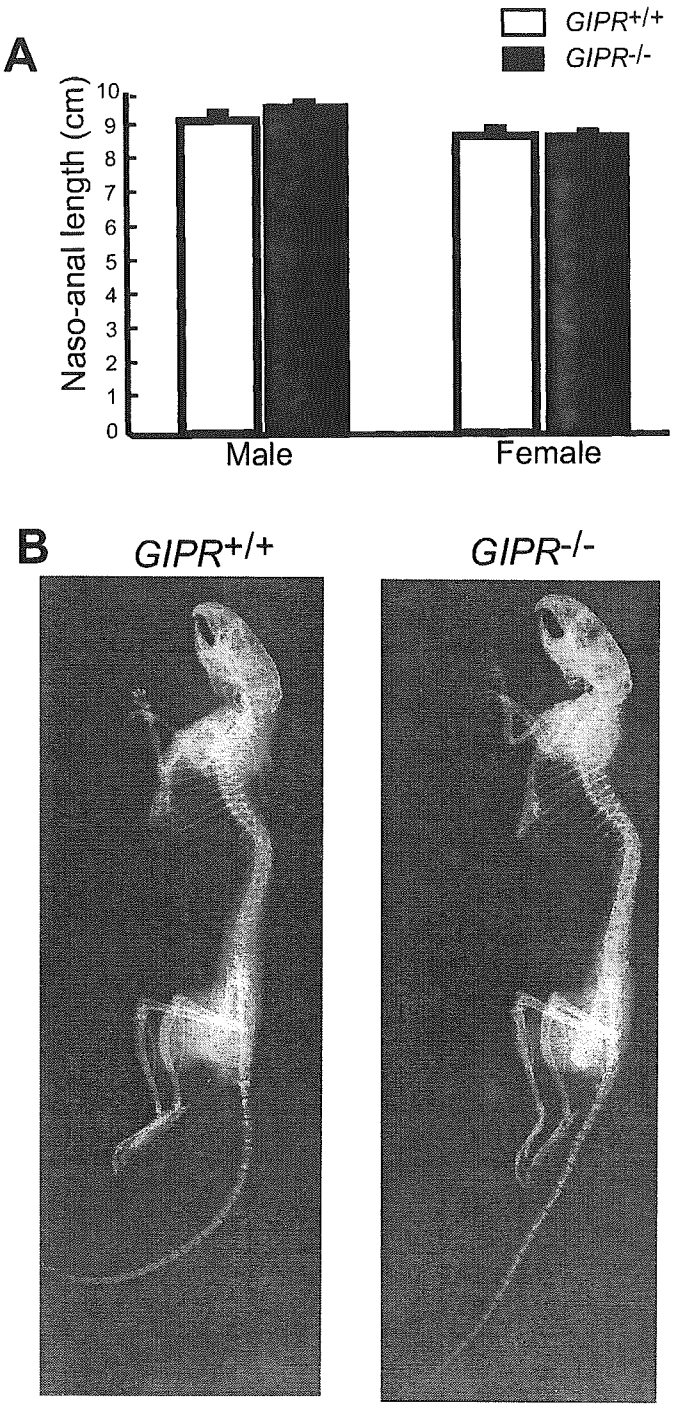

A
GIPR+/+
GIPR-/-
Naso-anal length (cm)
0
1
2
3
4
5
6
7
8
9
10
Male
Female
B
GIPR+/+
GIPR-/-

Figure 2

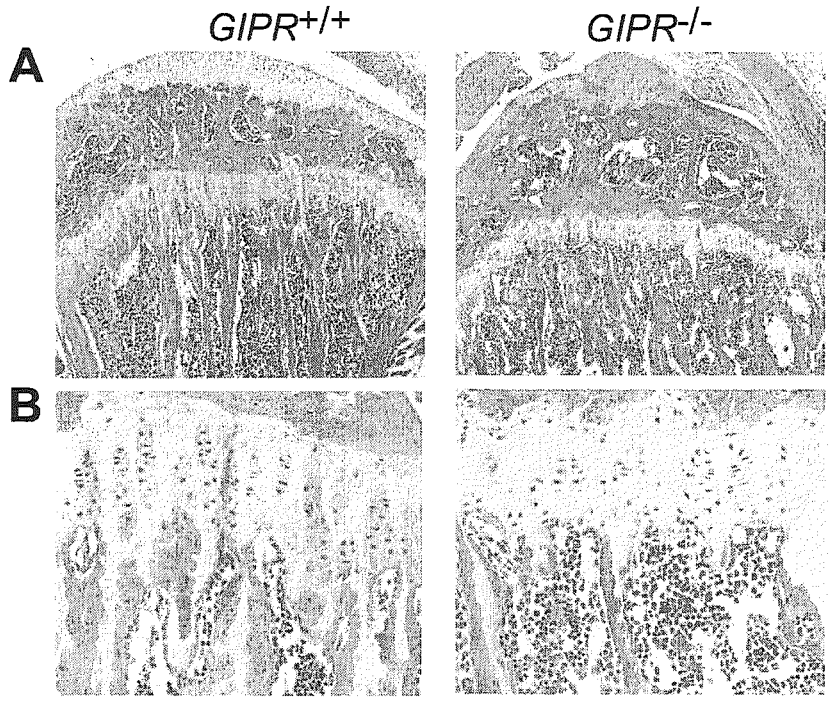


Figure 3

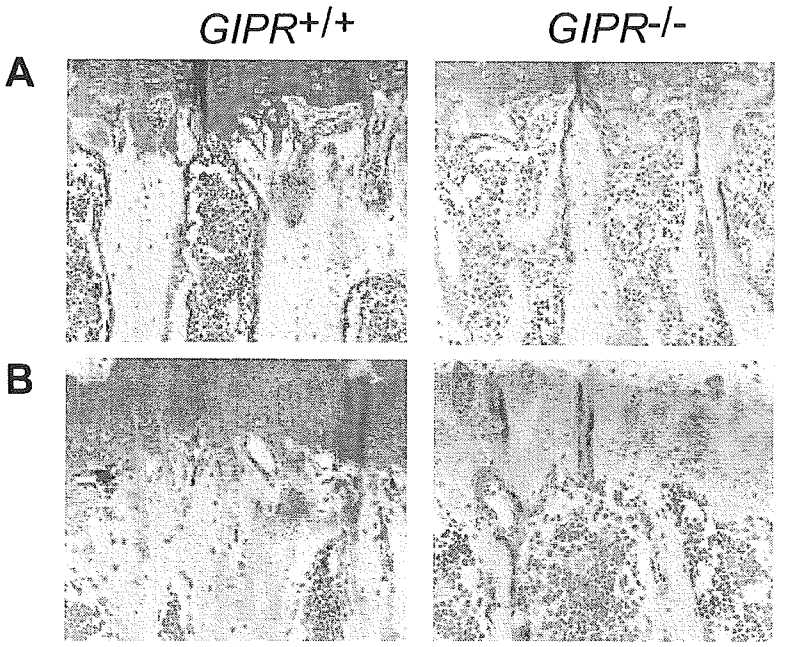


Figure 4

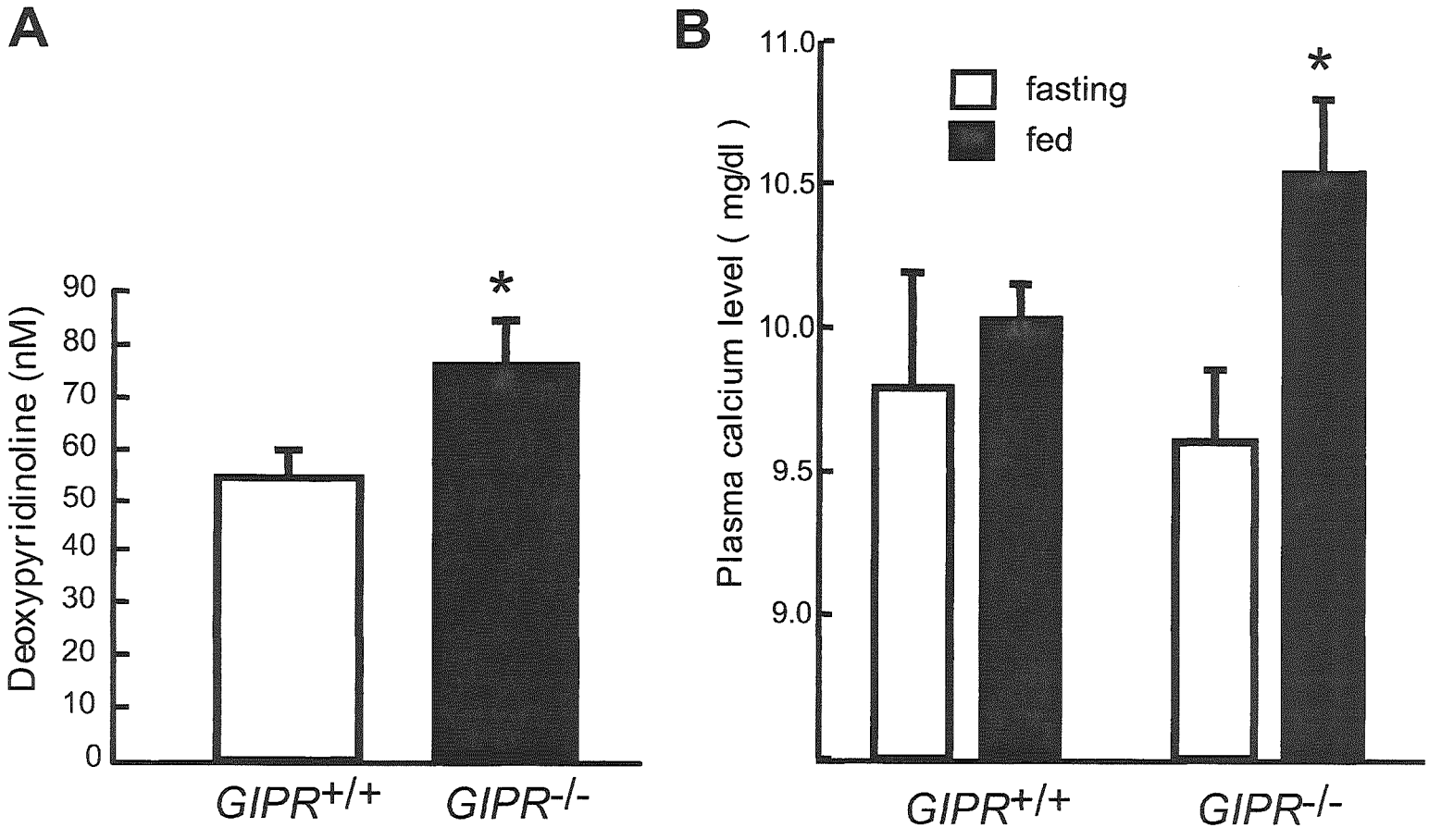
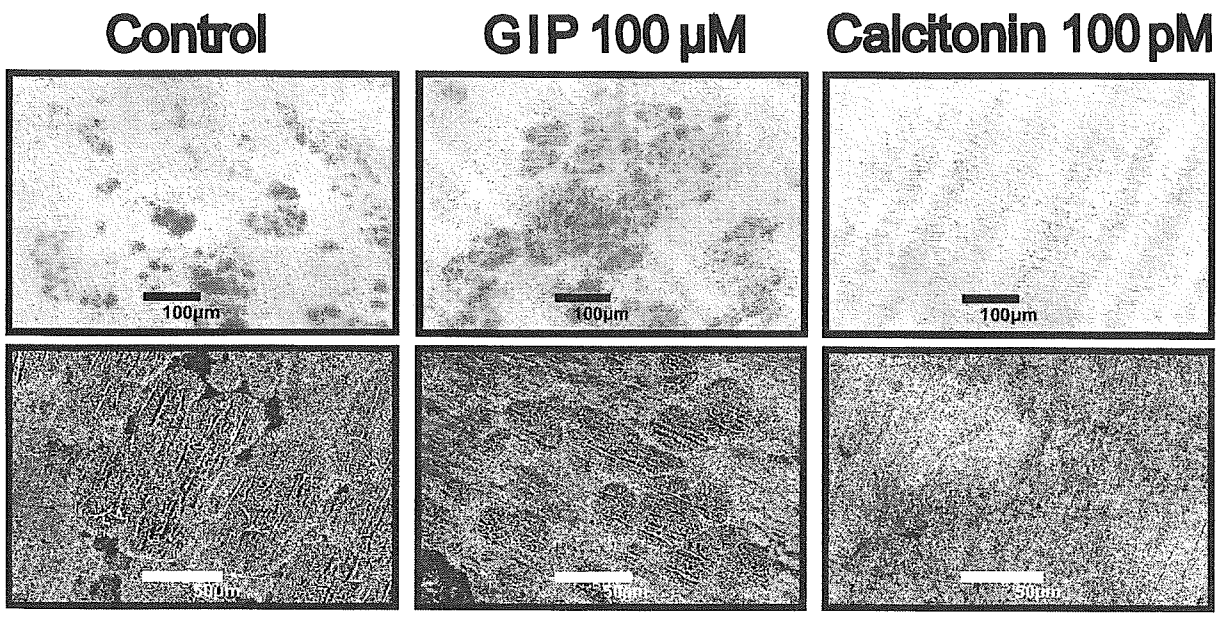

A
Deoxypyridinoline (nM)
90
80
70
60
50
40
30
20
10
0
*
GIPR+/+
GIPR-/-
B
Plasma calcium level (mg/dl)
11.0
10.5
10.0
9.5
9.0
fasting
fed
*
GIPR+/+
GIPR-/-

Figure 5

A


Control
GIP 100 µM
Calcitonin 100 pM
100µm
100µm
100µm

B

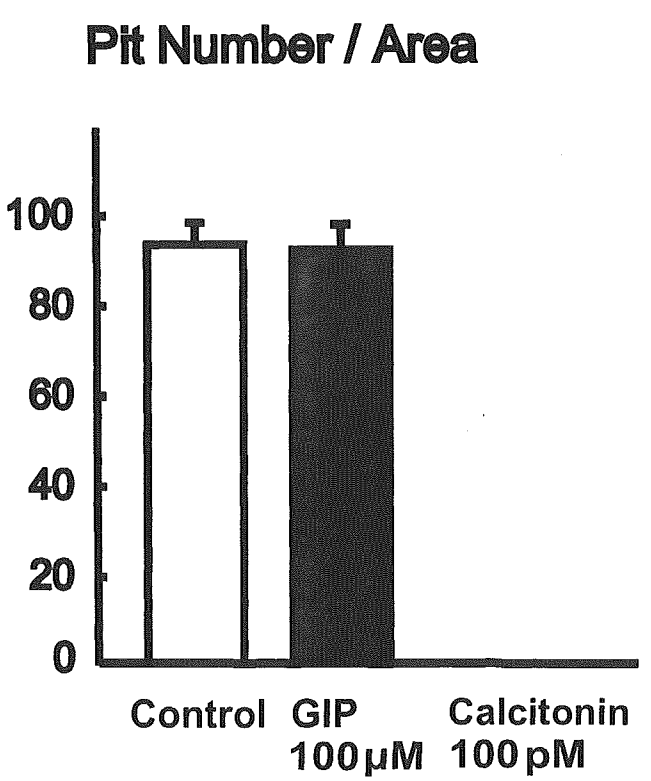

Pit Number / Area
100
80
60
40
20
0
Control
GIP 100 µM
Calcitonin 100 pM

Figure 6

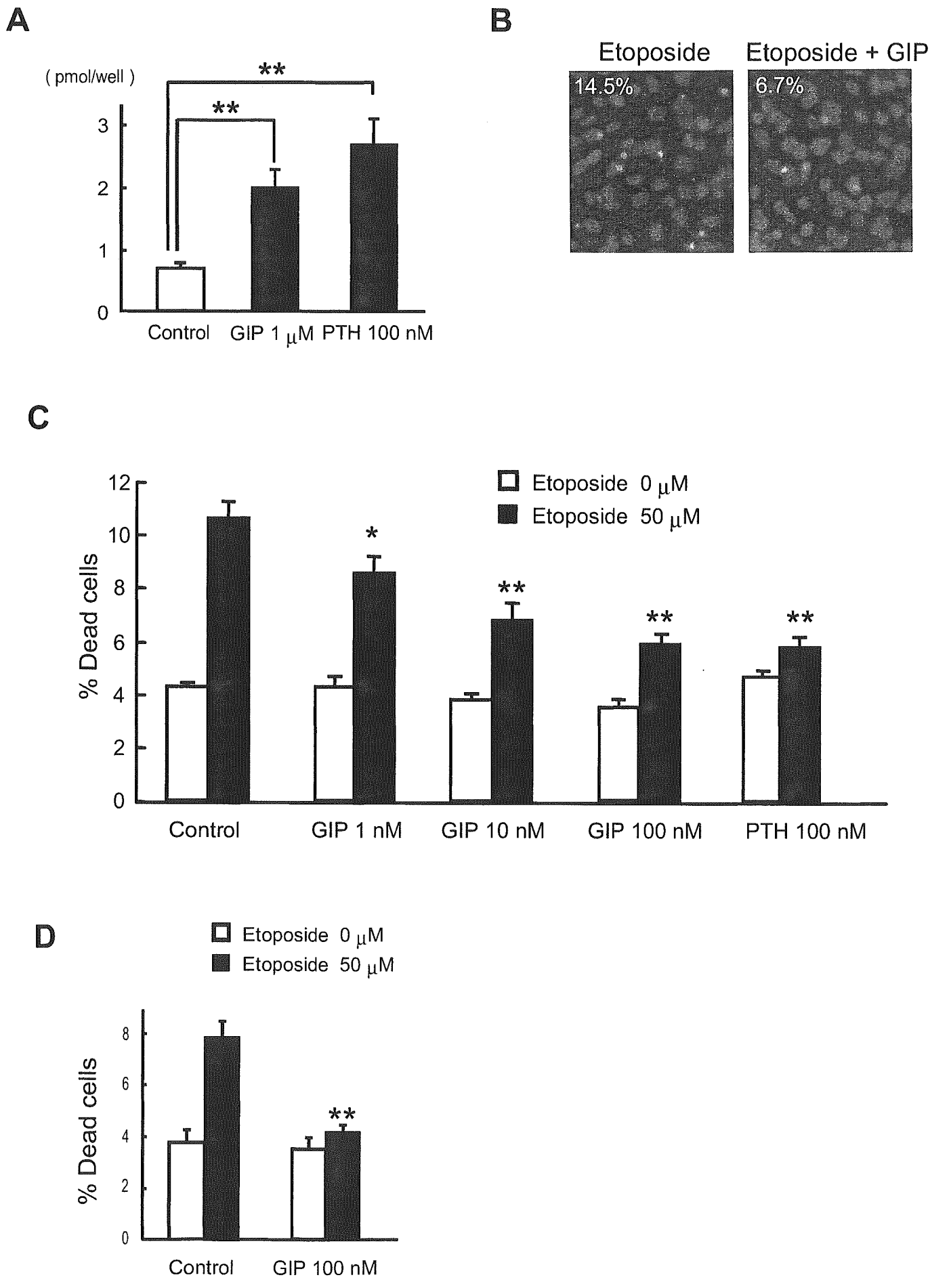
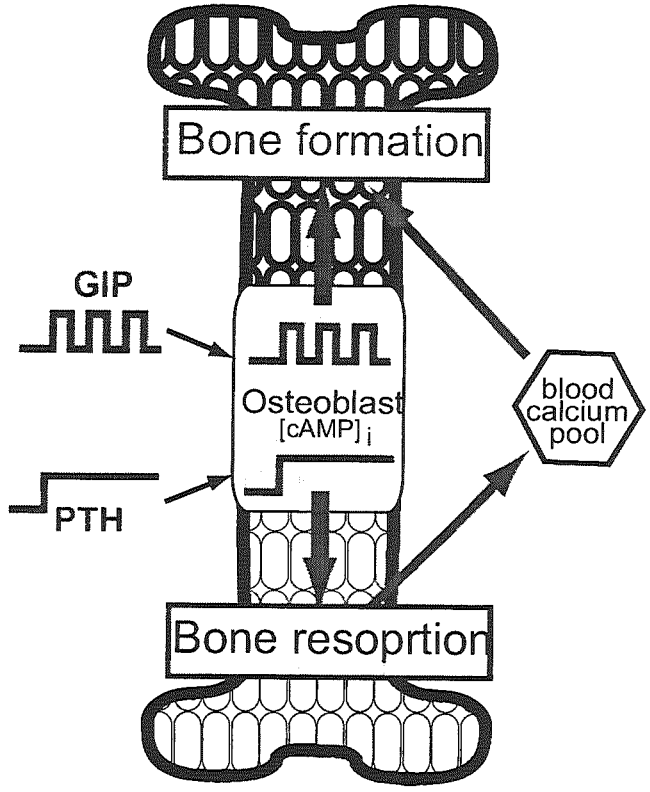

A
(pmol/well)
**
**
3
2
1
0
Control
GIP 1 μM
PTH 100 nM
B
Etoposide
Etoposide + GIP
14.5%
6.7%
C
Etoposide 0 μM
Etoposide 50 μM
% Dead cells
12
10
8
6
4
2
0
*
**
**
**
Control
GIP 1 nM
GIP 10 nM
GIP 100 nM
PTH 100 nM
D
Etoposide 0 μM
Etoposide 50 μM
% Dead cells
8
6
4
2
0
**
Control
GIP 100 nM

Figure 7


Bone formation
GIP
Osteoblast
[cAMP]i
blood
calcium
pool
PTH
Bone resoprtion

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A single transplantation of the islets can produce glycemic stability and reduction of basal insulin requirement

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Abstract

We investigated glycemic stability and insulin requirement 1 month after a single transplantation of the islets from non-heart-beating donors or a living donor. Overall blood glucose levels decreased immediately after transplantation. The *M*-value and mean amplitude of glycemic excursions (MAGE) decreased significantly from 53.0 (range, 8.9–91.0) to 4.2 (0.6–8.8, $P < 0.05$) and from 8.5 mM (4.8–11.7) to 3.3 mM (2.0–4.5, $P < 0.05$), respectively. The values after transplantation were lower than the first quartile of 102 type 2 diabetic control patients. The estimated HbA_{1c} level decreased significantly from 7.9% (5.7–10.9) to 5.4% (4.7–5.9, $P < 0.05$). The supplement of basal insulin decreased 43% from 0.31 units/kg/day (0.16–0.37) to 0.18 units/kg/day (0–0.22, $P < 0.05$), while that of stimulated insulin did not decrease significantly, from 0.28 units/kg/day (0.13–0.51) to 0.21 units/kg/day (0–0.41). Thus, only one islet transplantation can be sufficient to attain metabolic stability, probably by effective supply of basal insulin secretion, sufficient to avoid life-threatening severe hypoglycemia and prevent or delay the progress of secondary complications of diabetes by decreasing the HbA_{1c} level.

Keywords: A single transplantation of the islets; Non-heart-beating donor; Living donor; Metabolic stability

1. Introduction

Diabetes mellitus (DM) is a clinically and genetically heterogeneous group of disorders classified mainly into type 1 and type 2 diabetes. Type 1 diabetes is caused by β-cell destruction that often results in their complete loss and insulin-dependent diabetes mellitus

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(insulin-dependency). As both basal and stimulated insulin secretion from pancreatic β-cells is completely abolished in the majority of patients with type 1 diabetes, the blood glucose level remains unstable despite all effort to use optimal exogenous insulin.

In diabetic patients, blood glucose levels are considerably high or low in comparison with optimal levels, and the amplitude of glycemic excursions are large. The former is assessed by the *M*-value [1] and the latter by the mean amplitude of glycemic excursions (MAGE) [2]. In diabetic patients with metabolic instability, the *M*-value and MAGE are significantly high [1–3]. Such patients are at increased risk of progression of diabetic complications [4], life-threatening hypoglycemia unawareness and even "dead-in-bed syndrome" [5].

Since the success of islet transplantation with the corticosteroid-free protocol from Edmonton [6], islet transplantation has become more common radical therapy for type 1 diabetes mellitus [7]. The Edmonton protocol, in addition to optimizing islet isolation and modifying the immunosuppressive regimen, includes multiple infusions of islets from different donors. This therapy generally aims at insulin independence as well as the avoidance of severe hypoglycemia. Under present conditions, when cadeveric donors are used, multiple injections are required to attain insulin independence except in one report [8]. However, in many countries, the donor pool is extremely small, making it impractical to aim at insulin independence. Accordingly, we have investigated the effect of a single transplantation of the islets on glycemic lability.

Using even a non-heart-beating donor, a single transplantation of the islets can achieve metabolic stability probably by effective supply of basal insulin secretion regardless of the achievement of insulin independence, suggesting the therapeutic value of a single transplantation of the islets for patients with insulin-dependency in cases of donor shortage.

2. Methods

2.1. Subjects

Five patients (three women, two men) (patients one to five) (median age, 44 years; range, 36–58) who had had type 1 diabetes mellitus for a median of 21 years (range, 14–29) and a 27-year-old woman (Patient 6) who had had pancreatic diabetes for 12 years underwent islet transplantation in Kyoto University Hospital between April 7 2004 and January 19 2005. Diagnostic basis for type 1 diabetes in five of the six recipients was completely depleted insulin secretion (negative C-peptide [<0.1 ng/ml] prestimulation and post glucagon stimulation) and clinical course. The donors of five patients (Patients one to five) with type 1 diabetes mellitus were non-heart-beating donors, and a donor of a patient (Patient 6) with pancreatic diabetes was a living donor who was a mother of the patient. Each patient received islet transplantation from one or two pancreases [9,10].

The study was approved by the ethics committee of Kyoto University Graduate School and Faculty of Medicine.

2.2. Assessment of glycemic control

The *M*-value [1] and the mean amplitude of glycemic excursions (MAGE) [2], the indexes of glycemic lability, of the six patients were measured before and 1 month after a first islet transplantation.

There is a predictable relationship between plasma glucose and HbA_{1c}. Thus, the estimated HbA_{1c} levels were calculated by the corresponding seven-point plasma glucose profiles (premeal, postmeal and bedtime) before and 1 month after the first transplantation: estimated HbA_{1c} = mean plasma glucose (mg/dl)/35.6 + 1.87 [11,12].

The *M*-value, MAGE and HbA_{1c} levels of 52 type 1 diabetic patients (31 women and 21 men) (median age, 43 years; range, 16–79) and 102 type 2 diabetic patients (43 women and 59 men) (median age, 65 years; range, 28–79) admitted to Kyoto University Hospital from January 2002 to June 2005 were also examined.

2.3. Exogenous insulin requirement

We defined the total amount of premeal regular insulin or rapid-acting insulin analogue as the supplemental dose of stimulated insulin and that of neutral protamine Hagedorn (NPH) insulin, long-acting insulin analogue, or basal dose of continuous subcutaneous insulin infusion (CSII) as the supplement of basal insulin. The supplemental dose of basal and stimulated insulin were compared before and 1 month after the first islet transplantation.

2.4. Statistical analyses

Results are expressed as medians and ranges. Wilcoxon signed-rank test was used to compare the *M*-value, MAGE, the estimated HbA_{1c} levels and the amount of exogenous insulin before and 1 month after islet transplantation.

Mann–Whitney's *U*-test was used to compare the *M*-value, MAGE, and the HbA_{1c} levels of type 1 and type 2 diabetic control patients. Significance was taken at a *P* value of <0.05.

3. Results

3.1. Glycemic lability is higher in type 1 diabetic patients

The median HbA_{1c} level of 102 type two diabetic patients who had been admitted to Kyoto University

Hospital was 8.6% (range, 5.3–14.5%). Their *M*-value and MAGE at discharge was 12.2 (range, 1.2–66.2) and 5.2 mM (range, 0.8–12.7 mM), respectively (Fig. 1A and B). The median HbA_{1c} level of 52 type 1 diabetic patients admitted to Kyoto University Hospital was 8.3% (range, 5.0–16.3%), not significantly different from that of type 2 diabetic patients. The *M*-value and MAGE of 52 type 1 diabetic patients at discharge was 22.0 (range, 1.3–127.6, $P < 0.05$) and 6.7 mM (range, 2.8–16.6 mM, $P < 0.05$), respectively, significantly higher than those of type 2 patients (Fig. 1A and B).

Glycemic lability was improved after the first islet transplantation. The *M*-value (Fig. 2A) and MAGE (Fig. 2B) before islet transplantation was 53.0 (range, 8.9–91.0) and 8.5 mM (range, 4.8–11.7 mM), respectively. These medians were higher than the third quartiles of the 52 type 1 diabetic control patients. The *M*-value (Fig. 2A) decreased significantly to 4.2 (range, 0.6–8.8, $P < 0.05$), and MAGE (Fig. 2B) also significantly decreased to 3.3 mM (range, 2.0–4.5 mM, $P < 0.05$) 1 month after islet transplantation. These values were lower than the first quartile of the 102 type 2 diabetic control patients.

The estimated HbA_{1c} level before islet transplantation was 7.9% (range, 5.7–10.9%). The estimated HbA_{1c} level 1 month after islet transplantation decreased significantly to 5.4% (range, 4.7–5.9%, $P < 0.05$). There was no worsening of the secondary complications of diabetes due to the decrease of HbA_{1c} levels in all six patients.

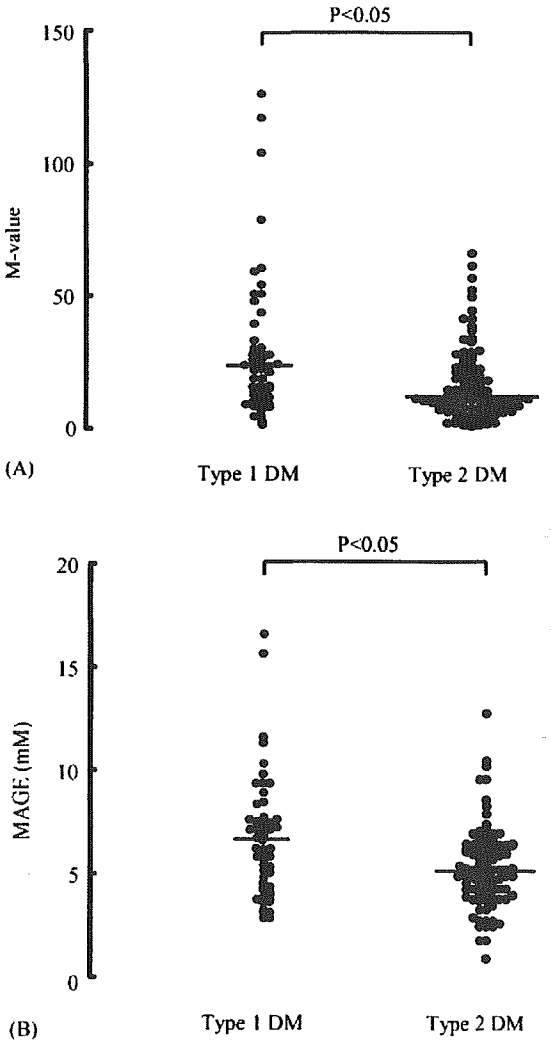

Fig. 1. (A) *M*-value of 52 type 1 diabetic patients and 102 type 2 diabetic patients at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians. (B) MAGE of 52 type 1 diabetic patients and 102 type 2 diabetic patients at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians.

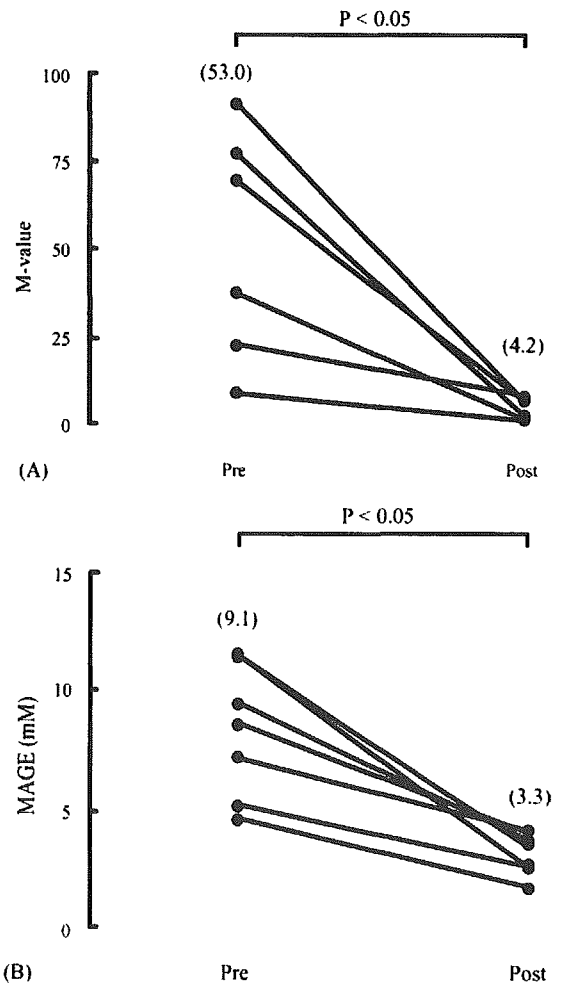

Fig. 2. (A) *M*-value before and 1 month after islet transplantation. Median values in parentheses. (B) MAGE before and 1 month after islet transplantation. Median values in parentheses.

3.2. Daily exogenous insulin use before and after islet transplantation

The total amount of exogenous insulin decreased gradually in all cases, stabilizing 1 month after transplantation. The supplemental dose of stimulated insulin (Fig. 3A) did not change significantly from 0.28 units/kg/day (range, 0.13–0.51 units/kg/day) to 0.21 units/kg/day (range, 0–0.41 units/kg/day); the supplement of basal insulin (Fig. 3B) decreased significantly by a median of 43% from 0.31 units/kg/day (range, 0.16–0.37 units/kg/day) to 0.18 units/kg/day (range, 0–0.22 units/kg/day, $P < 0.05$).

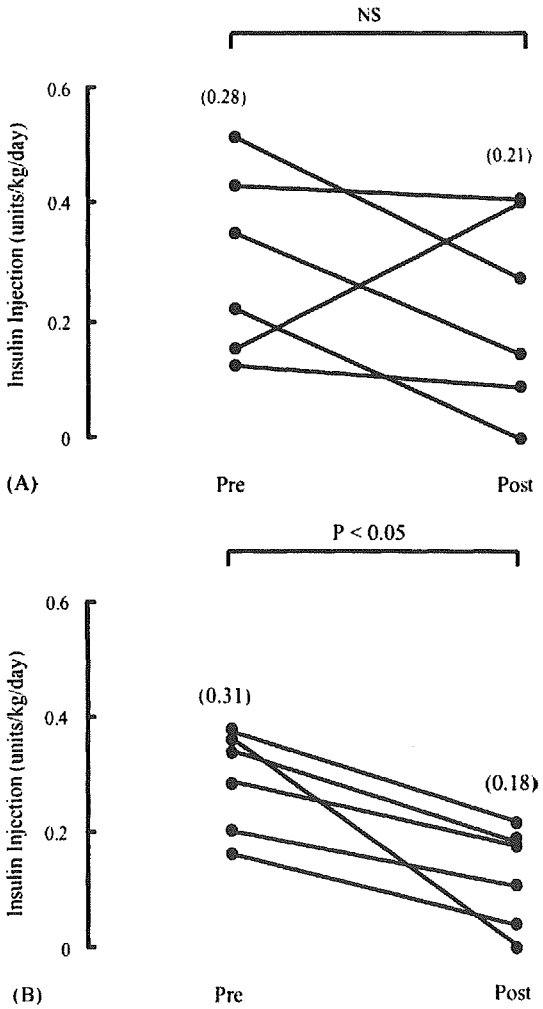

Fig. 3. (A) Daily supplement of stimulated insulin (units/kg/day) before and 1 month after islet transplantation. Median values in parentheses. (B) Daily supplement of basal insulin (units/kg/day) before and 1 month after islet transplantation. Median values in parentheses.

4. Discussion

Intensive insulin therapy can decrease HbA_{1c} and thereby delay the onset and slow progression of secondary complications of diabetes in both type 1 and type 2 diabetic patients [13,14]. However, our results showed that, in many patients with type 1 diabetes, blood glucose levels remained unstable despite optimal insulin therapy in hospital. The levels remained considerably high or low in comparison with the optimal levels, assessed by the *M*-value, and the amplitude of glycemic excursions remained large, assessed by MAGE, even though the HbA_{1c} levels of type 1 and type 2 diabetic patients on admission were nearly the same. In addition, before islet transplantation, all six patients exhibited hypoglycemia unawareness and life-threatening hypoglycemia despite all effort to optimize insulin therapy. Like this, in type 1 diabetic patients, it is quite difficult to achieve good glycemic control with exogenous insulin even if it is optimized.

Islet transplantation has become an alternative therapy for type 1 diabetes, the Edmonton protocol of multiple transplantations from different donors aiming at insulin independence as well as the avoidance of severe hypoglycemia. However, in some countries, the donor pool is extremely small, and many patients are on waiting lists. We evaluated the effects on glycemic lability in patients with insulin-dependency 1 month after a single transplantation of the islets, when the total amount of exogenous insulin became stable.

Glycemic lability in all six patients was improved significantly 1 month after the first islet transplantation. The *M*-value and MAGE became better than in most of the type 2 diabetic control patients. This indicates that a gain of endogenous insulin secretion resulted in non-insulin-dependent status. Furthermore, episodes of severe hypoglycemia have not occurred for at least 6 months after islet transplantation [10], even though the overall blood glucose level continues to be considerably lower than that before transplantation.

Only 5 years have passed since the Edmonton protocol was started, and the loss of insulin independence in many patients several years after transplantation has been reported [15]. Thus, the duration of insulin-independent status established by transplantation is uncertain. However, maintaining the blood glucose level close to the normal range for a certain period by transplantation can be beneficial: the epidemiology of diabetes interventions and complications (EDIC) study [16–20] indicates a reduced risk of onset and progression of diabetic complications for

several years, even after glycemic control becomes fair or poor due to graft failure.

As glycemic lability in type 1 diabetes is due to a complete loss of both basal and stimulated insulin secretion from β-cells, the supplemental dose of basal and stimulated insulin required before and after islet transplantation was compared. The supplement of basal insulin of all six patients decreased significantly 1 month after transplantation, indicating that a single transplantation of the islets is an effective therapy to supply basal insulin secretion.

The treat-to-target trial and related trials have shown that the uniform supplementation of basal insulin throughout the day significantly reduces risk of hypoglycemia and achieves good glycemic control in type 2 diabetic patients [21–23]. Our findings apparently support this. The supplemental dose of stimulated insulin was not changed significantly, partly due to the toxic effects on the islets of the immunosuppressive drugs. Indeed, we have shown that tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity [24]. In addition, sirolimus significantly impairs glucose-induced insulin secretion in islets [25,26]. Accordingly, the development of a new immunosuppressive regimen less toxic to β-cells may be required.

These results demonstrate that only a single transplantation of the islets using even a non-heart-beating donor can be sufficient to achieve metabolic stability, avoid life-threatening severe hypoglycemia, and improve the quality of life in patients with insulin-dependency regardless of the achievement of insulin independence. Thus, a single transplantation of the islets using one donor may enable a greater number of diabetic patients to benefit from islet transplantation therapy where donor shortage is a serious problem.

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