In the present study, therefore, we measured the plasma levels of MG and 3-deoxyglucosone (DG),[20,21] another α-oxoaldehyde, in 50 type 2 diabetic patients using electrospray ionization-liquid chromatography-mass spectrometry[5] and assessed the correlations between the baseline levels of MG or DG and the percentage of changes in the following clinical parameters linked to diabetic macroangiopathy or microangiopathy between baseline and a 5-year follow-up examination: intima-media thickness (IMT), pulse wave velocity (PWV), systolic blood pressure (BP; SBP), the amount of urinary albumin excretion (ACR), and estimated glomerular filtration rate (eGFR). It has been reported that IMT, PWV, SBP, ACR, and eGFR are predictive of future vascular events and that DG is a factor closely related to the advancement of microangiopathy.[20] It is, thus, important to clarify the relationship between these parameters and MG.

This study was carried out to verify our hypothesis that elevated levels of MG and DG are predictive of the development or progression of diabetic angiopathy. We, therefore, examined the correlation between baseline values of MG and DG and diabetic renovascular complications at a 5-year follow-up in patients with type 2 diabetes mellitus. The present study provides the first clear evidence that MG is a predictor of intima-media thickening, vascular stiffening, and elevation of BP in type 2 diabetics.

Methods

This research is a 5-year prospective follow-up study, targeting 50 type 2 diabetes patients who visited our outpatient department during the 3 months from April to June 2003 and who gave their consent to participate in the study. From July to September 2003, we collected early morning initial urine samples from the subjects and collected blood samples in the early morning after fasting. The blood was immediately centrifuged after being drawn to collect the plasma and then immediately stored frozen at a temperature of −80°C. After the samples of all 50 of the subjects were collected, we measured MG and DG at the same time.

The following parameters were measured at baseline: height, body weight, BP, serum creatinine (Cre), triglycerides (TGs), total cholesterol, high-density lipoprotein cholesterol, glycohemoglobin A1c (HbA1c), high-sensitive C-reactive protein, atrial natriuretic peptide, brain natriuretic peptide, adiponectin, tumor necrosis factor α-α (TNFα), monocyte chemoattractant protein (MCP) 1, interferon-inducible protein 10, interleukin (IL) 6, IL-18, vessel endothelial growth factor, urinary pentosidine, urinary 8-epi-prostaglandin F2α, 8-hydroxydeoxyguanosine (8-OHdG), ACR, type IV collagen, IMT, PWV, ankle brachial index (ABI), and ocular fundus data. The plasma levels of MG and DG were measured simultaneously. We also measured the IMT, PWV, Cre, ACR, and SBP values and calculated eGFR, which was calculated by the following formula: $eGFR = 194]Cre^{-1094} \times age^{-0.287}(female \times 0.739)$, at a follow-up examination performed 5 years after baseline, and examined the potential correlations between MG and DG and the 5-year changes in each of these 5 parameters. The present study was conducted after obtaining informed consent from all of the subjects, and the study protocol was approved by the ethics committees of Tohoku University Hospital.

Measurements

Quantification of MG and DG

MG and DG levels were assayed by derivatization with *o*-phenylenediamine and electrospray ionization-liquid chromatography-mass spectrometry of the resulting quinoxalines, as reported previously.[5] Briefly, plasma samples were deproteinized with perchloric acid, and 2,3-dimethylquinoxaline as an internal standard and *o*-phenylenediamine as a derivatizing agent were added to the supernatant. The samples were incubated at 4°C for 22 hours and then applied to a prepared C18 SPE column and filtrated through 0.2-μm filters into sample vials. Derivatized MG and DG were analyzed by high-performance liquid chromatography (Agilent 1100 series, Agilent Technologies) and electrospray ionization-mass spectrometry using a time-of-flight mass spectrometer (AccuTOF JMS-T100LC, JEOL). Derivatives were resolved by reverse-phase chromatography on a C18 Column (Cadenza CD-C18, 2.0×150 mm, Imtakt).

We modified the analytic conditions of liquid chromatography-mass spectrometry to obtain more precise data. The gradient speed of the mobile phase was slowed (from 6 to 10 minutes), and the mass/charge ratio (*m/z*) was detected more precisely (from *m/z* 145.00 to *m/z* 145.07 for MG and from *m/z* 235.00 to *m/z* 235.11 for DG). According to this modified method, the linear calibration curves relating peak area ratio with MG and DG concentrations were observed in the range of 50 to 3200 nmol/L (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org).

To evaluate interference in the assay of MG and DG in plasma samples, we compared a simple calibration curve with a standard addition curve prepared by adding increasing amounts of MG and DG to plasma samples. The slopes of the standard addition curves from plasma samples (Figure S2) were significantly different from the slopes of the simple calibration curves (Figure S1). However, the obtained slopes from 3 different healthy volunteers were very similar (Figure S2). Therefore, the average value of the slopes obtained from the standard addition curves with healthy control plasma was used to determine the MG and DG levels in plasma samples. According to this modified method, the mean plasma MG and DG levels in 10 healthy volunteers were 137±17 and 363±33 nmol/L, respectively.

The plasma MG levels derived using this modified method were lower than those we derived previously using the original previous data derived from the previous method,[5] but significant correlations were observed between the previous and modified methods for 30 plasma samples: 10 from healthy controls and 20 from patients undergoing dialysis ($P<0.0001$; Figure S3). The raw data from subjects in this study were evaluated by the previous method, so in this article we presented the data converted with the regression equation, as shown in Figure S3. The intraday coefficients of variation for the assay of MG and DG with the modified method were 1.9% and 2.0% (n=5), and the interday coefficients of variation were 4.3% and 12.0% (n=5), respectively.

Quantification of Oxidative Stress Markers and Inflammatory Markers

Plasma adiponectin, MCP-1, interferon-inducible protein 10, IL-6, IL-18, TNFα, and vessel endothelial growth factor were measured via ELISA using, respectively, an Adiponectin ELISA kit (Ohtsuka Pharmaceutical Co, Ltd), an MCP-1 ELISA kit (R&D Systems), a human IP-10 ELISA kit (R&D Systems, Inc), an IL-6 ELISA kit (R&D Systems), a human IL-18 ELISA kit (Medical and Biological Laboratories), an Ultra Sensitivity TNFα ELISA kit (BioSource International), and a human vessel endothelial growth factor Immunoassay kit (R&D Systems Inc). Urinary 8-epi-prostaglandin F2α and 8-OHdG were measured using an 8-isoprostane enzyme immunoassay kit (Cayman Chemical Co) and ELISA kit (Japan Institute for the Control of Aging), respectively, whereas urinary pentosidine was measured using high-performance liquid chromatography. Both were corrected according to the level of urinary creatinine excretion.

Vascular Evaluation

The PWV and ABI were measured using Form PWV/ABI, version 112 (Colin Electronics Co, Ltd), and IMT, by the ATL Ultramark HDI 5000 Ultrasound System (Bothell).

Statistical Study

The data for patients whose actual measurement values (or log-converted values) showed normal distributions were recorded as the mean±SEM, and the data for patients who did not show a normal distribution were recorded as the median (range). The comparisons between the baseline and 5-year follow-up values of IMT, PWV,

SBP, ACR, and eGFR were carried out using the Student *t* test (for IMT, PWV, SBP, and eGFR) or the Wilcoxon signed-ranked test (for ACR). The correlation at baseline between MG and the various parameters was studied using single regression analysis, and the correlation between the percentage changes in IMT, PWV, SBP, ACR, and eGFR over the 5-year interval and either MG or DG was also investigated using single regression analysis. Finally, we performed a multiple regression analysis using the percentage changes in IMT, PWV, ACR, SBP, and eGFR over the 5-year period as the dependent or objective variables and the values of MG, DG, HbA1c, TG, body mass index (BMI), diabetic duration, and SBP at the baseline as the independent variables. Correlations were determined by the Spearman rank correlation test, with P values of <0.05 regarded as significant.

Results

The baseline characteristics of the patients in this study, that is, the clinical information, the serum and urinary levels of the various parameters measured, and the main drugs administered, are shown in Table 1. At the initiation of the study, a weak but significant correlation was observed between each of MG and DG (r=0.19; P<0.05), BMI (r=0.18; P<0.05), SBP (r=0.26; P<0.05), DBP (r=0.30; P<0.05), Cre (r=0.25; P<0.05), eGFR (r=0.35; P<0.05), or TG (r=0.29; P<0.05). No correlation was observed between MG and the other parameters at baseline. Specifically, MG showed no significant correlation to markers for oxidative stress (8-OHdG, 8-epi-prostaglandin F2α, and pentosidine), inflammation (MCP-1, IL-6, IL-18, high-sensitive C-reactive protein, and TNFα), or macroangiopathy (IMT, PWV, and ABI). Unexpectedly, MG was not significantly correlated with either clinical parameters linked to diabetic macroangiopathy or clinical parameters linked to microangiopathy (ACR) or to biomarkers of oxidative stress or inflammation. This shows that MG was not directly involved in oxidative stress, inflammation, or arteriosclerosis of these patients at the time of the baseline examination. In addition, this may, at least in part, reflect the fact that the patients in our study had already taken a variety of drugs at the initiation of this study that might have influenced the progression of angiopathy, oxidative stress, and inflammation, such as renin-angiotensin system inhibitors (RASIs; n=34), biguanide (n=28), and statins (n=14). On the other hand, at the baseline, 16 subjects were found to be RASI (−), 22 to be biguanide (−), and 5 to be both RASI (−) and biguanide (−). At the follow-up 5 years later, 7 subjects were RASI (−), 14 were biguanide (−), and none were both RASI (−) and biguanide (−). All of the subjects had been following a modified diet and an exercise regimen before taking part in this study, and there were no changes in these efforts during the test period. Despite the variety of treatments, the patients progressively developed hypertension and renal cardiovascular complications over the next 5 years: SBP was increased from 138.7±2.7 to 141.8±2.7 mm Hg, IMT from 1.51±0.12 to 1.60±0.12 mm, PWV from 1725.4±52.2 to 1792.7±53.1 cm/s, and ACR from 126.4 (range: 2.7 to 3060.6) to 468.1 (4.7 to 4696.4) mg/g of Cre, and eGFR was decreased from 68.12±4.37 to 49.04±3.43 mL/min. We assessed the correlations between the baseline levels of MG or DG and the percentage changes after 5 years in IMT, PWV, SBP, and ACR or eGFR. The values of IMT, PWV, SBP, and eGFR all increased during the 5-year period. MG correlated significantly with the percentage of changes of IMT (Figure S4), SBP (Figure S5), eGFR (Figure S6), and PWV (Figure S7). The percentage of increase of SBP correlated significantly

Table 1. Baseline Characteristics of Study Subjects

Clinical Information	Units	Data
Variable		
Age	y	61.3±2.0
Male/female	n	19/31
BMI	kg/m^2	24.4±0.6
SBP	mm Hg	138.8±2.8
DBP	mm Hg	78.7±1.8
HbA1c	%	6.8±0.2
Creatinine	μmol/L	0.9±0.1
eGFR	mL/min	68.2±4.37
Triglyceride	mmol/L	1.33±0.13
Total cholesterol	mmol/L	4.78±0.11
HDL-C	mmol/L	1.37±0.05
MG	nmol/L	233.2±6.5
DG	nmol/L	549.0±27.8
Urinary examination		
ACR	mg/g of Cre	126 (2.7 to 3060.6)
IV-collagen	μg/g of Cre	5.6±0.5
Pentosidine	nmol/g of Cre	48.8±3.1
8-OHdG	μg/g of Cre	8.9±0.6
8-epi-PGF	ng/g of Cre	218 (13.9 to 777.0)
Heart failure		
ANP	pg/mL	26.2 (7.4 to 697.1)
BNP	pg/mL	31.0 (4.0 to 375.8)
Inflammation		
IL-6	pg/mL	0.7 (0.2 to 22.8)
IL-18	pg/mL	271.6±17.7
MCP-1	pg/mL	184.5±11.1
hsCRP	mg/dL	0.1 (0.005 to 0.800)
Atherosclerosis		
Max IMT	mm	1.5±0.1
PWV	cm/s	1725.4±52.2
ABI		1.1±0.02
VEGF	pg/mL	53.3 (15.6 to 252.0)
Adipocytokines		
Adiponectin	μg/mL	10.2 (3.2 to 48.5)
TNFα	pg/mL	1.7±0.3
Retinopathy		19
Treatments		
Renin angiotensin system inhibitors		34
Calcium channel blockers		36
Insulin/sulf only urea/pioglitazone		21/18/21
Biguanides/α-glucosidase inhibitors		28/20
Statin/fibrate		14/9

VEGF indicates vessel endothelial growth factor; HDL-C, high-density lipoprotein cholesterol; PGF, prostaglandin F2α; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; hsCRP, high-sensitive C-reactive protein.

Table 2. The Correlation Between the Percentage Changes During the 5-Year Period of IMT, PWV, SBP, ACR, and eGFR and the MG and DG (Using Simple Linear Regression Analysis)

Variable				
Dependent	Independent	R^2	r	P
% change of IMT	MG	0.3932	0.6271	<0.01
	DG	0.0803	0.2835	NS
% change of PWV	MG	0.3246	0.5697	<0.01
	DG	0.001	0.0316	NS
% change of ACR	MG	0.0979	0.3129	<0.05
	DG	0.1787	0.4227	<0.01
% change of SBP	MG	0.257	0.5069	<0.01
	DG	0.0013	−0.0363	NS
% change of eGFR	MG	0.1294	0.3597	<0.01
	DG	0.0061	0.0781	NS

NS indicates not significant.

with the percentage of increase of PWV (Figure S8). Please see the online Data Supplement.

As summarized in Table 2, MG showed a statistically significant correlation with all of these parameters. By contrast, DG was significantly correlated only with the percentage of change in ACR. However, the multiple regression analysis showed that MG was an independent risk factor for the percentage of change of IMT, PWV, and SBP but not for that of ACR and eGFR (Table 3). DG was an independent risk factor for the percentage of change of ACR (Table 3).

Discussion

This study provides for the first time evidence that MG is a predictor in type 2 diabetes mellitus of intima-media thickening, of vascular stiffening, and of elevation of SBP, suggesting its clinical usefulness as a biomarker for diabetic macroangiopathy. Postprandial hyperglycemia increases MG and advances macroangiopathy. Therefore, MG is believed to increase the subsequent advancement of macroangiopathy in type 2 diabetes mellitus, especially in patients who have postprandial hyperglycemia.[15,16] Postprandial hyperglycemia-induced epigenetic changes and increased inflammatory subunit expression are prevented by reducing mitochondrial superoxide production or superoxide-induced α-oxoaldehydes, such as MG.[22] These results highlight the dramatic and long-lasting effects that short-term hyperglycemic spikes can have on vascular cells and suggest that transient spikes of hyperglycemia may be HbA1c-independent risk factors for diabetic complications. Postprandial hyperglycemia increases MG in the vascular cell by suppressing glyoxylase 1, which degrades MG activity. A rise in MG appears to be closely related to the decreased activity of glyoxylase 1 in the vascular endothelium, and this is related to metabolic memory. However, our study did not evaluate this glyoxylase 1. In the future, evaluation of glyoxylase-1 activity will be necessary.

Moreover, by suppressing the increase of MG, biguanide suppresses the advancement of macroangiopathy. (Biguanide is believed to suppress macroangiopathy not by improving postprandial hyperglycemia but by suppressing the increase of MG itself.) Our contention that MG predicts the develop-

Table 3. A Multiple Regression Analysis, Using the Percentage Changes in IMT, PWV, ACR, SBP, and Cre Over the 5-Year Period as the Dependent or Objective Variables and the Values of MG, DG, HbA1c, TG, BMI, DD, and SBP at the Baseline as the Independent Variables

Dependent Variable	Independent Variable	MG	DG	HbA1c	TG	BMI	DD	SBP
% change of IMT	β	0.21	0.02	1.16	0.04	−0.06	0.04	0.01
	95% CI	0.11	−0.01	−2.57	−0.02	−1.19	−0.48	−0.23
		0.31	0.04	4.88	0.11	1.07	0.55	0.25
	P	<0.01	0.12	0.53	0.17	0.92	0.89	0.91
% change of PWV	β	0.08	−0.00	−0.26	0.00	−0.11	−0.23	0.05
	95% CI	0.03	−0.01	−1.84	−0.02	−0.59	−0.45	−0.22
		0.14	0.01	1.31	0.03	0.36	−0.01	0.31
	P	<0.01	0.45	0.74	0.84	0.63	0.04	0.73
% change of SBP	β	0.13	−0.01	−0.64	−0.02	−0.05	−0.02	−0.20
	95% CI	0.09	−0.02	−2.21	−0.04	−0.53	−0.24	−0.30
		0.17	0.01	0.94	0.01	0.43	0.20	−0.10
	P	<0.01	0.30	0.42	0.20	0.84	0.83	<0.01
% change of ACR	β	4.03	1.37	−134.86	−1.09	3.67	−14.28	2.31
	95% CI	−0.73	0.26	−315.99	−4.12	−51.34	−39.49	−9.37
		8.79	2.48	46.27	1.95	58.68	10.94	13.99
	P	0.10	0.02	0.14	0.48	0.89	0.26	0.69
% change of eGFR	β	−0.10	−0.01	0.03	−0.06	−0.36	−0.29	0.03
	95% CI	−0.21	−0.03	−4.12	−0.13	−1.62	−0.87	−0.24
		0.01	0.02	4.17	0.01	0.90	0.29	0.30
	P	0.07	0.58	0.99	0.12	0.57	0.32	0.82

DD indicates diabetic duration.

ment of diabetic macroangiopathy in type 2 diabetics is in good agreement with previous observations that postprandial hyperglycemia, a factor contributing to the development of macroangiopathy, dramatically increases the production of MG and that biguanide, an agent effectively suppressing macroangiopathy independent of its blood glucose lowering, significantly lowers the production of MG.[17–19] In this study, many patients had taken biguanides both at the baseline (28 subjects) and 5 years later (36 subjects). A previous study by Beisswenger et al[18] demonstrated the biguanides lower plasma MG in diabetic patients. However, no such effect was observed in the present study: there was no statistically significant difference in plasma MG levels between patients with and those without biguanide treatment ($P=0.658$). One plausible explanation for this finding would be that the dose of biguanides administered to patients in Japan is suboptimal, at only 750 mg/d for metformin and only 150 mg/d for buformin.[18]

MG has been shown to be an independent variable affecting the percentage of change in SBP. MG has indeed been linked to the progression of hypertension in diabetic models through increases in vascular resistance, insulin resistance, and salt sensitivity and by the retention of body fluid volume.[2,3,8] Previous studies by us and others have demonstrated that administration of MG induces a rise in BP in experimental animals, which is significantly suppressed by administration of angiotensin receptor blockers or *N*-acetyl cysteine (an antioxidant agent).[3,23,24] In blood vessels under diabetic conditions, MG primarily accumulates in endothelial cells.[7,11] We reported recently that MG increases oxidative stress in vascular endothelial cells and induces vascular disorders.[4] MG indeed triggers IMT hypertrophy and PWV increase by an increase in local AGEs and oxidative stress.[1–3] MG is an independent risk factor of the increase of vascular stiffness (PWV) and the increase of the vascular thickness (IMT). Moreover, MG increases the salt sensitivity.[8] These are better explanations as to why the levels of MG should predict systolic and not diastolic pressure. We reported previously that administration of MG induces a rise in BP, increases ACR, and expands renal sclerotic lesions, all of which can be significantly suppressed by administration of angiotensin receptor blockers.[25] Treatment with RASIs therefore appears to limit the organ damage caused by MG,[23,24] and it has been indicated that angiotensin receptor blocker prevents MG-induced apoptosis by inhibiting caspase 3 activation, which might explain at least in part the beneficial effects of the angiotensin receptor blocker against diabetes-related cardiovascular diseases.[26] In our present study, the majority of patients (34 of 50 at the baseline and 43 of 50 after 5 years) had taken RASIs, making it difficult to analyze the effects of RASIs by subgrouping the patients. Mori et al[27] observed previously that MG induces hypertension and cardiorenal injury in Dahl salt-sensitive rats with a normal diet through the angiotensin II–mediated oxidative stress pathway. They found that, in the MG-treated rats, enhanced renal expressions of *N*ε-carboxyethyl-lysine (an AGE), 8-OHdG (a marker of oxidative stress), ED-1–positive cells (a marker of inflammation), and NAD(P)H oxidase activity occurred in parallel with a rise in SBP. However, in our study, the level of AGEs (eg, *N*ε-carboxyethyl-lysine) was not measured, so it is unclear whether MG directly causes vascular injury or whether MG changes into AGEs and causes vascular injury. An analysis that measures both AGEs and MG will be needed.

MG is not an independent risk factor of ACR: only DG is an independent risk factor.[20,21] The explanation for this remains unknown but may be attributed to the fact that the sources of MG and DG are not necessarily identical. In our study, we did not investigate the association of plasma levels of DG with diabetic retinopathy or neuropathy. It would be of interest to examine in a future study whether DG could be a useful biomarker for diabetic microangiopathy. MG is produced not only by hyperglycemia but also by a variety of proteins and conditions, such as TG.[1,2,11] It accumulates inside the vascular endothelial cells, where it induces primarily macroangiopathy. On the other hand, DG is produced primarily by hyperglycemia and induces microangiopathy. We believe that this fact may explain why blood glucose improvement suppresses microangiopathy but not macroangiopathy. Despite an early loss of glycemic differences, a continued reduction in microvascular risk and emergent risk reductions for myocardial infarction and death from any cause were observed during 10 years of posttrial follow-up in the United Kingdom Prospective Diabetes Study-80.[28,29] Early and rigorous blood glucose control thus has either a metabolic memory effect or a legacy effect of suppressing the onset of vascular disorders for extended periods. The possible mechanism of such effects remains unclear, although Holman et al[28] suggested that increased formation of AGEs may play an underlying role.[30] The increased levels of MG observed in individuals with diabetes mellitus are not merely the result of short-term changes in glucose or MG but may reflect long-term alterations to tissue proteins.

In this context, it is of interest that MG, a precursor for AGEs, at the baseline is an independent risk factor for the percentage changes after 5 years of IMT, PWV, and BP. MG could be a target for future study to elucidate the biochemical mechanisms of such a legacy effect.

Perspectives

This study provides evidence that MG is a predictor of intima-media thickening, vascular stiffening, and elevation of BP in type 2 diabetics, suggesting its clinical usefulness as a biomarker for diabetic macroangiopathy. Medical agents interfering with α-oxoaldehydes, such as MG, or decreasing their production may have potential therapeutic benefits for diabetic angiopathies.

Limitations

This is an observational study in which diverse interventions and treatments were carried out over a 5-year period and, thus, cannot directly verify the cause-effect relationship of MG in the development of diabetic angiopathy. Therefore, there is a possibility that numerous factors have been modified. Because this study has numerous factors (eg, oxidative stress and inflammations markers) that were not measured after that period, this analysis is insufficient.

It is quite possible that higher levels of MG simply reflect lesser renal excretion of this substance. In fact, the plasma

levels of creatinine were correlated with MG at the onset of this study. Because urinary MG levels were not measured in this study, the possibility of a rise in MG levels being caused by a decline in renal function cannot be ruled out.

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Disclosures

None.

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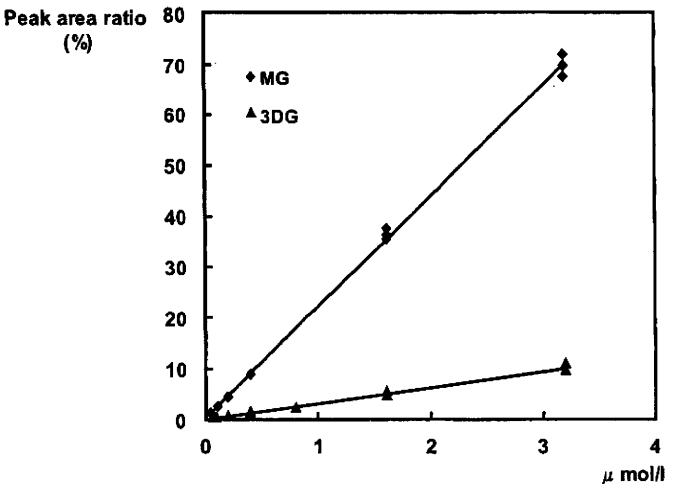
Methylglyoxal is a predictor in type 2 diabetic patients of intima-media thickening and elevation of blood pressure

Supplementary Figure

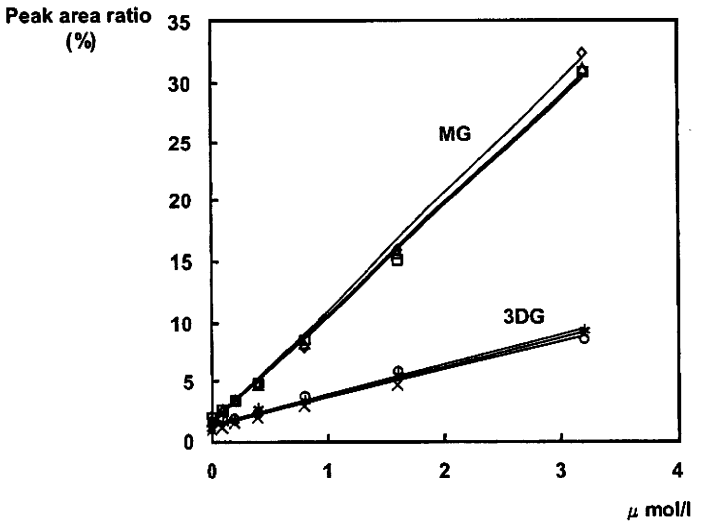
S1: Simple calibration curves for methylglyoxal (MG) and 3-deoxyglucosone (DG) assay. Regression equations of the standard curves were y=21.886x+0.1532 (r=0.999) and y=3.1635x-0.0196 (r=0.997) for MG and DG, respectively. Each sample was evaluated three times.

S1



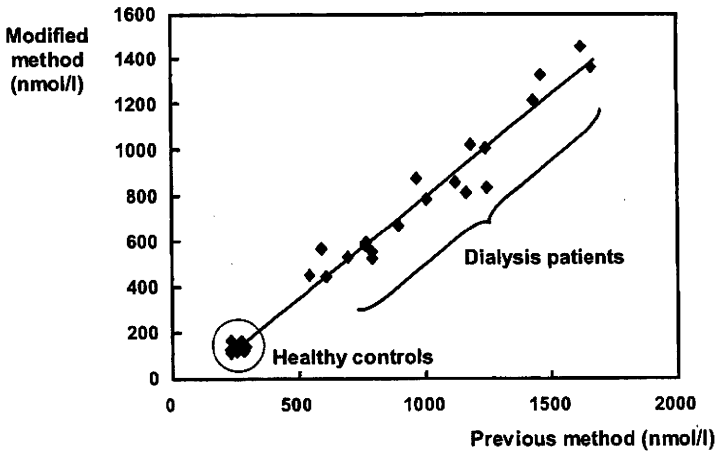
S2: Correlation between peak area ratio and concentration of MG and DG added to three plasma samples obtained from three different healthy controls. Regression equations for MG obtained from subject 1, 2 and 3 were y=9.6267x+1.0232 (r=0.999), y=9.0468x+1.4169(r=0.999) and y=9.2006x+1.2523 (r=0.999), respectively. Those for 3DG were y=2.5219x+1.0209 (r=0.999), y=2.5714x+1.2922 (r=0.999) and y=2.3623x+1.4629 (r=0.994), respectively.

S2



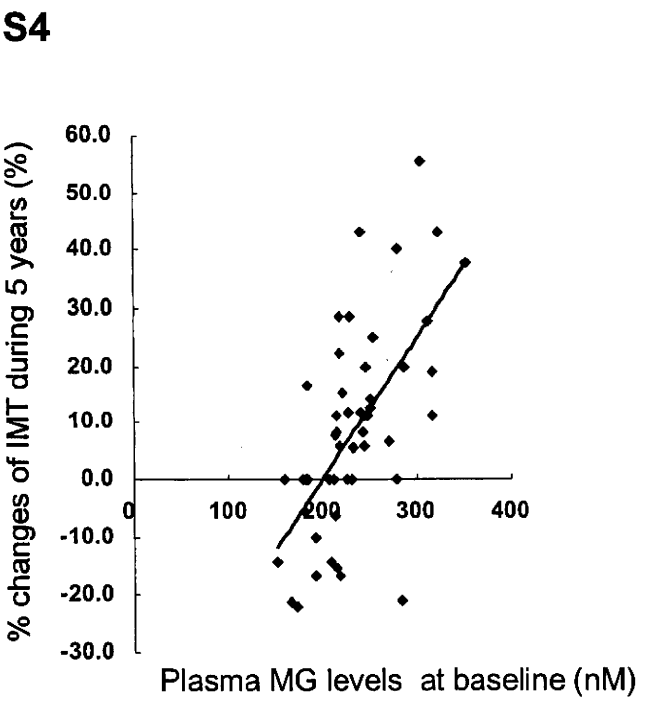
S3: Correlation between values obtained with previous and modified methods for MG assay. Twenty dialysis patients and ten healthy controls were evaluated. Regression equation was y=0.8934x-92.02 and correlation efficient was 0.987.

S3

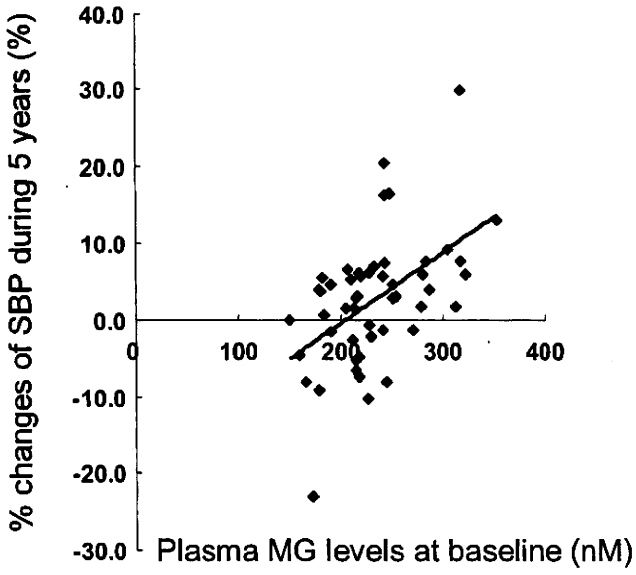


Supplementary Figure

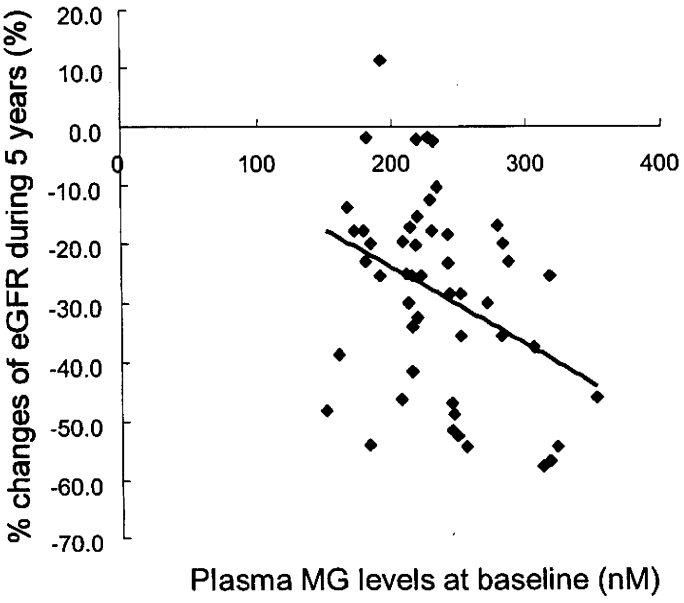
The values of intima-media thickness (IMT), systolic blood pressure (SBP) increased, and estimated glomerular filtration rate (eGFR), decreased during the 5-year period. Methylglyoxal (MG) correlated significantly to the % changes of IMT (S4): $y = 0.2449x - 48.684$, $r = 0.6271$, $p < 0.01$, SBP (S5): $y = 0.0929x - 19.081$, $r = 0.5069$, $p < 0.01$, and eGFR (S6): $y = -0.1302x + 1.9887$, $r=0.3597$, $p < 0.01$.



S5

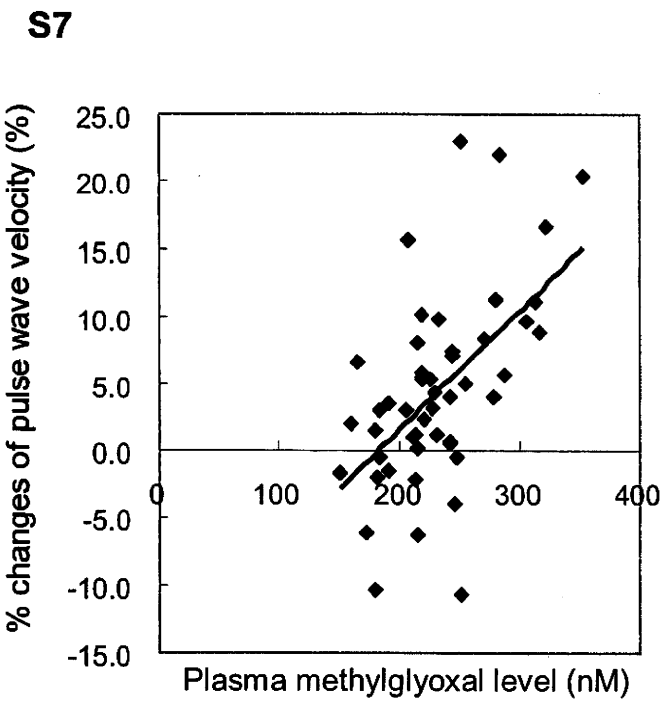


S6

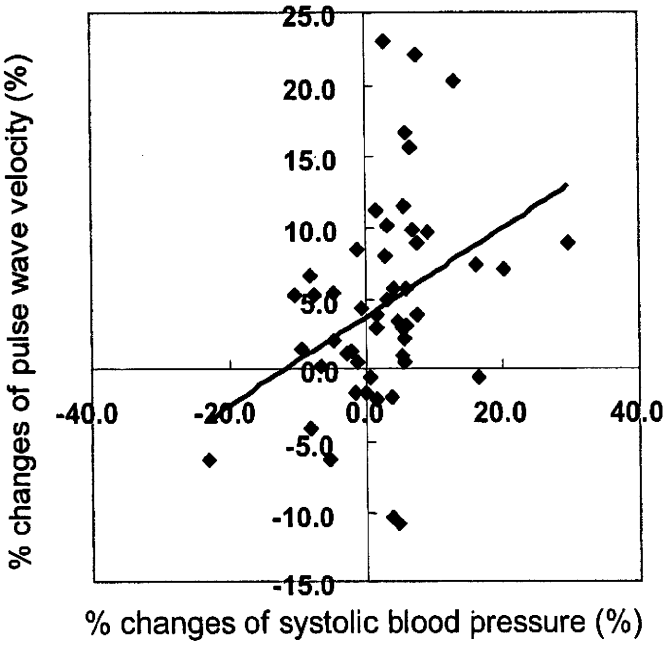


Supplementary Figure

The values of pulse wave velocity (PWV), systolic blood pressure (SBP) increased during the 5-year period. Methylglyoxal (MG) correlated significantly to the percent changes of PWV (S7): $y = 0.0892x - 16.296$, $r = 0.5697$, $p < 0.01$, percent change of SBP correlated significantly to the percent changes of PWV (S8): $y = 0.3092x + 3.7201$, $r = 0.3617$, $p < 0.01$.



S8



平成２２年度厚生労働科学研究費補助金
（循環器疾患等生活習慣病対策総合研究事業）
研究分担報告書

糖尿病に対する新規細胞移植治療の開発

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研究要旨　メタボリックシンドロームは糖尿病、血管病のハイリスクであることから適切な診断と予防が必要である。しかしながら、糖尿病発症はわが国のみならず世界中で頻度が増加し、高齢化地域では合併症を考慮した基礎インスリン補充の開発も考える必要がある。そこで、糖尿病を対象に患者脂肪組織由来の初代培養細胞（前脂肪細胞）に補充目的遺伝子を導入しこれを自家移植する普遍的な遺伝子細胞治療法を樹立することを目的に、本研究は、腎不全、視力障害などの重篤な合併症を引き越す家族性レシチンコレステロールアシルトランスフェラーゼ(LCAT)欠損症における長期に安定したLCAT補充に関わる新規の治療法の開発に着手した。昨年度までにLCAT遺伝子導入ヒト脂肪細胞は、GMP製造、移植細胞の品質試験にもとづいて調整し、遺伝子導入特性や遺伝子導入後の細胞の性状について*in vitro* 及び動物実験で検討し、前脂肪細胞の特性が遺伝子治療用細胞として適していることを見出し、さらにその安全性に関する知見を蓄積してきた。本年度は、薬効に関する評価、移植用scaffoldの検討を実施した。遺伝子導入前脂肪細胞の分泌するLCAT蛋白の機能評価としてLCAT欠損症患者血清への*in vitro*添加試験により、LCAT機能欠損によるHDLの成熟障害が改善された。移植時の細胞のscaffoldとしてフィブリンゲルに着目、マウス移植実験で血中へのLCAT蛋白補充を指標において評価した結果、マウス実験で用いられるマトリゲルと同等の性能を見出した。本移植技術の臨床応用に向け厚生労働省へ本計画を申請し厚生科学審議会による審議を受け、その指摘事項に対応する追加検討を実施している。今後、移植技術を至適化し、本治療法の安全性とLCAT欠損マウスモデルで有効性評価及び生着性を確証し本治療法の臨床適用を予定する。臨床で有効性・安全性を確立した後、糖尿病への適応研究を目指す。

A. 研究目的

糖尿病は日本、米国に加えてアジア地域における多くの人々の生活レベルを規定する疾患である。したがって、そのハイリスクであるメタボリックシンドロームの診断と適切な予防法が重要である。しかしながら、糖尿病を発症した場合、とりわけ高齢者では、合併症を考慮した基礎インスリン補充が求められる。

われわれは、単離・培養及び遺伝子導入の容易さに加えて、特異的に脂肪細胞に分化しがん化などの形質転換の報告がない前脂肪細胞と、分裂細胞への遺伝子導入効率が高く、長期にわたる蛋白質発現が可能なレトロウイルスベクターとの組み合わせに着目し、脂肪細胞移植を用いた新規の蛋白補充技術のコンセプトを糖尿病モデルマウスとヒトインスリン遺伝子導入マウス前脂肪細胞を用いて明らかにし、この成果に基づき糖尿病の新規治療法として、インスリン分泌脂肪細胞の移植治療法の開発を目的とした研究を進めてきた。平成22年度は、このような背景の中で、遺伝子導入脂肪細胞移植技術を、酵素欠乏症に対する普遍的治療法として適応するため、治療用組換え蛋白が存在せず、

有効な治療法の存在しない先天性酵素欠乏症である家族性LCAT欠損症に対する治療法を臨床導入することを目指した研究を実施した。

B & C. 研究方法と結果

1．ヒトLCAT遺伝子導入前脂肪細胞が分泌するLCAT蛋白の機能発現評価

天井培養法により調製した前脂肪細胞に対して、レトロウイルスベクターを用いてhLCAT遺伝子を導入し、得られた細胞より培養上清中に分泌されたLCAT蛋白についてその遊離型コレステロールのエステル化能を3H-コレステロールを含む人工基質をもちいて評価した。その結果、導入されたhLCAT遺伝子コピー数に依存したエステル化活性が認められた。また、遺伝子導入法の工夫により、さらに高コピー数の遺伝子導入が可能となっており、臨床効果の発揮に治療用蛋白の更なる分泌が要求される病態にも将来的に応用可能であることが示唆された。

このLCAT欠損症は現在まで報告されているだけでも80種類以上の遺伝子変異が存在するが、この治療技術を臨床応用するためには、本治療法による効果が期待できるかどうかを評価し得る薬効解析システムを構築する必要がある。

われわれは、LCAT欠損症患者血清と健常人血清のリポ蛋白分布の違いに着目し、患者血清に前脂肪細胞が分泌したLCAT蛋白を添加し、添加後のHDLの成熟の度合いを2次元電気泳動とApoA-Iに対するウェスタンブロット法を組み合わせて評価した。その結果、魚眼病（T123I変異）患者血清において、ApoA-I含有HDLが添加したLCATの用量依存的に高分子側にシフトすること、すなわちHDLの成熟に寄与することを明らかにした（図1、Mol. Genet. Metab. 2011）。このことは、前脂肪細胞が分泌するLCAT蛋白が、患者血清中に補充された際に薬効が期待できることを示している。現在、この解析系を患者適合性評価試験として確立するべく、LCAT欠損症15症例を有するアムステルダム大学アカデミックメディカルセンター（Kastelein教授）との国際共同研究を進めている。

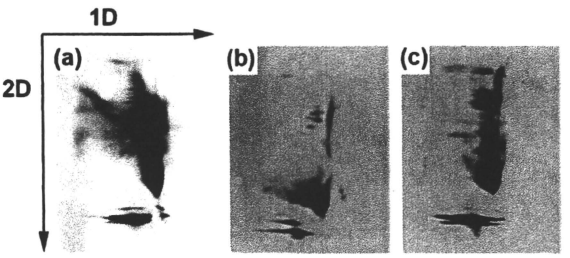

図1　患者血清でのリポ蛋白（Apo-AI）分布改善効果

(a) 健常人血清、(b) 魚眼病患者血清、未処置、(c) 魚眼病患者血清、hLCAT添加24時間処理、について2次元電気泳動後、ApoA-Iに対するウェスタンブロットを行った。

2．移植細胞の機能評価（LCAT蛋白の機能発現評価）と移植用のscaffoldの検討

移植後の持続的なLCAT産生を確保するための、移植細胞の生着率向上を目的とする製剤化検討を実施した。特に、移植細胞懸濁液に混合するscaffoldとして臨床での使用が可能なフィブリンゲルに着目した。マウス脂肪組織より、LCAT遺伝子導入マウス前脂肪細胞を調製、フィブリンゲルと共にマウスへの移植実験を行い、経時的に血清および移植細胞を回収した。マウス移植モデルにてLCAT蛋白の血中への分泌を評価した。その結果、フィブリンゲルは細胞の生存とその後のLCAT蛋白の血中への補充に寄与することが明らかとなった。同時に、マウスでの移植実験でscaffoldとして頻用されるマトリゲルと同等の血中分泌が認められ、フィブリンゲルが遺伝子治療臨床研究で使用可能なscaffoldであることが示唆された（図2、Exp. Mol. Med. 2011）。現在、フィブリンゲルを用いた移植効率の至適化検討を実施している。

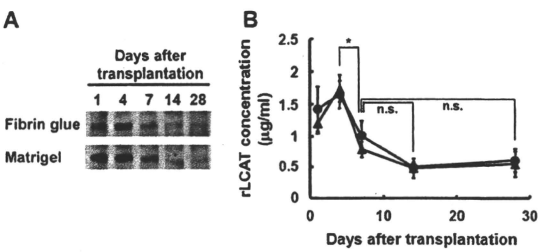

図2　フィブリンゲルの生着、LCAT分泌促進効果

フィブリンゲルとマトリゲルをscaffoldとしてhLCAT遺伝子導入マウス前脂肪細胞をB6マウスに皮下移植し、血中へのhLCAT補充について

経時的に検討した。A、マウス１個体で経時的に採血を実施し、血中のhLCATを検出した。B、マトリゲル移植群（●）とフィブリンゲル移植群（▲）について血中hLCAT分泌量の比較を行った。

３．家族性LCAT欠損症患者を対象とした臨床研究

平成22年4月、厚生労働省へ遺伝子治療実施計画を、それ申請し厚生科学審議会により審議された。6月、厚生科学審議会作業委員会で本臨床研究実施計画の概要を説明し、質問・照会事項を7月に受理し、それに対する回答書を10月に提出し追加検討を開始した。平成23年度は、改訂した臨床研究実施計画書を提出する予定である。臨床研究は千葉大学医学部附属病院で実施予定である。

(倫理面への配慮)

移植細胞の薬効薬理および生着性に関する研究は、千葉大学大学院医学研究院の規定に従い、国で定められている、ヒト生体由来細胞を用いた実験、組換えDNA実験、動物取り扱いに関する指針に従い、千葉大学で開催される各委員会で実験許可を受けて実施した。移植細胞の調製業務は、「遺伝子治療用医薬品の品質及び安全性の確保に関する指針」、「ヒト（自己）由来細胞・組織加工医薬品等の製造管理・品質管理の考え方について」、「医薬品の臨床試験の実施の基準に関する省令」に基づく「治験薬の製造管理及び品質管理基準および治験薬の製造施設の構造設備基準（治験薬GMP）について」を満たす製造設備及び手順に遵守し製造した。臨床研究の実施に先立ち、臨床研究に関する計画書などについて、千葉大学医学部附属病院遺伝子治療審査委員会において、科学的、倫理的な観点から審議を受け、承認を受けた。さらに臨床研究計画書について厚生科学審議会での審議の後、厚生労働大臣の承認を受けた上で臨床研究を開始する。

DおよびE．考察および結論

本研究は、根本的治療法のない家族性LCAT欠損症、さらには糖尿病を対象として、遺伝子導入脂肪細胞の自己移植という新規の補充療法を実用化するトランスレーショナル研究である。現在の糖尿病に対するインスリン注射は頻回の投与が必須であり、今後のさらなる高齢化社会への移行と共に、それによる患者QOLの低下が非常に重要な課題である。これらの課題を克服できる本治療法の特徴は、すでに形成外科臨床領域で行われている脂肪吸引、脂肪移植を応用して遺伝子導入脂肪細胞を製品化し、自己移植により目的蛋白を長期にわたり安定して補充するという、これまで医療経済的に蛋白補充が困難であったまれな難治性疾患に広く応用することが可能な新規技術であることである。さらに我が国の糖尿病患者は推定2,000万人を超えると考えられており、本治療法の導入による医療経済効果は計り知れない。

しかしながら、自家移植した前脂肪細胞は、その生存が認められるとはいえ、移植後の減少は避けられず、治療目的に応じた移植条件の至適化検討が求められる。外来遺伝子を導入した移植細胞での治療目的蛋白質の持続的発現は動物及びヒトで一部見られているが、その安定した薬効発現のためには、*in vitro* 及び *in vivo* での多面的検討が必要である。

本研究により、LCAT搭載レトロウイルスベクター及び移植細胞のGMP製造法と品質試験法を確立した。また、*in vitro* 及び動物でのそれらの安全性を確認してきた。さらに、hLCAT遺伝子導入前脂肪細胞が分泌するLCAT蛋白が、LCAT欠損症患者血清中で障害されているHDLの成熟を健常人のそれに近づける改善効果があることを明らかにした。また移植における遺伝子導入前脂肪細胞の生着向上にフィブリンゲルが使用できることが示唆された。今後、移植後の持続的な治療蛋白産生を確保するための細胞移植技術の至適化を行い臨床研究の実施準備に着手する。厚生科学審議会追加検討事項の1つにLCAT遺伝子欠損マウスでの薬効評価試験があり、これまで検討してきた移植生着技術を応用予定である。また、インスリン遺伝子導入脂肪細胞移植治療開発においては、移植後の細胞からの治療蛋白発現の変化について精査する必要がある。これらの基礎検討を実施し安全性配慮と法令等遵守のもと本治療法の安全性並びに有効性評価及び生着性を検証する臨床研究を実施する予定である。

F．健康危険情報

特記事項なし

G. 研究発表

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H. 知的財産権の出願、登録状況

「遺伝子治療用脂肪細胞の調製のための外来遺伝子の導入に適した細胞集団かどうかを判定する方法」

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別紙4

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Aoyagi Y, Kuroda M, Asada S, Bujo H, Tanaka S, Konno S, Tanio M, Ishii I, Aso M, Saito S	Fibrin glue increases the cell survival and the transduced gene product secretion of the ceiling culture-derived adipocytes transplanted in mice	Exp. Mol. Med.	43	161-167	2011
Kuroda M, Aoyagi Y, Asada S, Bujo H, Tanaka S, Konno S, Tanio M, Ishii I, Machida K, Matsumoto F, Satoh K, Aso M, Saito Y.	Ceiling culture-derived proliferative adipocytes are a possible delivery vehicle for LCAT protein replacement therapy.	The Open Gene Ther. J.	4	1-10	2011
Asada S, Kuroda M, Aoyagi Y, Bujo H, Tanaka S, Konno S, Tanio M, Ishii I, Aso M, Saito Y.	Disturbed apolipoproteinA-I containing lipoproteins in fish eye disease is improved by lecithin: cholesterol acyltransferase produced by the gene-transduced adipocytes	Mol. Genet. Metab.	102(2)	229-231	2011
黒田正幸、武城英明	脂肪細胞による新規蛋白質補充療法ーLCAT欠損症遺伝子治療臨床研究ー	医学のあゆみ	233	1246-1247	2010

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Fibrin glue increases the cell survival and the transduced gene product secretion of the ceiling culture-derived adipocytes transplanted in mice

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Abbreviations: ccdPA, ceiling culture-derived proliferative adipocytes; LCAT, phosphatidylcholine-sterol O-acyltransferase; PPAR, peroxisome proliferator-activated receptor

Abstract

The development of clinically applicable scaffolds is important for the application of cell transplantation in various human diseases. The aims of this study are to evaluate fibrin glue in a novel protein replacement therapy using proliferative adipocytes and to develop a mouse model system to monitor the delivery of the transgene product into the blood and the fate of the transduced cells after transplantation. Proliferative adipocytes from mouse adipose tissue were transduced by a retroviral vector harboring the human lecithin-cholesterol acyltransferase (*lcat*) gene, and were subcutaneously transplanted into mice combined with fibrin glue. The *lcat* gene transduction efficiency and the subsequent secretion of the product in mouse adipocytes were enhanced using a protamine concentration of 500 μg/ml. Adipogenesis induction did not significantly affect the *lcat* gene-transduced cell survival after transplantation. Immunohistochemistry showed the ectopic enzyme production to persist for 28 days in the subcutaneously transplanted gene-transduced adipocytes. The increased viability of transplanted cells with fibrin glue was accompanied with the decrease in apoptotic cell death. The immunodetectable serum LCAT levels in mice implanted with the fibrin glue were comparable with those observed in mice implanted with Matrigel, indicating that the transplanted *lcat* gene-transduced adipocytes survived and functioned in the transplanted spaces with fibrin glue as well as with Matrigel for 28 days. Thus, this *in vivo* system using fibrin is expected to serve as a good model to further improve the transplanted cell/scaffold conditions for the stable and durable cell-based replacement of defective proteins in patients with LCAT deficiency.

Keywords: adipocytes; enzyme replacement therapy; fibrin tissue adhesive; phosphatidylcholine-sterol O-acyltransferase; tissue scaffolds; transplantation

Introduction

Aspirated fat is a common source of autologous tissue transplantation for the correction of tissue defects in plastic and reconstructive surgery (Billings and May, 1989; Patrick, 2000, 2001). Recent studies have shown that the preadipocytes in aspirated fat are multipotential and implicated in the source of cell-based therapies (Stashower *et al.*, 1999; Zuk *et al.*, 2001; Gimble *et al.*, 2007). One such potential is the high capability for exogenous gene transduction and the secretion of transgene products (Ito *et al.*, 2005). We have recently identified human ceiling culture-derived proliferative adipocytes (h-ccdPA) in subcutaneous adipose tissue, and proposed the application of gene-transduced h-ccdPA to the long-lasting replacement therapy for a variety of inherited or acquired gene-defective diseases (Asada *et al.*, 2011; Kuroda *et al.*, 2011).

A key factor in the protein delivery system via the autotransplantation of various types of gene-transduced cells is the regulation of the survival and the secretory function of these cells at the transplanted space. We have shown that the nutri-

tional condition of the recipient is one of the important factors for the survival and the gene expression of adipocytes in the fat graft after subcutaneous transplantation in mice (Matsumoto *et al.*, 2002). In addition, the secretion of vascular endothelial growth factor (a bioactive molecule secreted from the vascular system) around the transplanted graft in recipients is also important for long-term cell survival (Yamaguchi *et al.*, 2005). Particularly, recent studies have highlighted the importance of various cytokines for the regulation of cell function and the surrounding matrix conditions (Kimura *et al.*, 2003; Cho *et al.*, 2006; Torio-Padron *et al.*, 2007b; Kuramochi *et al.*, 2008; Ning *et al.*, 2009). Together with the consideration of cytokine delivery for the transplanted cells, the development of scaffolds for transplantation contributes to the early construction of the surrounding matrix around the transplanted site. Insulin gene-transduced cells transplanted with Matrigel as a scaffold have been shown to survive as insulin-secreting adipocytes for three months after transplantation (Ito *et al.*, 2005). It is therefore critical to set up an appropriate clinically applicable scaffold for the adipocyte transplantation into patients, which allows not only a longer survival of the implanted cells but also guarantees a longer-lasting secretion of the therapeutic gene product into the blood stream.

In this study, we have optimized the gene transduction conditions for the most effective retroviral vector-mediated gene transduction using ceiling culture-derived proliferative adipocytes from mouse adipose tissue (m-ccdPA). We established a mouse model for the transplantation with the expanded human enzyme gene-transduced m-ccdPA for the evaluation of protein delivery in the serum of the mice. Using an *in vivo* model, we analyzed the effect of fibrin glue (Malafaya *et al.*, 2007; Mano *et al.*, 2007; Neuss *et al.*, 2008) as a clinically applicable scaffold on the efficacy of the circulating enzyme delivery.

Results

Establishment of human *lcat* gene transduced m-ccdPA (m-ccdPA/*lcat*)

We have recently established h-ccdPA, which secretes functionally active hLCAT, a key circulating enzyme for serum cholesterol esterification, and proposed a novel cell-based gene therapy by the subcutaneous transplantation of the cells for the long-lasting replacement of the protein in the patients with LCAT deficiency (Kuroda *et al.*, 2011). In order to establish the most suitable mouse model for the evaluation of the effect of the scaffold on the survival and function of the transplanted adipocytes, we first prepared m-ccdPA for the *lcat* gene transduction as donor cells for the recipient mice. The biochemical characterization showed that the prepared m-ccdPA have morphological features and surface antigen expression patterns similar to those of h-ccdPA (unpublished data). Our preliminary experiments showed that the transduced m-ccdPA secreted a much lower amount of hLCAT than the h-ccdPA when the average copy number

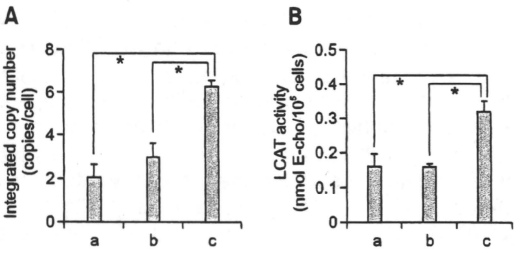

Figure 1. Enhanced gene transduction efficiency in m-ccdPA. Integrated copy numbers (A) and LCAT activity in the culture medium (B) after retroviral vector-mediated human *lcat* gene transduction were analyzed. Single round (b) and two rounds (a, c) of exposure to CGT_hLCATRV in the presence of 8 (a) or 500 μg/ml (b, c) of PS. Transductions with 8 μg/ml (a) and 500 μg/ml (b, c) of PS were performed overnight and one hour, respectively. Data are presented as the mean ± SD (n=3). *P < 0.05.

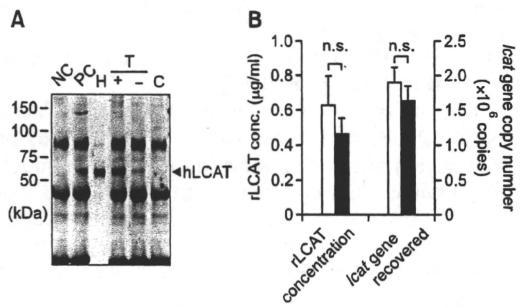

Figure 2. Detection of hLCAT and survival of human *lcat* gene after transplantation of *lcat* gene-expressing m-ccdPA. Human *lcat* gene-transduced mouse ccdPA (5×10^6 cells) were subcutaneously transplanted in nude mouse with fibrin glue as a scaffold. (A) Existence of hLCAT protein in mice sera was detected by IP-Western experiments. 15 μg human high density lipoprotein (HDL) was loaded for quantification of signals (H). Mouse serum with (PC) or without (NC) 15 μg HDL were subjected to IP-Western. The gene-transduced (T) m-ccdPA were transplanted with (+) or without (-) fibrin glue. Sera (100 μl) from the mice and mice transplanted with un-transduced (C) m-ccdPA were subjected to IP-Western analysis. (B) Human *lcat* gene-transduced mouse ccdPA (5×10^6 cells) were transplanted after three days of culture with (open bars) or without (closed bars) adipogenic differentiation medium. The serum concentrations of the hlcat protein were quantified by densitometric analysis (left), and the human *lcat* gene was quantified in excised implants (right).