Table 1 Association study for the candidate susceptibility genes for type 2 diabetes selected by multistage screening in the Japanese population

SNP ID	*Chr*	*Gene*	*Risk allele*	*Panel*	*RAF (DM)*	*RAF (NC)*	*P*	*OR*	*95% CI*
rs2250402	15	*EIF2AK4*	C	Replication-Japanese	0.23	0.20	0.04	1.17	1.01–1.36
				Replication-Chinese	0.24	0.23	0.41	1.05	0.93–1.19
				Meta-analysis			0.05	1.10	1.00–1.20
rs2307027	12	*KRT4*	C	Replication-Japanese	0.18	0.17	0.17	1.12	0.95–1.32
				Replication-Chinese	0.14	0.13	0.16	1.11	0.96–1.29
				Meta-analysis			0.05	1.12	1.00–1.25
rs3741872	12	*FAM60A*	C	Replication-Japanese	0.25	0.24	0.18	1.11	0.96–1.28
				Replication-Chinese	0.23	0.22	0.21	1.08	0.96–1.22
				Meta-analysis			0.07	1.09	0.99–1.20
rs574628	20	*ANGPT4*	G	Replication-Japanese	0.60	0.61	0.46	0.95	0.84–1.08
				Replication-Chinese	0.65	0.65	0.59	1.03	0.93–1.15
				Meta-analysis			0.96	1.00	0.92–1.08
rs2233647	6	*SPDEF*	G	Replication-Japanese	0.86	0.87	0.70	0.97	0.81–1.16
				Replication-Chinese	0.94	0.93	0.54	1.07	0.87–1.31
				Meta-analysis			0.90	1.01	0.88–1.16
rs3785233	16	*A2BP1*	C	Replication-Japanese	0.18	0.16	0.19	1.12	0.95–1.32
				Replication-Chinese	0.13	0.12	0.10	1.14	0.97–1.34
				Meta-analysis			0.04	1.13	1.01–1.27
rs2075931	1	Intergenic	A	Replication-Japanese	0.67	0.66	0.85	1.01	0.89–1.16
				Replication-Chinese	0.73	0.74	0.27	0.94	0.84–1.05
				Meta-analysis			0.48	0.97	0.89–1.06

Abbreviations: Chr, chromosome; OR, odds ratio for risk allele frequency.
Assignment of risk alleles was based on the original study.[15] Numbers of cases versus control subjects in the replication-Japanese and replication-Chinese panels were 1000 versus 1000 and 1416 versus 1577, respectively. RAF (DM) and RAF (NC) denote risk allele frequencies in cases and controls, respectively. *P* values were calculated for allele frequency. Meta-analysis was performed by the Mantel–Haenszel method (fixed-effects models). *P*-values for the test of heterogeneity among panels joined in the Mantel–Haenszel tests were all >0.05.

panels were included in the meta-analyses, these two loci, as well as the SNPs in *EIF2AK4* (rs2250402) and *FAM60A* (rs3741872), gave *P*-values of <0.001 and ORs between 1.15 and 1.18 (Supplementary Table 3). However, the *P*-values did not reach the proposed significance of GWAS ($=5\times10^{-7}$).

Selection of polymorphisms for the prediction model

To construct a reliable prediction model for diabetes, polymorphisms with strong evidence of association should be used. From the previous literature, we selected 15 genes (including one intergenic marker), that is, *SLC30A8*, *HHEX*, *LOC387761*, *EXT2*, *CDKN2A/B*, *GCKR*, *IGF2BP2*, *CDKAL1*, *FTO*,[1–5] *TCF7L2*,[22] *KCNJ11*,[23] *PPARG*,[24] *WFS1*,[25] *HNF1B*[26] and *KCNQ1*,[15] as candidate genes to be included in both gene–gene interaction analysis and construction of a prediction model. Starting from 23 SNPs in these 15 genes, we selected 11 SNPs in 11 genes according to the following process. There is sufficient evidence of the associations of *KCNQ1* and *TCF7L2* genes with diabetes as supported by replication studies in the Japanese population.[6,15,27] In addition, *SLC30A8*, *HHEX*, *CDKN2A/B*, *IGF2BP2* and *CDKAL1* associated with the disease in the European population were found in our earlier study to be associated with the disease in the Japanese population as well.[7–9]

To further extract genes with strong evidence of the association with diabetes, we attempted to replicate the associations reported earlier using our own data (analysis panel with 2424 cases and 2424 controls). For the 19 SNPs in *SLC30A8*, *HHEX*, *LOC387761*, *EXT2*, *CDKN2A/B*, *GCKR*, *IGF2BP2*, *CDKAL1*, *FTO*, *TCF7L2*, *KCNJ11*, *PPARG* and *KCNQ1*, we extracted genotyping data from our earlier studies[6–9,15,27–29] and, if necessary, genotyped additional subjects to obtain a data set for 2424 cases and 2424 controls of the Japanese population (analysis panel). The SNPs in *WFS1* (rs6446482, rs734312) and *HNF1B* (rs7501939, rs4430796) were genotyped for this study in the same individuals. SNPs with *P*-values for the test of deviation from the Hardy–Weinberg equilibrium of <0.01 were excluded for further analysis. When two SNPs were located in the same genomic region, the one with the lower *P*-value for the association test was selected for further analysis. *GCKR*, for which we earlier reported the marginal association with type 2 diabetes,[7] was found to be associated with the disease in this enlarged Japanese panel ($P=1.7\times10^{-5}$; Supplementary Table 4). *KCNJ11* and *PPARG*, which have been included in the genes associated with diabetes in Caucasians, showed marginal associations ($P=0.066$ and $P=0.075$, respectively; Supplementary Table 4) in our panel. Two SNPs in *WFS1* and two SNPs in *HNF1B* were newly genotyped in the analysis panel. Although no association was apparent between *WFS1* and type 2 diabetes, both SNPs in *HNF1B* exhibited *P*-values of <0.05 (Supplementary Table 4). From these data, we included 11 SNPs in 11 genes as described above for the source of genotype data to be analyzed in both the examination of gene–gene interaction and the prediction of phenotypes.

Gene–gene interaction

We evaluated multiplicative gene–gene interaction for each pair of the 11 loci as described in Materials and methods. Two combinations, rs1801282 (*PPARG*) ×rs1470579 (*IGF2BP2*) (nominal $P=0.0025$) and rs1801282×rs3802177 (*SLC30A8*) (nominal $P=0.018$), showed *P*-values of less than 0.05 (Supplementary Figure 1). However, these *P*-values were not significant when Bonferroni's correction for multiple testing was applied (significance level, $0.05/55=9.1\times10^{-4}$). Although *PPARG* and *IGF2BP2* are located on the same chromosome (3p25 and 3q28, respectively), it is unlikely that loci on different arms of the same chromosome show significant linkage disequilibrium. *SLC30A8* is located on a different chromosome (8q24.11) from

PPARG. The reason why nominal *P*-values of these combinations showed less than 0.05 may be because of the low minor allele frequency of rs1801282.

Cumulative risk assessment for type 2 diabetes on the basis of susceptibility genes

As there was no evidence of gene–gene interaction between 11 SNPs of 11 genes, *SLC30A8*, *HHEX*, *CDKN2A/B*, *GCKR*, *IGF2BP2*, *CDKAL1*, *TCF7L2*, *KCNJ11*, *PPARG*, *KCNQ1* and *HNF1B*, they were included in the prediction model as independent variables with the additive effect (additive effect in the liability and multiplicative effect in the odds) without interaction terms. Effective numbers of cases and controls whose genotypes for the 11 loci were successfully obtained were 2316 and 2370, respectively. The Cochran–Armitage trend test gave a *P*-value of 4.7×10^{-56} for the trend in the increase in the odds for cases relative to controls with an increasing number of risk alleles for the 11 susceptibility loci (Supplementary Table 5). We then estimated ORs for type 2 diabetes in subjects with different numbers of risk alleles on the basis of the multiplicative model by logistic regression analysis with adjustment for age, sex and BMI. The ORs for type 2 diabetes in subjects with 7–18 risk alleles in comparison with those harboring 0–6 risk alleles are shown in Figure 1. An increase of one risk allele resulted in an average increase in the odds of 1.29 (95% CI=1.25–1.33, $P=5.4\times10^{-53}$, logistic regression analysis).

To predict disease status for type 2 diabetes in a given individual, we constructed a prediction model on the basis of the number of risk alleles or the liability value calculated from the number of risk alleles as well as age, sex and BMI. The coefficients to calculate the liability value were estimated with the logistic regression model. To estimate the predictive power of the model, we generated ROC curves as described in Materials and methods. The AUC was 0.63 when only the number of risk alleles was used for the prediction. When age, sex and BMI were also included, the AUC increased to 0.72 (Figure 2). Meanwhile, an AUC value for the ROC curve based on only age, sex and BMI was 0.68, which was better than that based on only the number of risk alleles (data now shown). The model incorporating age, sex and BMI as well as the number of risk alleles thus showed moderate power for the prediction of type 2 diabetes. The best accuracy was 0.66 at the threshold between non-diabetic and diabetic status of 0.52 (non-diabetic status=0, diabetic status=1), for which the specificity and the sensitivity were 0.71 and 0.61, respectively.

DISCUSSION

By the validation of the results from our multistage GWAS, we detected only marginal associations of *EIF2AK4*, *KRT4* and *A2BP1* with type 2 diabetes in meta-analyses with two subject panels of Japanese or Chinese individuals. Relations of *KRT4* (keratin 4 gene) and *A2BP1* (ataxin-2-binding protein 1 gene, also known as *FOX1*) to glucose or lipid metabolism are unknown. Deletion of *EIF2AK4* (eukaryotic translation initiation factor 2 alpha kinase 4 gene, also known as *GCN2*) in mice resulted in liver steatosis during leucine deprivation as a result of unrepressed expression of lipogenic genes.[30] The functionally related gene, *EIF2AK3* (also known as *PERK* or *PEK*), has been shown to cause diabetes mellitus both in humans (Wolcott–Rallison syndrome, OMIM604032) and in rodent models.[31,32] Taken together, *EIF2AK4* may be a good candidate for the diabetes susceptibility gene. The sample size required for a statistical power of 0.80 with equal numbers of cases and controls is 10 505 when the frequency of the risk allele, OR and type I error probability are assumed to be 0.20, 1.10 (the value for *EIF2AK4* in the meta-analysis in Table 1) and 0.05, respectively. Further studies of these genes in other Asian populations as well as in other ethnic groups are needed for confirmation of their association with type 2 diabetes. Given this uncertainty, we did not include these genes in the assessments of cumulative risk and gene–gene interaction.

Among tens of type 2 diabetes susceptibility genes identified by recent GWASs in Caucasians, the associations of six genes, that is, *TCF7L2*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *SLC30A8* and *HHEX*, have been replicated in Asian populations as well as in populations of European ancestry. A recent meta-analysis in Japanese subjects also supported the associations.[12] In this study, we performed replication study, and, on the basis of the results, we added five more genes, that is, *KCNJ11*, *PPARG*, *GCKR*, *KCNQ1* and *HNF1B*, for the cumulative risk assessment for type 2 diabetes. Thus, the SNPs of *HNF1B*, which were earlier associated with type 2 diabetes in Chinese as well as in Caucasians,[26] showed the association with the disease in the Japanese

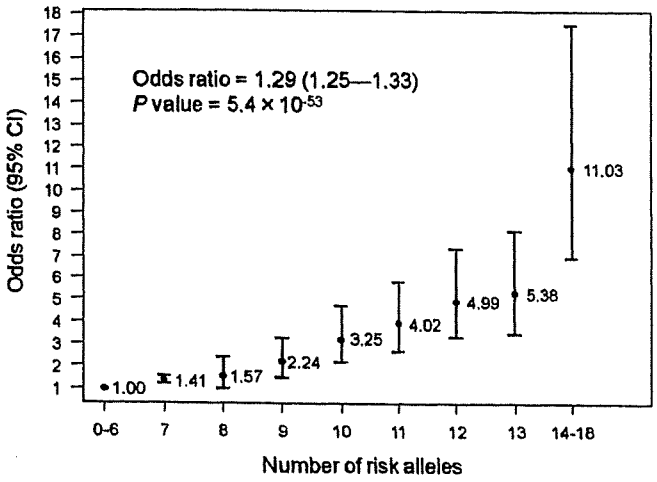

Figure 1 Odds ratios for subjects with different numbers of risk alleles for 11 susceptibility loci for type 2 diabetes. The cumulative effect of the 11 loci on type 2 diabetes was tested by counting the number of risk alleles associated with type 2 diabetes with a logistic regression model with adjustment for age, sex and BMI. The ORs for subjects with each number of risk alleles are expressed relative to individuals with 0–6 risk alleles.

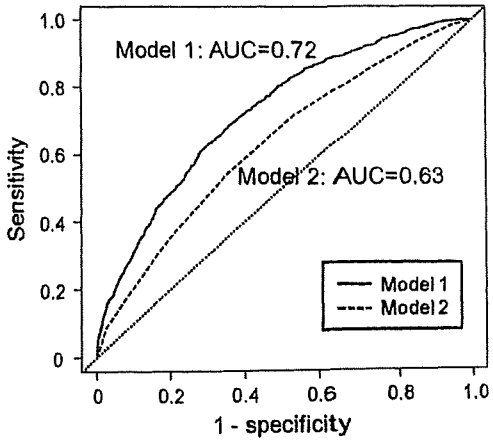

Figure 2 ROC curves for the prediction model on the basis of the number of risk alleles for 11 susceptibility loci for type 2 diabetes. The prediction model for type 2 diabetes was constructed using the logistic regression model, and ROC curves for the model were generated. In model 1, the number of risk alleles was used as an independent variable together with age, sex and BMI as covariates, whereas only the number of risk alleles was used as an independent variable in model 2.

population in this study. In addition, the *C* allele of rs780094 in *GCKR* was associated with increased risk of type 2 diabetes in this study, which is consistent with a recent study in Caucasians.[33] The associations of *KCNJ11* and *PPARG* with diabetes were marginal in this study; however, they were included for the prediction model, as the associations were replicated in some studies of Caucasians.

Our gene–gene interaction analysis showed no significant interaction for any of the 55 possible pairs of genes when corrected for multiple testing. When the significance level was set at 0.05, two pairs were judged to be significant. However, such gene–gene interactions were not supported from the functional point of view. A large-scale study may provide more convincing evidence for such interactions.

As no confirmatory evidence for gene–gene interaction was observed, we treated the 11 genes as independent variables in the prediction model. The addition of one risk allele was estimated to increase the odds by an average of 1.29 according to the multiplicative model. This value is similar to that (1.24) estimated for type 2 diabetes in Caucasians.[17] Two earlier cumulative risk assessments for type 2 diabetes in Asian populations with relatively small numbers of associated loci yielded values of 1.17 and 1.24 for the fold increase in risk for each additional risk allele.[11,34] In our prediction model for type 2 diabetes, the AUC for the ROC curve was lower than that in the earlier study[17] based on 15 loci in Caucasians (0.72 and 0.86, respectively). However, the number of loci in our study (11 loci) was lower than that in the study for Caucasians. The inclusion of additional loci in our model should improve its ability to predict type 2 diabetes in Asian populations. Several reports of the prediction of type 2 diabetes using ~18 loci were recently described for populations of European ancestry.[35–38] A prediction based on 18 loci gave an AUC value of 0.80 for the ROC curve,[35] whereas the corresponding values for a population-based prospective study were 0.68,[36] 0.615[37] and 0.75.[38] They concluded that genetic variations associated with diabetes had a small effect on the ability to predict the development of type 2 diabetes as compared with clinical characteristics alone. In fact, the AUC value (0.72) based on both the genetic variations and the clinical characteristics was slightly better than that based on only the clinical characteristics (0.68). We admit that the evidence of the association with diabetes is a little weaker for *KCNJ11* and *PPARG* in the Japanese population than for the other nine genes. If *KCNJ11* and *PPARG* were excluded from the analysis, the AUC for the ROC curve in the prediction model incorporating age, sex and BMI remained unchanged at 0.72, probably because of the relatively large effects of *KCNQ1* and *TCF7L2*.

Finally, our prediction model for type 2 diabetes achieved limited success even though it has some value. Given that GWASs for diabetes in Asians have not been as extensive as those in Caucasians, many risk loci for diabetes in Asians remain most likely to be undiscovered. Considering that the average increase in OR conferred by each additional risk allele was similar between Caucasians and Japanese, incorporation of data from additional risk loci is most likely to increase the predictive power.

ACKNOWLEDGEMENTS

We thank all the subjects who joined this project; Sumio Sugano and Shoji Tsuji for support and helpful discussion throughout the project; and Megumi Yamaoka-Sageshima for technical assistance. This work was supported by a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan; a grant from the National Institute of Biomedical Innovation (NIBIO) of Japan; grants from the Ministry of Health, Labour and Welfare of Japan; a Grant-in-Aid for Scientific Research on Priority Areas (C), 'Medical Genome Science (Millennium Genome Project)', 'Applied Genomics', and 'Comprehensive Genomics', from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a grant from the Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER, Tokushima, Japan). The Hong Kong diabetes case–control study was supported by the Hong Kong Research Grants Committee Central Allocation Scheme CUHK 1/04C.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

INTERNAL MEDICINE

□ CASE REPORT □

Peginterferon (PEG-IFN) Plus Ribavirin Combination Therapy, but neither Interferon nor PGE-IFN Alone, Induced Type 1 Diabetes in a Patient with Chronic Hepatitis C

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Abstract

Interferon (IFN) therapies, including IFN, peginterferon (PEG-IFN) and ribavirin (RBV) plus PEG-IFN combination, are widely used for patients with chronic hepatitis C. We encountered a patient with chronic hepatitis C in whom previous IFN or PEG-IFN alone had not induced type 1 diabetes (T1D), while the addition of RBV to PEG-IFN did induce T1D. The patient had HLA types conferring highly susceptibility to T1D. Thus, adding RBV to PEG-IFN may render chronic hepatitis C patients, with T1D-susceptible HLA types, more prone to developing T1D than IFN or PEG-IFN alone. To prevent T1D development, we recommend HLA typing prior to initiating RBV plus PEG-IFN administration.

Key words: human leukocyte antigen, anti-glutamate acid decarboxylase (GAD) antibody, anti-insulinoma-associated antigen (IA)-2 antibody, autoimmune disease

(Inter Med 48: 1387-1390, 2009)
(DOI: 10.2169/internalmedicine.48.2220)

Introduction

Interferon (IFN) is used for chronic hepatitis C and B. Until recently, IFNα was the main option for treating chronic hepatitis C (1). Now, peginterferon (PEG-IFN, polyethylene glycol-binding IFN) combined with ribavirin (RBV), which augments IFN action, is a standard anti-viral therapy for chronic hepatitis C (2). However, IFN therapy can adversely impact the immune system and induce autoimmune diseases including type 1 diabetes (T1D) (3, 4). Not only IFN, but also PEG-IFN (5) and PEG-IFN plus RBV therapy (6, 7), can reportedly induce T1D. We encountered a patient with chronic hepatitis C in whom neither IFN nor PEG-IFN alone induced T1D, while RBV plus PEG-IFN did induce T1D with elevated anti-glutamate acid decarboxylase (GAD) and anti-insulinoma-associated antigen (IA)-2 antibodies. Herein, we emphasize the risk of T1D development with PEG-IFN plus RBV therapy.

Case Report

A woman was diagnosed as having chronic hepatitis C at the age of 53 and received IFNα 1 million IU/week for 6 months, and subsequently became negative for viral marker (HCV-RNA). At age 60, she was diagnosed as having type 2 diabetes; fasting plasma glucose (FPG) was 199 mg/dL and HbA1c 6.9%. Her HbA1c improved with glimepiride 1 mg/day. Because HCV-RNA was again increased, PEG-IFNα 180 μg/week was started at age 61. Due to a taste disorder, the dose was decreased to 90 μg/week two months later and continued for 9 months. During PEG-IFN administration, blood glucose control worsened, but adding buformin (150 mg/day) to her treatment regimen decreased HbA1c from

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Received for publication February 27, 2009; Accepted for publication April 6, 2009
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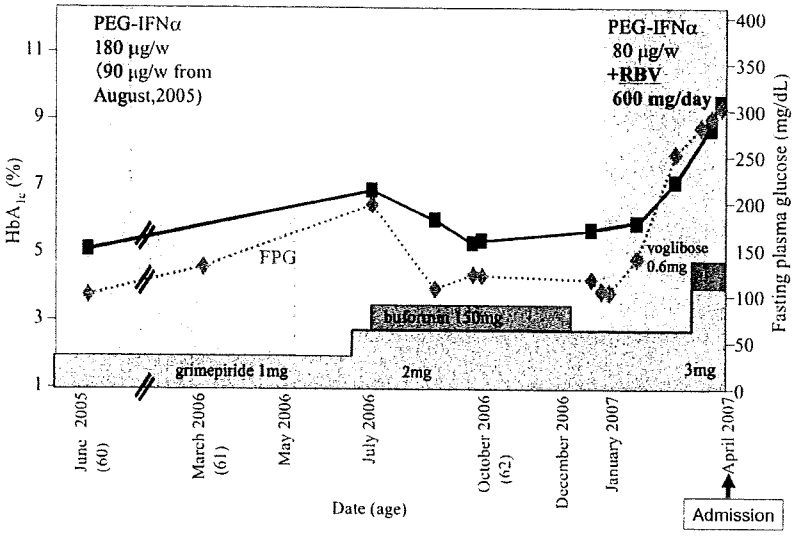

Figure 1. Clinical course: HbA_{1c} and fasting plasma glucose.

Table 1. Data on Admission

WBC	1600 /μL	T-Bil	0.9 mg/dL	BUN	11 mg/dL	Glu	303 mg/dL
Seg	53 %	AST	53 IU/L	Cr	0.4 mg/dL	HbA1c	9.5 %
Eos	2 %	ALT	60 IU/L	UA	2.3 mg/dL	Anti-GAD antibody	27.0 U/mL (0-1.4)
Baso	0 %	ALP	213 IU/L	Na	135 mEq/L	Anti-IA-2 antibody	3.0 U/mL (0-0.3)
Lym	34 %	γ-GTP	40 IU/L	K	4.1 mEq/L	Anti-insulin antibody	6.5 % (0-10)
Mon	11 %	LDH	195 IU/L	Cl	102 mEq/L	Fasting IRI	4.3 μU/mL(1.84-12.2)
RBC	326×10^4/μL	ZTT	10.2 U	Ca	8.7 mg/dL	Blood C-peptide	0.6 ng/mL (1.5-3.5)
Hb	10.7 g/dL	TTT	6.1 U	TG	54 mg/dL	Urinary C-peptide	25.0 μg/day (41-145)
Ht	31.3 %	CHE	304 IU/L	T-Cho	78 mg/dL	Free T3	2.6 pg/mL (2.5-4.3)
Plt	78×10^4/μL	CPK	25 IU/L	HDL-C	27 mg/dL	Free T4	0.91 ng/mL (1.76-1.65)
TP	6.7 g/dL			LDL-C	39 mg/dL	TSH	6.72 μIU/mL (0.31-4.69)
A/G	1.48					TRAb	2.1 % (<15%)
						TgAb	138.2 IU/mL (0-44)
						TPOAb	149.9 IU/mL (<0.72)

Normal ranges are in parenthesis

7.0% to 5.0%. At age 62, because HCV-RNA levels had not decreased, PEG-IFN (80 μg/week) plus RBV (600 mg/day) combination therapy was started. Glycemic control rapidly deteriorated; FPG and HbA1c were increased to 280 mg/dL and 8.8%, respectively, two months after the initiation of RBV therapy (Fig. 1). One month later, the patient was admitted to our hospital for blood glucose control. On admission, her body mass index was 20.8 kg/m^2, with no remarkable physical findings. Laboratory data included high blood glucose (FPG 303 mg/dL, HbA1c 9.5%) with slightly elevated hepatic transaminases (AST/ALT 53/60 IU/L). It was noteworthy that she was positive for both anti-GAD and anti-IA2 antibodies. Thyroid hormone levels were normal with slightly elevated TSH. Anti-thyroglobulin antibody (TbAb) and anti-thyroid peroxidase antibody (TPOAb) were positive (Table 1), suggesting autoimmune thyroiditis with subclinical hypothyroidism. Her HLA types included A24, DRB1*0405/0901, DQA1*0302 and DQB1*0401/0303, which confer high susceptibility to T1D. Based on positive autoantibodies against pancreatic islets, T1D was diagnosed. The PEG-IFN and RBV combination therapy was stopped and intensive insulin therapy was started, resulting in gradual improvement of blood glucose control with 35 units/day of insulin. Five months later, anti-GAD antibody remained positive (31.7 U/mL) with fair blood glucose control (HbA1c 5.5%) using 27 units/day of insulin.

Discussion

Since IFN was first reported to be effective for HCV infection in 1986 (8), IFN has been widely used for patients with chronic hepatitis C. However, autoimmune diseases, such as autoimmune thyroiditis (9), rheumatoid arthritis (10), autoimmune hepatitis (11), systemic lupus erythematosus (12) and T1D (13), reportedly develop with IFN therapy. In particular, several reports have documented the development of thyroid autoimmune disorders in cases receiving IFN plus RBV combination therapy (14, 15) and the present patient is likely such a case.

T1D is at least in part an autoimmune disease character-

ized by loss of pancreatic β cells with T lymphocyte infiltration of islets (16). IFNα activates T-helper (Th)1 lymphocytes which are $CD4^+$ and secrete interleukin-2, IFNγ and tumor necrosis factor β. These cytokines facilitate the generation of $CD8^+$ cytotoxic T cells which injure pancreatic β cells (17). In fact, IFNα is significantly up-regulated in patients with T1D (18). These findings suggest that IFNα is involved in β cell destruction and thereby in T1D development.

In 1992, it was documented for the first time that IFN therapy for chronic hepatitis C can induce T1D (13), and this was followed by similar case reports (reviewed in (19)). Subsequently, PEG-IFN therapy was also reported to induce T1D (5). Therefore, IFN administration is likely to affect Th1 immune reactions, leading to the development of T1D, as discussed above.

The present case was first diagnosed as having type 2 diabetes 7 years after IFN therapy. IFN therapy reportedly worsens insulin resistance, resulting in deterioration of glucose tolerance (20). In our case as well, blood glucose control deteriorated slightly during PEG-IFN therapy, though fair control of blood glucose was achieved with biguanide treatment but no insulin, indicating that the diabetes in this case was not clinically insulin-dependent T1D during this period. In contrast, after RBV was added to PEG-IFN, glucose control rapidly worsened with positive autoantibodies, i.e. anti-GAD and anti-IA2 antibodies, suggesting T1D onset. Although the possibility that IFN or PEG-IFN alone had induced T1D several years or months earlier can not be completely excluded in this patient, her clinical course (see Fig. 1) strongly suggests that the RBV addition was a trigger for T1D development.

It is likely that previous administrations of IFN and PEG-IFN alone had not induced T1D, while adding RBV to PEG-IFN had induced T1D in the same patient. These three anti-viral strategies for chronic hepatitis C can all reportedly induce T1D (3-7). To our knowledge, however, no studies have compared these three therapies in terms of the likelihood of T1D induction. The clinical course of our case strongly suggests that adding RBV renders patients, who have T1D-susceptible HLA, more prone to T1D development than either IFN or PEG-IFN alone. RBV is a guanosine analog which exerts immunological effects on Th1-like activation (21). Therefore, adding RBV to IFN therapy might augment the autoimmune response to IFN. We emphasize the importance of HLA typing, particularly prior to RBV addition, since the combination of PEG-IFN with RBV is now established as the first line therapy for chronic hepatitis C (22, 23). RBV administration should be avoided in patients with T1D-susceptible HLA.

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Islets 1:1, 73-75; July/August 2009; © 2009 Landes Bioscience

Identification of a novel mechanism regulating β-cell mass

Neuronal relay from the liver to pancreatic β-cells

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Key words: compensatory β-cell response, β-cell proliferation, regenerative medicine, diabetes, insulin, inter-organ metabolic communication, neuronal relay, insulin resistance

Submitted: 03/31/09
Accepted: 04/01/09

Previously published online:
www.landesbioscience.com/journals/islets/article/8615

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Addendum to: Imai J, Katagiri H, Yamada T, Ishigaki Y, Suzuki T, Kudo H, et al. Regulation of pancreatic β-cell mass by neuronal signals from the liver. Science 2008; 322:1250–4; PMID: 19023081; DOI: 10.1126/science.1163971.

Recent studies have demonstrated that β-cell replication plays a central role in maintaining adult β-cell mass. β-cell proliferative activity changes dynamically to meet systemic needs throughout life. One condition in which β-cell proliferation is enhanced is obesity-related insulin resistance. However, the mechanism underlying this compensatory β-cell response is not well understood. We have identified a neuronal relay, originating in the liver, which enhances both insulin secretion and pancreatic β-cell proliferation. Blockade of this neural relay in murine obesity models inhibited pancreatic islet expansion during obesity development, showing this inter-organ communication system to be physiologically involved in compensatory β-cell proliferation. While there is controversy about which mechanism, proliferation of pre-existing β-cells or production of new β-cells from progenitor cells, plays the dominant role in maintaining or regulating β-cell mass, we herein provide an example that proliferation of pre-existing β-cells contributes to a β-cell increment in obesity-related insulin resistance. Furthermore, we have shown the potential for clinical application of this inter-organ system as a therapeutic target for insulin-deficient diabetes.

Although pancreatic β-cells were once thought to be incapable of significant proliferation due to being in a terminal differentiation state, recently derived evidence has revealed that β-cell replication plays a central role in maintaining adult β-cell mass.[1] In addition, rates of β-cell proliferation change dynamically according to metabolic demand throughout life.[2] Proliferation of β-cells occurs at a high rate during the late embryonic stage, but begins to decline postnatally. During adulthood, though β-cells continue proliferating, the rate of proliferation gradually declines with age.[3] However, even during adulthood, proliferation rates of β-cells can increase considerably under specific conditions such as pregnancy or obesity. Enhancement of β-cell proliferation under such conditions is regarded as a compensatory mechanism in response to increasing systemic insulin demand. Although important roles of insulin[4] and glucose[5] in this β-cell compensation have been suggested, the mechanism underlying this process is not well understood. In fact, insulin-resistant animals[6,7] and human subjects[8] reportedly exhibit islet hyperplasia and/or hyperinsulinemia prior to the onset of detectable hyperglycemia, suggesting the existence of as yet unknown mechanisms enhancing compensatory β-cell proliferation in response to obesity-related insulin resistance.

Metabolism does not occur independently in multiple organs, but rather in a coordinated and regulated manner. This coordinated metabolic regulation requires inter-organ metabolic communication. During this decade, growing evidence has suggested an essential role of this inter-organ communication system in maintaining systemic metabolism, and that impairment of this system apparently contributes to the pathogenesis of metabolic disorders.[9] While humoral factors such as cytokines or nutrients are known to be important mediators of inter-organ

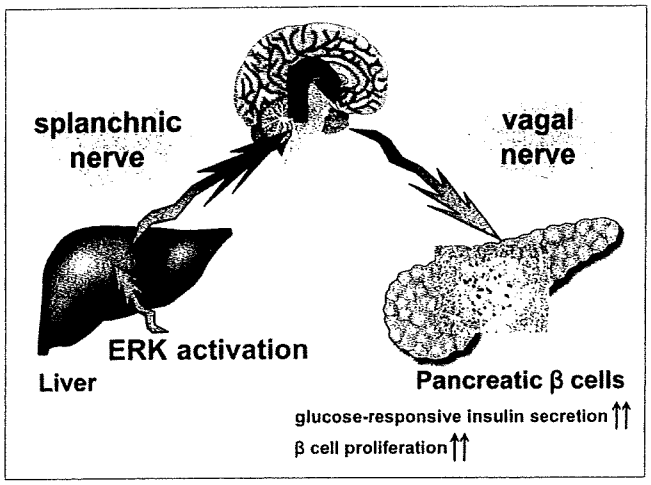

Figure 1. Schematic model of the neuronal relay originating in the liver.

communication, recent reports, including ours, have identified that neuronal signaling, consisting of both afferent and efferent autonomic nerves, also plays important roles in this system.[10] For instance, neuronal afferent signals from visceral adipose tissue modulate food intake,[11] those from the liver regulate systemic energy expenditure,[12] and sympathetic nerves regulate adiponectin synthesis in white adipose tissues.[13] In addition to these mechanisms, we have further unraveled that pancreatic β-cells are also regulated by in the nerve-mediated inter-organ communication system.[14]

To elucidate the mechanisms underlying these compensatory responses of β-cells, we expressed several genes, known to be upregulated or activated in the livers of obesity models, in the livers of lean mice. Among them, we found that hepatic extracellular signal-regulated kinase (ERK), phosphorylation of which is reportedly enhanced in the liver of a murine obesity model,[15,16] plays an important role in compensatory β-cell responses. To elucidate the metabolic roles of hepatic ERK activation, we expressed the constitutively active mutant of mitogen-activated protein kinase/ERK kinase (MEK-1) in the liver using an adenoviral gene transduction system.[17] Intriguingly, liver-selective ERK activation induced insulin hypersecretion and β-cell proliferation. These pancreatic effects of hepatic ERK activation were inhibited by splanchnic afferent blockade, pancreatic vagus dissection or midbrain transection. These results indicate that a neuronal relay system, consisting of the afferent splanchnic nerve, the central nervous system and the efferent vagus, mediates inter-organ (liver-to-pancreas) communication (**Fig. 1**). In addition, blockade of this neuronal relay at each of several steps in murine obesity models inhibited pancreatic islet expansion during obesity development, showing that this novel inter-organ communication system is physiologically involved in compensatory β-cell proliferation. Furthermore, it is noteworthy that, when applied to murine models of insulin-deficient diabetes, hepatic activation of ERK signaling induced β-cell regeneration and thereby improved diabetes.

This inter-organ machinery has been shown to physiologically elicit compensatory β-cell responses to obesity-related insulin resistance, and may thus function as a diabetes prevention system during obesity development. When systemic insulin demand is increased, it may be reasonable for animals to enhance glucose-responsive insulin secretion from preexisting β-cells in the early period and, subsequently to augment β-cell mass to later sustain insulin secretion. It is noteworthy that these sequential responses of β-cells are very similar to responses observed after activation of the hepatic ERK pathway, enhancing insulin secretion and augmenting β-cell mass during the early and late periods after hepatic ERK activation, respectively. Thus, activation of this inter-organ machinery is a possible candidate for the currently unknown trigger initiating compensatory β-cell responses.

We do not deny the possibility that progenitor cells contribute to the β-cell increment under certain conditions. In fact, we reported that bone marrow transplantation following pharmacological β-cell injury increased pancreatic islets in the vicinity of pancreatic ducts with a substantial increment in proliferating ductal cells,[18] suggesting generation of new islets from ductal progenitor cells. However, after activation of the hepatic ERK pathway, β-cell proliferation was tremendously increased within islets, suggesting that self-duplication of pre-existing β-cells is the main source of β-cell increments in this system. We performed further experiments to explore whether new β-cells were derived from progenitor cells or pre-existing β-cells proliferating in the context of inter-organ communication. We activated the hepatic ERK pathways of mice in which β-cells had been more thoroughly obliterated than in the experiments described in our original article, by administering a higher dose of streptozotocin. In these mice, pancreatic insulin contents were only slightly increased (from 6 to 9 ng/mg pancreas), indicating that the therapeutic effect of hepatic ERK activation depends on the remaining β-cell mass. Thus, increments in pancreatic insulin contents appear to be attributable to self-duplication of pre-existing β-cells. There is controversy about which mechanism, proliferation of pre-existing β-cells[1,19] or the production of new β-cells from progenitor cells,[20] plays the major role in regulating β-cell mass under both physiological and pathological conditions. We have provided an example, supporting the notion that proliferation of pre-existing β-cells contributes to β-cell increments, at least under one major pathological condition, obesity-related insulin resistance.

We would like to emphasize the potential therapeutic application of this inter-organ communication to insulin-deficient diabetes. Type 1 diabetes mellitus is characterized by progressive loss of β-cells, leading to a life-long dependence on insulin treatments. Recently, β-cells were also reported to be decreased in type 2 diabetes.[21] In such patients, one potential underlying mechanism is β-cell apoptosis induced by endoplasmic reticulum (ER) stress.[22-24] In this study, we succeeded in improving both types of insulin-deficient

diabetes in animal models. Generation of insulin-producing cells from a patient's own tissues may represent a strategy for the treatment of insulin-deficient diabetes, i.e., enhancing proliferation of pre-existing β-cells by activating endogenous neural machinery. Since ERK activation might elicit undesirable effects including tumor formation, further studies are needed to develop strategies for activating this neural pathway safely and selectively, other than hepatic ERK activation.

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Case Report

Eradication of insulin resistance

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Lancet 2009; 374: 264

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In July, 2007, an 84-year-old Japanese man, with a body-mass index of 23 kg/m², presented in a cold sweat and shivering. Clinical examination was unremarkable. His blood glucose was low (3·1 mmol/L). He was not taking any medications. Platelet count (285×10⁹ per L) and HbA_{1c} (5·0%) were normal. His symptoms resolved and he was discharged. At follow-up 1 month later, his platelet count had fallen to 56×10⁹ per L and his HbA_{1c} had risen (figure). Despite treatment with several antidiabetic agents, including acarbose, pioglitazone, and metformin, HbA_{1c} increased to 9·0% 9 months after the first visit, and he was still experiencing frequent hypoglycaemic attacks. Antibodies against insulin were not detected, but antibodies against the insulin receptor were present. Our patient's serum inhibited binding of iodine-125-labelled insulin to the insulin receptor of IM-9 cells by 69·1% at a 1 to 4 dilution. Fasting plasma insulin was very high (137·9 mU/L), indicating severe insulin resistance. Type B insulin resistance syndrome was diagnosed.

In the mean time, thrombocytopenia also worsened, with the platelet count falling to 10×10⁹ per L. Antibodies against platelets (PA IgG) were detected (302 fg/platelet). Leucocytes, erythrocytes, inflammatory markers, and complement factors were all within normal limits. Bone-marrow aspirate showed no evidence of megakaryocyte hypoplasia. There were no findings suggesting collagen diseases. CT showed no evidence of pancreatic tumour, liver cirrhosis, or splenomegaly. On the basis of these findings, immune thrombocytopenic purpura (ITP) was diagnosed. *Helicobacter pylori* infection was detected by the carbon-14 urea breath test and eradication therapy (amoxicillin, lansoprazole, and clarithromycin) was given for 7 days. Following *H pylori* eradication (confirmed by breath test) platelet count increased and HbA_{1c} decreased. 6 months after *H pylori* eradication PA IgG decreased (80 fg/platelet), insulin receptor antibodies were not detectable, HbA_{1c} normalised, and hypoglycaemic episodes no longer occurred. Notably, fasting plasma insulin decreased to 10·1 mU/L, confirming striking improvement of insulin resistance. When last seen in February, 2009, our patient was not taking glucose-lowering drugs; anti-IR antibodies were still undetectable, and HbA_{1c} was normal (4·8%). Our patient had not had any new hypoglycaemic symptoms.

Type B insulin resistance syndrome is a rare cause of diabetes with severe insulin resistance and is caused by polyclonal immunoglobulin G antibodies directed against the insulin receptor. These antibodies block insulin binding to the receptor, resulting in hyperglycaemia. Paradoxically, hypoglycaemia, particularly while fasting, is occasionally associated with this disorder. Type B insulin resistance syndrome is frequently associated with other autoimmune diseases.[1] Our patient's clinical course strongly suggests that type B insulin resistance syndrome and ITP developed simultaneously and that both improved with *H pylori* eradication, which is the recommended treatment for ITP.[2] In this case, *H pylori* eradication also ameliorated type B insulin resistance syndrome. There is increasing evidence that *H pylori* infection is directly involved in modulating host immune responses.[3] Furthermore, *H pylori* eradication reportedly ameliorates some immunological disorders, including antiphospholipid antibody syndrome and rheumatoid arthritis.[4,5] Our case suggests an *H pylori* infection-related pathological mechanism underlying type B insulin resistance syndrome. There is no established effective therapy for type B insulin resistance syndrome. Indeed, it was very difficult to manage our patient's diabetes, which was also associated with occasional hypoglycaemia; treatment was no longer necessary after *H pylori* eradication. In cases of type B insulin resistance syndrome, testing for *H pylori* infection may be worthwhile, with a view to treating the infection if present.

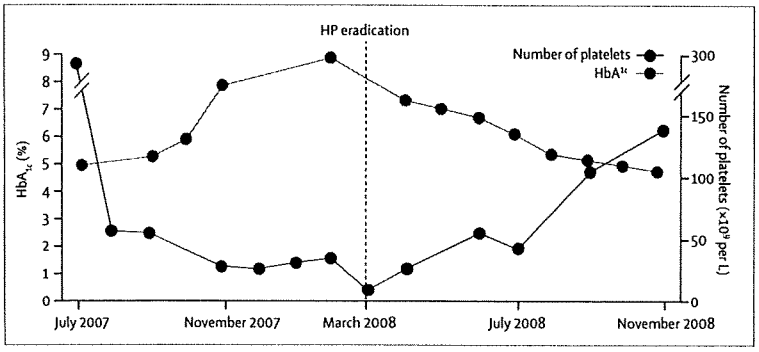

Figure: Clinical course

Contributors

All authors were involved in caring for the patient. JI, TY, YO, and HK wrote the report. JI and TY contributed equally.

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Circulating oxidized LDL: a biomarker and a pathogenic factor

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Current Opinion in Lipidology 2009, 20:363–369

Purpose of review

Oxidized LDL (oxLDL) contributes to many atherogenic steps in the vascular wall, but the significance of oxLDL in circulating blood remains unclear. Recent progress in procedures for measuring both human and murine oxLDL has provided growing evidence of the importance of circulating oxLDL.

Recent findings

Circulating oxLDL is elevated in patients with advanced atherosclerosis, such as coronary heart disease and ischemic stroke, and also reflects early atherosclerotic changes and metabolic disorders including diabetes and obesity. In-vitro exposure to oxLDL increased mononuclear cell nuclear factor-κB activity, suggesting a pathogenic role of circulating oxLDL in exacerbation of oxidative stress. In addition, adenoviral administration of secreted scavenger receptor-A1, which functions as a decoy, suppresses foam cell formation in LDL receptor-deficient mice via a blockade of modified LDL incorporation into macrophages. Furthermore, when lectin-like oxLDL receptor-1 was ectopically expressed in the liver, circulating oxLDL was reduced, resulting in complete prevention of atherosclerotic progression in apolipoprotein E-deficient mice. Thus, circulating oxLDL impacts atherogenic formation.

Summary

The roles of circulating oxLDL in atherosclerotic pathogenesis are now attracting considerable attention. OxLDL removal from circulating blood is a promising therapeutic strategy against atherosclerosis.

Keywords

atherosclerosis, inflammation, oxidative stress, oxidized LDL, scavenger receptors

Curr Opin Lipidol 20:363–369

0957-9672

Introduction

The mechanisms underlying the pathogenesis of atherosclerosis are extremely complex and are affected by interactions among several biological pathways, including those of inflammation [1], metabolic disorders [2] and oxidative stress [3]. Oxidative modification of LDL is regarded as a key step in the formation of atherosclerosis [4]. Oxidized LDL (oxLDL) has been proposed to be involved in many atherogenic steps in the vascular wall such as endothelial dysfunction [5], migration of macrophages and smooth muscle cells [6] and release of inflammatory cytokines [7]. Importantly, oxLDL is incorporated into macrophages, leading to macrophage transformation into foam cells and atherosclerotic plaque formation [8]. Furthermore, oxLDL itself reportedly induces oxidative stress in endothelial cells, smooth muscle cells and macrophages, resulting in a vicious cycle of atherosclerotic progression [9]. Oxidation of LDL particles is thought to occur primarily in vascular walls rather than in plasma, which is strongly antioxidant enriched [10], and fully oxLDL is reportedly cleared from circulating blood mainly by hepatic Kuppfer cells [11]. However, recent progress in enzyme assay procedures has provided direct evidence of the presence of oxLDL in circulating blood [12–14]. Although the amounts of circulating oxLDL represent a very small fraction of total LDL [15], growing evidence indicates a relationship between circulating oxLDL and various pathogenic processes of cardiovascular disease [16]. Thus, the significance of circulating oxLDL has been established as a biomarker of atherosclerosis. In addition, several recent studies [17,18,19••,20••] revealed the pathogenic roles of circulating oxLDL in atherosclerosis. This review summarizes the relevant clinical studies on circulating oxLDL as a biomarker, as well as several animal studies, which raise the possibility of circulating oxLDL being a pathogenic factor in atherosclerosis.

Measurement of circulating oxidized LDL

OxLDL is a mixture of lipoproteins with various degrees of heterogeneous modifications such as oxidation of phospholipids, modification of apolipoprotein B (apoB) with malondialdehyde and aggregation of apoB.

Table 1 Procedures of sandwich ELISA for direct oxidized LDL assays

	Human oxLDL		
Oxidation-specific antibodies	DLH3	E06	4E6
Capture antibody	DLH3	MB47	4E6
Recognition	Oxidized phosphatidylcholines	Human apoB-100	Modified apoB-100
Detecting antibody	Sheep polyclonal	E06	Mouse monoclonal
Recognition	Human apoB-100	Oxidized phospholipids	Human apoB-100
Reference	[13]	[12]	[14]

	Murine oxLDL	
Oxidation specificity	LOX-1	DLH3
Capture molecule	Recombinant LOX-1	DLH3
Recognition	LOX-1 ligands	Oxidized phosphatidylcholines
Detecting antibody	Chicken monoclonal	Rabbit polyclonal
Recognition	Mouse and human apoB	Mouse apoB-48
Reference	[22]	[20••]

apo, apolipoprotein; LOX-1, lectin-like oxLDL receptor-1; oxLDL, oxidized LDL.

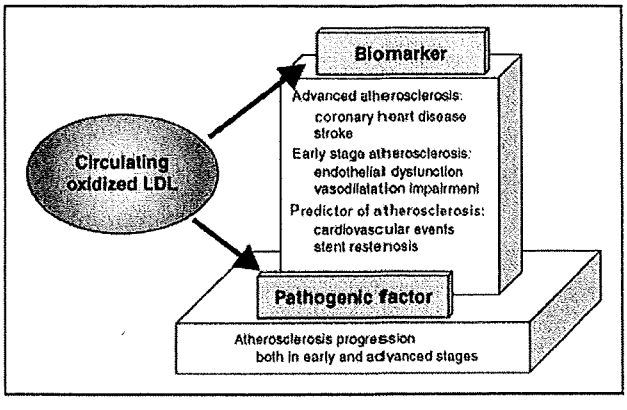
Currently, three ELISAs, using murine mAbs detecting different epitopes, details of which were described in another review [21], are widely used [12,13,22] for the measurement of circulating oxLDL (Table 1). However, these measuring methods did not allow us to analyze murine oxLDL, as antibodies against human apoB have no reactivity to murine apoB. Recently, two innovative novel immunochemical methods have been developed for measuring murine circulating oxLDL levels (Table 1). The sandwich ELISA using a chicken mAb for apoB and recombinant lectin-like oxLDL receptor-1 (LOX-1) protein, an oxLDL receptor [23], now allows measurement of circulating murine oxLDL. In another study [20••], a rabbit polyclonal antibody was raised for mouse apoB-48, a major component of murine LDL, instead of the antibody for human apoB-100, and the ELISA can be used in combination with an antibody against oxidized phospholipids, DLH3. These novel methods enabled us to measure circulating oxLDL in experimental animals, promoting active investigation of the pathogenic roles of circulating oxLDL.

Biomarker for atherosclerosis

According to oxLDL measurements in humans, evidence indicating the involvement of circulating oxLDL in cardiovascular diseases is growing [16]. The net values of these ELISA results are not comparable among studies because different antibodies detecting a variety of oxidative sites of LDL were used. However, similar associations of circulating oxLDL levels with certain pathological conditions are consistent among these clinical studies. Circulating oxLDL values were significantly elevated in patients with coronary heart disease [24], especially acute coronary syndrome [25,26]. Plasma levels of circulating oxLDL were also associated with carotid atherosclerosis [27] and ischemic stroke [28]. In addition, circulating oxLDL was increased in patients with renal failure receiving hemodialysis [14] and was shown to be a prognostic marker of cardiovascular events in cardiac transplant patients [29]. These results indicate the significance of circulating oxLDL as a biomarker of advanced atherosclerosis (Fig. 1).

Furthermore, the levels of circulating oxLDL are likely to reflect early changes promoting atherosclerosis, such as endothelial dysfunction [30] and impairment of vasodilatation responses [31,32], in individuals without apparent atherosclerosis (Fig. 1). In addition, recently, several studies have raised the possibility that circulating oxLDL levels can be used for the prediction of future cardiovascular events. Increased levels of circulating oxLDL were shown to be a strong predictor of cardiovascular events [33–35] as well as of stent restenosis in patients who had

Figure 1 The pathogenic roles of circulating oxidized LDL in atherosclerotic development may underlie the established roles of circulating oxidized LDL as a biomarker of early and advanced stages of atherosclerosis



received coronary intervention [36], although prospective studies involving a large number of patients will be needed to show circulating oxLDL to be an independent biomarker for predicting cardiovascular events.

Biomarker for metabolic disorders

Accumulating clinical observations have revealed intriguing aspects of circulating oxLDL, serving as a biomarker for metabolic disorders in addition to atherosclerosis. Several studies have demonstrated the significant elevation of circulating oxLDL values in the patients with diabetes mellitus [37] and insulin resistance [38]. Circulating oxLDL values also correlate positively with degrees of obesity [24,39–41]. Body weight reduction after gastric banding surgery decreased circulating oxLDL levels, which had been elevated in association with obesity [42]. The body weight reduction induced by dietary restriction also decreased plasma levels of circulating oxLDL in postmenopausal women [43]. Importantly, in several studies [41,43], the circulating oxLDL level showed a much stronger association with the degree of obesity than did the LDL level.

Interestingly, circulating oxLDL was reported to be positively associated with the incidence of metabolic syndrome [44]. A major factor underlying the strong relationship between oxLDL levels and metabolic disorders is considered to be the enhancement of oxidative stress in visceral adiposity. Although the mechanism underlying obesity-induced oxidative stress has not been fully elucidated, several studies [45,46] have suggested that a low degree of inflammation in obesity involves macrophage infiltration into visceral adipose tissue, leading to the increased secretion of inflammatory cytokines such as TNF-α, monocyte chemoattractant protein-1 and IL-6. Chronic inflammation exacerbates systemic oxidative stress. In addition, excess energy storage in adipose tissue may suppress expression of superoxide dismutase, which prevents oxidative stress [47]. These mechanisms together may promote oxidation of LDL particles.

Leptin, a major adipokine, appears to be involved in the greater oxidative stress in obese patients. Previous studies [48,49] demonstrated a direct effect of leptin on the generation of reactive oxygen species in endothelial cells. In fact, Porreca *et al.* [43] reported changes in circulating oxLDL values induced by body weight reductions to be strongly associated with changes in plasma leptin levels. In addition to these mechanisms, increasing oxidative stress, small dense LDL particles, which are more prone to oxidation than intermediate LDL, are reportedly increased in obese patients [50]. Taken together, these lines of evidence indicate that a high concentration of circulating oxLDL is apparently associated with metabolic disorders via various mechanisms attributable to visceral adiposity-induced oxidative stress.

Sites of LDL oxidization

Where is LDL oxidized? As circulating oxLDL temporarily increases during the acute phase of myocardial infarction or stroke, and then gradually declines to normal levels during pathological improvement [36,51], oxLDL was believed to be released from ruptured plaques into the circulation at the time of infarction occurrence. However, if the sites of LDL oxidation were limited to atherosclerotic plaques, circulating oxLDL would not be elevated in individuals without apparent atherosclerosis. Interestingly, circulating oxLDL is temporarily elevated prior to atherosclerotic progression in apoE-deficient mice [20••]. The atherosclerotic lesion area was remarkably increased from 28 to 40 weeks of age. In contrast, circulating oxLDL transiently increased at 20 weeks of age and then gradually decreased through 40 weeks of age. These results suggest that circulating oxLDL, which increases prior to atherosclerotic development, plays a pathogenic role in the early stage of atherosclerosis. Although the sites at which LDL is oxidized and those from which oxLDL is released into circulating blood are essentially unknown, arterial medial tissue under oxidative stress has been hypothesized to be a major site of LDL oxidation before and in the early stage of atherosclerosis [20••].

Indirect evidence of circulating oxidized LDL as the pathogenic factor in atherosclerosis

OxLDL is well known to be a major pathogenic factor in atherosclerotic formation, when oxLDL is localized at the vascular wall [52]. On the contrary, whether oxLDL in plasma has biological effects remains unclear. Several research groups have endeavored to explore the pathogenic roles of circulating oxLDL in the formation of atherosclerosis.

In the Watanabe heritable hyperlipidemic rabbits, plasma levels of oxLDL, which were measured as ligands for LOX-1, were higher than in control rabbits as early as 2 months of age, but antioxidant supplementation reduced plasma levels of oxLDL without altering plasma total cholesterol (TC), accompanied by suppression of atherosclerosis development. This result indirectly suggested a pathogenic role of LDL oxidation in the progression of atherosclerosis [53].

Several human studies also suggest a possible pathogenic role of circulating oxLDL. An intriguing association between circulating oxLDL and inflammation was reported in patients with angina pectoris [54]. The authors measured plasma oxLDL levels and circulating

NF-κB in peripheral blood mononuclear cells. The angina pectoris patients had higher levels of both circulating oxLDL and NF-κB activity than controls. Interestingly, in-vitro addition of either high-dose oxLDL or serum from the patients with unstable angina increased the NF-κB activity of mononuclear cells via the LOX-1 pathway. These findings suggested that NF-κB activation was induced, at least partially, by circulating oxLDL, leading to increased oxidative stress in the angina pectoris patients.

In addition, antibodies against oxLDL reported to play important roles in atherogenic regulation [55]. Antibodies to oxLDL have been found in human and rabbit plasma [56], as well as in atherosclerotic lesions of humans [57]. Most studies have shown elevated antibody titers to oxLDL, especially IgG, to be related to the degree of atherosclerotic progression. Paradoxically, though interestingly, in another study [15], circulating oxLDL levels correlated negatively to the IgG titers against oxLDL. An inverse relationship between IgM titers and atherosclerotic disease has also been reported [58,59]. In addition, inducing production of IgM antibody to oxLDL in LDL receptor-deficient mice decreased the extent of atherosclerosis [60]. Furthermore, intriguingly, treatment with a recombinant human IgG1 antibody against a malondialdehyde-modified apoB-100 peptide sequence, a specific oxLDL epitope, has been shown to reduce the level of circulating oxLDL [61] and to induce regression of preexisting lesions in LDL receptor-deficient mice overexpressing human apoB-100 [62]. These results together suggest that oxLDL antibodies play a role in maintaining low levels of circulating oxLDL. Production of immune complexes against oxLDL might prevent the development of atherosclerosis, at least partly, due to inhibition of oxLDL incorporation into macrophages [11].

Direct evidence of oxidized LDL as the pathogenic factor in atherosclerosis

To evaluate the importance of plasma-modified LDL, including oxLDL, in atherosclerotic progression, several experimental animals in which scavenger receptors were manipulated have been generated. Class A scavenger receptors (SR-A), the first cloned and now well investigated scavenger receptor family [63], play a role in the incorporation of modified LDL into macrophages, leading to foam cell formation. Whitman *et al.* [64] established the mouse model of macrophage-specific SR-A1 overexpression, and using the bone marrow transplantation technique, SR-A1 overexpression in macrophages was induced in hypercholesterolemic LDL receptor-deficient mice, resulting in inhibition of aortic atherosclerosis. However, as apoB-containing lipoproteins, including total LDL, were decreased in this study, whether intermediate LDL or modified LDL is important for the development of atherosclerosis remains unclear.

Next, to clarify the role of circulating modified LDL in the pathogenesis of atherosclerosis, Laukkanen *et al.* [17] constructed a secreted type of SR-A1 as a fusion protein consisting of a bovine growth hormone signal sequence and the extracellular domain of human SR-A1. The secreted SR-A1 functioned as a 'decoy' blocking the incorporation of modified LDL into macrophages. Adenoviral administration of secreted SR-A1 delayed the clearance of modified LDL, resulting in suppression of foam cell formation in macrophages as well as prevention of atherosclerotic lesion in LDL receptor-deficient mice [18]. Thus, blockade of the incorporation of modified LDL into macrophages may exert a beneficial effect in prevention of atherosclerosis.

Recently, we directly demonstrated the atherogenic impact of oxLDL removal from circulating blood [19••]. LOX-1 is one of the scavenger receptors and incorporates oxLDL selectively among modified types of LDL. To examine the effects of oxLDL removal from circulating blood on atherosclerotic progression, we expressed LOX-1 ectopically in the livers of apolipoprotein E (apoE)-deficient mice, using an adenoviral gene transfer system. LOX-1 expressed in the liver successfully functioned as a receptor of circulating oxLDL, thereby reducing values of circulating oxLDL, with no significant changes in plasma TC, triglyceride or LDL cholesterol (LDL-C) levels. This transient reduction in circulating oxLDL completely prevented atherosclerotic progression in apoE-deficient mice. In addition, hepatic LOX-1 expression markedly suppressed oxidative stress and inflammation in the whole body, especially in the aorta. Furthermore, smooth muscle cell deposition in the surface areas of atherosclerotic plaques was increased, possibly leading to plaque stability [19••].

These studies provide direct evidence that circulating oxLDL plays important roles in atherogenesis via mechanisms involving both direct (inhibition of foam cell formation) and indirect (antioxidative stress) effects. Thus, oxLDL removal is a promising therapeutic strategy against atherosclerosis (Fig. 1).

Reduction in circulating oxidized LDL

Then, which procedures can reduce circulating oxLDL? In general, plasma levels of circulating oxLDL correlate significantly with total levels of LDL-C [65]. Statin therapy is an established approach to decreasing LDL-C. As expected, administration of statins also reduced circulating oxLDL values, which depended on the degree

of LDL-C reduction [66–68]. However, according to the Multicenter InSync Randomized Clinical Evaluation (MIRACLE) trial [69], administration of atrovastatin to patients with coronary heart disease elevated the plasma ratio of oxLDL:apoB. Thus, oxidative phospholipids might be condensed in LDL particles after statin therapy.

Several trials [70–75] of antioxidant therapies designed to inhibit the oxidation step of LDL have been reported, but effectiveness against atherosclerosis is controversial. In murine models, administration of antioxidants effectively reduces atherosclerosis [72]. However, the majority of clinical trials yielded negative results [75]. This may at least partly be due to insufficient antioxidant effects of natural and synthetic compounds when administered to humans. For instance, in a randomized placebo-controlled study [76] in healthy adults, daily administration of high-dose vitamin E did not affect the breakdown of lipid peroxidation products. Moreover, high doses of these antioxidants reportedly have adverse effects [75], including the pro-oxidant effects of vitamin E at high doses [77]. Therefore, clinical applications of antioxidants seem to be limited at present. The development of novel strategies for lowering oxLDL itself is highly anticipated.

Conclusion

Over the past decade, investigations of circulating oxLDL have progressed dramatically. The important role of circulating oxLDL as a biomarker of cardiovascular disease has been largely established. However, further extensive examinations will be required to explore predictive values for future atherosclerotic events. In addition, the pathogenic roles of circulating oxLDL in the formation of atherosclerosis are now being elucidated. The pathogenic involvement of circulating oxLDL may account for its significance as a biomarker of not only advanced atherosclerosis but also the early-stage atherosclerosis (Fig. 1). As oxLDL may induce inflammation as well as oxidative stress, the roles of oxLDL, not only in the vascular wall but also in circulating blood, are now attracting considerable attention from investigators working on the pathogenesis of atherosclerosis. Moreover, circulating oxLDL removal is a promising strategy for the treatment of atherosclerosis.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research to H. Katagiri (B2, 15390282) and Y. Oka (A2, 19209034) from the Ministry of Education, Science, Sports and Culture of Japan and a Grant-in-Aid for Scientific Research (H19-genome-005) to Y. Oka from the Ministry of Health, Labor and Welfare of Japan. This work was also supported by the Global Centers of Excellence Program for 'Network Medicine', with funding to Y. Oka from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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ORIGINAL ARTICLE

Adiponectin Upregulates Ferritin Heavy Chain in Skeletal Muscle Cells

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OBJECTIVE—Adiponectin is an adipocyte-derived protein that acts to reduce insulin resistance in the liver and muscle and also inhibits atherosclerosis. Although adiponectin reportedly enhances AMP-activated protein kinase and inhibits tumor necrosis factor-α action downstream from the adiponectin signal, the precise physiological mechanisms by which adiponectin acts on skeletal muscles remain unknown.

RESEARCH DESIGN AND METHODS—We treated murine primary skeletal muscle cells with recombinant full-length human adiponectin for 12 h and searched, using two-dimensional electrophoresis, for proteins upregulated more than threefold by adiponectin compared with untreated cells.

RESULTS—We found one protein that was increased 6.3-fold with adiponectin incubation. MALDI-TOF (matrix-assisted laser desorption/ionization–top of flight) mass spectrometric analysis identified this protein as ferritin heavy chain (FHC). When murine primary skeletal muscle cells were treated with adiponectin, IκB-α phosphorylation was observed, suggesting that adiponectin stimulates nuclear factor (NF)-κB activity. In addition, FHC upregulation by adiponectin was inhibited by NF-κB inhibitors. These results suggest NF-κB activation to be involved in FHC upregulation by adiponectin. Other NF-κB target genes, manganese superoxide dismutase (*MnSOD*) and inducible nitric oxide synthase (*iNOS*), were also increased by adiponectin treatment. We performed a reactive oxygen species (ROS) assay using CM-H_2DCFDA fluorescence and found that ROS-reducing effects of adiponectin were abrogated by FHC or MnSOD small-interfering RNA induction.

CONCLUSIONS—We have demonstrated that adiponectin upregulates FHC in murine skeletal muscle tissues, suggesting that FHC elevation might partially explain how adiponectin protects against oxidative stress in skeletal muscles. ***Diabetes* 58:61–70, 2009**

Adipocytes have been recognized to secrete a variety of proteins, such as tumor necrosis factor (TNF)-α, adipsin, plasminogen activator inhibitor-1, leptin, resistin, and adiponectin. These proteins are termed adipokines and are likely to physiologically exert a variety of hormonal actions (1). Among these proteins, adiponectin is exclusively expressed in adipose tissue and consists of an NH_2-terminal collagenous domain and a COOH-terminal globular domain (2). Adiponectin belongs to the soluble collagen superfamily and has structural homology with collagens VIII and X, complement factor C1q (3), and the TNF family (2,4). Circulating adiponectin is extremely abundant (~15 μg/ml), and adiponectin forms various oligomeric complexes, including low (LMW), medium (MMW), and high (HMW) molecular weight species. Adiponectin exerts antidiabetes effects on muscles and the liver through AMP-activated protein kinase activation (5) and antiatherosclerotic effects by inhibiting monocyte adhesion to endothelial cells and lipid accumulation into macrophages (6,7). Thus, adiponectin increases glucose uptake and fatty acid oxidation in muscles via the type 1 adiponectin receptor (8), and decreases hepatic gluconeogenesis via the type 2 adiponectin receptor (8,9). On the other hand, nuclear factor (NF)-κB but not AMP-activated protein kinase activity was demonstrated to be enhanced by MMW or HMW adiponectin in muscles (10). According to recent studies (9–14), HMW adiponectin appears to be more important for the antidiabetes and antiatherosclerotic effects than the other two oligomeric complexes. Though the physiological role of HMW adiponectin in improving insulin resistance or reducing oxidative stress is clearly significant, the precise mechanisms by which adiponectin acts on skeletal muscles remain unknown.

Therefore, in the present study, we investigated adiponectin function in primary cultured skeletal muscle cells by comparing protein expressions in untreated cells using two-dimentional electrophoresis. A marked increase in FHC protein was observed with adiponectin incubation. FHC is one of two subunits of ferritin, the other being ferritin light chain (FLC) (15), and has ferroxidase activity, which is required for iron sequestration (16). FHC was reported to suppress reactive oxygen species (ROS) production (17), which may explain the ROS-reducing effects of adiponectin. FHC upregulation followed by an enhanced ROS-reducing effect is suggested to be a novel mechanism by which adiponectin acts directly against oxidative stress.

RESEARCH DESIGN AND METHODS

Cell culture and chemicals. Murine primary cultured skeletal muscle cells were purchased from Cell Garage (Tokyo, Japan) in cultured flasks, and maintained in DMEM containing 10% fetal bovine serum (FBS). We switched the medium to DMEM containing 2% horse serum, which promotes differentiation of myocytes into myotubes, and continued the incubation for 5 days before the experiments. Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (Baltimore, MD) as cryopreserved cells. After thawing, the cells were plated in collagen-coated culture flasks and cultured to confluence in EBM-2 medium (Lonza, Walkersville, MD) containing the indicated ligands, 2% FBS, and antibiotics (BulletKit EGM-2, cat. no. CC-3162). C2C12 myoblasts were maintained in DMEM containing 10% FBS at 37°C in 5%

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Received 25 May 2007 and accepted 8 October 2008.
Published ahead of print at http://diabetes.diabetesjournals.org on 17 October 2008. DOI: 10.2337/db07-0690.