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Identification of a novel mechanism regulating β-cell mass

Neuronal relay from the liver to pancreatic β-cells

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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.

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

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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

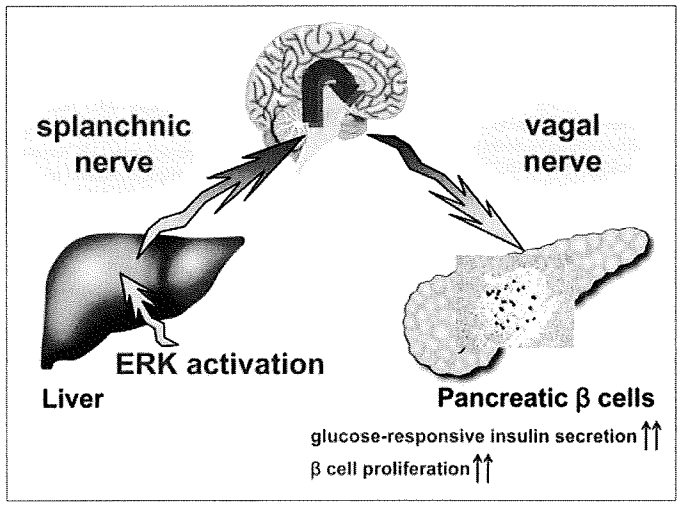

Figure 1. Schematic model of the neuronal relay originating in the liver.

such as cytokines or nutrients are known to be important mediators of inter-organ 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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Eradication of insulin resistance

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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 (285×10^9 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 56×10^9 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 10×10^9 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.

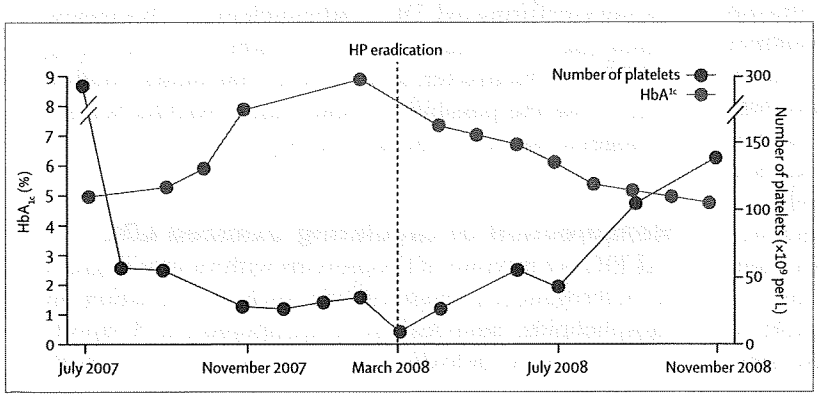

Figure: **Clinical course**

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.

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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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

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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.

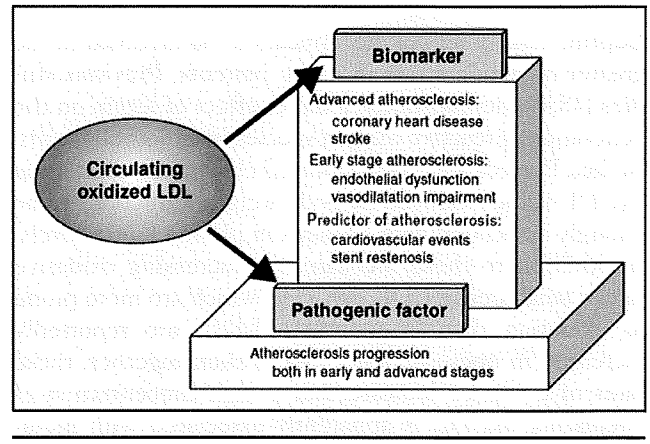
Currently, three ELISAs, using murine mAbs detecting different epitopes, details of which were described in another review [21], are widely used [12,13,22] for the measurement of circulating oxLDL (Table 1). However, these measuring methods did not allow us to analyze murine oxLDL, as antibodies against 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.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 428).

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Fruit, vegetable and bean intake and mortality from cardiovascular disease among Japanese men and women: the JACC Study

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To examine the association of plant-based food intakes with CVD and total mortality among Japanese. In the Japan Collaborative Cohort Study for Evaluation of Cancer Risk, 25 206 men and 34 279 women aged 40–79 years, whose fruit, vegetable and bean intakes were assessed by questionnaire at baseline in 1988–90, were followed for 13 years. Deaths from total stroke, stroke subtypes, CHD and total CVD, according to the International Classification for Diseases 10th Revision, were registered. During 756 054 person-years of follow-up, there were 559 deaths from total stroke, 258 from CHD, 1207 from total CVD and 4514 from total mortality for men, and for women, 494, 194, 1036 and 3092, respectively. Fruit intake was inversely associated with mortality from total stroke (the multivariable hazard ratio (HR (95 % CI)) in the highest *v.* lowest quartiles = 0·67 (0·55, 0·81)), total CVD (HR = 0·75 (0·66, 0·85)) and total mortality (HR = 0·86 (0·80, 0·92)). Vegetable intake was inversely associated with total CVD (HR = 0·88 (0·78, 0·99)). Bean intake was inversely associated with other CVD (HR = 0·79 (0·64, 0·98)), total CVD (HR = 0·84 (0·74, 0·95)) and total mortality (HR = 0·90 (0·84, 0·96)). Further adjustment for other plant-based foods did not alter the association of fruit intake with mortality from total stroke, total CVD and total mortality, but attenuated the associations of vegetables and beans with mortality risk. In conclusion, intakes of plant-based foods, particularly fruit intake, were associated with reduced mortality from CVD and all causes among Japanese men and women.

Fruits: Vegetables: Beans: CVD: Mortality

Protective effects of plant-based foods against CVD have been suggested by prospective cohort studies in Western countries[1–6]. Fruit and vegetable intakes were associated with reduced risks of stroke and CHD[1,2], and nut intake was associated with a reduced risk of CHD[3–6]. These potential effects of plant-based foods need to be examined for Japanese, because of their different profiles of CVD and diet. In Japan, the incidence of stroke is higher than that of CHD, and the proportion of haemorrhagic stroke among the stroke subtypes is higher than in Western countries[7,8]. The Japanese habitually consume more beans than the Westerners, and soyabeans, in particular, have recently been highlighted as a protective factor for CVD in Western countries[9,10].

So far, several Japanese studies have shown inverse associations of the fruit, vegetable or bean intake with the risk of stroke[11–14]. The Hiroshima/Nagasaki Life Span Study showed a protective association of both fruit and vegetable intakes with mortality from both ischaemic stroke and intra-parenchymal haemorrhage[11]. The Shibata Study showed a protective association of vegetable intake with the incidence of total stroke[12].

Abbreviation: HR, hazard ratio.

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The Japan Public Health Center-based Prospective Study showed a protective association of fruit intake with the incidence of CVD for Japanese men and women combined[13], and also showed a protective association of soya intake with the incidence of cerebral infarction and mortality from CVD for women[14]. The Takayama Study also showed a protective association of vegetable intake with mortality from CVD for Japanese women[15]. The Japan Collaborative Cohort Study for Evaluation of Cancer Risk previously reported the association between plant-based food intakes and mortality from CVD, cancer and all causes[16], but these associations were not adjusted for major confounding factors.

Therefore, no study has examined whether plant-based foods were associated with CVD, their subtypes and total mortality systematically in Japan. Such a study in Japanese is also of value because plant-based food intakes are positively correlated with saturated fat intake in Japanese[15] unlike Western populations[17,18], and confounding factors may be different from Western studies. We hypothesised that higher plant-based food intake had beneficial effects for the prevention of CVD and their subtypes in general Japanese populations, and we comprehensively examined the associations of the fruit, vegetable and bean intake with mortality from stroke, stroke subtypes, CHD, total CVD and all causes in a 13-year cohort study of approximately 60 000 Japanese men and women.

Experimental methods

Subjects

The Japan Collaborative Cohort Study sponsored by Monbusho, the Ministry of Education, Science, Sports and Culture, began in 1988–90 when 110 792 individuals (46 465 men and 64 327 women) aged 40–79 years living in forty-five communities across Japan participated in municipal health screening examinations and completed a self-administered questionnaire about their lifestyles (habits of smoking and drinking, physical activity, hours of sleep, education and mental stress) and medical histories (hypertension, diabetes, CVD and cancer). Informed consent was obtained before completing the questionnaire. We excluded 2576 men and 3288 women from the analysis because of previous history of stroke, CHD or cancer at baseline. Persons (18 683 men and 26 760 women) with missing information regarding the intake of fruits, vegetables and beans were also excluded, and a total of 25 206 men and 34 279 women were used for the analysis. There was no substantial difference in mortality rates between persons who gave the valid dietary information and those who did not; the multivariable hazard ratios (HR (95 % CI)) for respondents *v.* non-respondents were 1·08 (0·98, 1·21) for total stroke, 0·98 (0·83, 1·14) for CHD, 1·04 (0·97, 1·12) for total CVD and 1·02 (0·98, 1·07) for all causes. No material differences were also found between the respondents and non-respondents for BMI, history of hypertension, history of diabetes, smoking, ethanol intake and other cardiovascular risk characteristics.

Dietary assessment

The self-administered FFQ was conducted to estimate the consumption of thirty-three foods during the past year[19]. The food items were beef, pork, ham or sausage, chicken, liver, eggs, milk, yogurt, cheese, butter, margarine, deep-fried foods or tempura, fried vegetables, fresh fish, steamed fish paste, dried fish or salted fish, spinach or garland chrysanthemum, carrot or pumpkin, tomatoes, cabbage or head lettuce, Chinese cabbage, edible wild plants, fungi, potatoes, algae, pickles, preserved foods using soya sauce, boiled beans, tofu, citrus fruits, fruits excluding citrus varieties, fresh fruit juice in summer and sweets. Each food had a five-level precoded answer: 'rarely eat'; 'once or twice per month'; 'once or twice per week'; 'three or four times per week'; 'almost daily'. Then, we converted the answers 'rarely eat' to 0, 'once or twice per month' to 0·375, 'once or twice per week' to 1·5, 'three or four times per week' to 3·5 and 'almost daily' to 7 servings per d to estimate the average weekly intake of each fruit (citrus fruits, fruits excluding citrus varieties and fresh fruit juice in summer), vegetable (spinach or garland chrysanthemum, carrot or pumpkin, tomatoes, cabbage or head lettuce and Chinese cabbage) and beans (tofu, i.e. soyabean curd, and boiled beans) for each participant. The average weekly intakes of individual foods were combined to compute the total fruit, vegetable and bean intakes.

The reproducibility of the dietary data was confirmed by comparing two questionnaires administered 1 year apart for eighty-five subjects (eight men and seventy-seven women)[19]. The median (range) values of the Spearman correlation coefficients were 0·57 (0·55, 0·58) for three items of fruits, 0·63 (0·43, 0·66) for five items of vegetables and 0·62 (0·59, 0·64) for two items of beans. The validity of the data was confirmed by comparing the data from the questionnaire with those from four 3-d dietary records for the eighty-five subjects, collected approximately 3–4 months apart[19]. The median values of the Spearman correlation coefficients were 0·26 (0·24, 0·39) for three items of fruits, 0·33 (0·18, 0·45) for five items of vegetables and 0·40 (0·30, 0·50) for two items of beans. The intakes of selective nutrients, i.e. cholesterol, saturated, *n*-3 polyunsaturated and sodium intake, were calculated and adjusted using the residual method, and used as potential confounding factors for the analysis.

Mortality surveillance

For mortality surveillance in each community, investigators systematically reviewed death certificates, all of which were filed in the public-health centre in the area of residency. Mortality data were sent centrally to the Ministry of Health and Welfare and the underlying causes of deaths were coded for the National Vital Statistics according to the International Classification for Diseases, 9th Revision from 1988 to 1994 and 10th Revision from 1995 to 2003. Registration of death is required by the Family Registration Law in Japan, and is believed to be completed across the country. Therefore, all deaths that occurred in the cohort were ascertained by death certificates from the public-health centres, except for subjects who died after they moved from their original community, in which case the subject was treated as censored. The follow-up was conducted until the end of 2003 and the average follow-up period for the participants was 12·7 years.

Cause-specific mortality was defined separately for total stroke (International Classification for Diseases-9 codes

Table 1. Age- and sex-adjusted mean values or prevalence of cardiovascular risk factors according to quartiles of the frequency of the fruit, vegetable and bean intakes*

	Quartiles of fruit intake				*P* for trend	Quartiles of vegetable intake				*P* for trend	Quartiles of bean intake				*P* for trend
	Q1	Q2	Q3	Q4		Q1	Q2	Q3	Q4		Q1	Q2	Q3	Q4	
Servings per week	0·9	2·3	3·9	5·9	–	1·2	2·3	3·4	5·2	–	0·8	1·8	3·0	4·5	–
Number of subjects	14 967	14 066	17 607	12 845	–	14 768	15 213	14 142	15 362	–	15 212	15 573	12 321	16 379	–
Age (years)	55·9	56·4	56·6	56·1	<0·001	55·3	55·5	56·4	57·8	<0·001	54·9	55·5	57·0	57·7	<0·001
Women (%)	43·8	55·6	63·3	68·2	<0·001	46·3	56·8	61·4	65·9	<0·001	51·5	56·3	57·3	64·7	<0·001
BMI (kg/m^2)	22·8	22·8	22·8	22·9	0·07	22·9	22·8	22·8	22·9	0·16	22·8	22·8	22·8	22·9	0·24
History of hypertension (%)	20·4	20·0	19·6	19·5	0·04	20·0	20·3	19·8	19·4	0·08	19·9	20·7	20·0	19·1	0·01
History of diabetes (%)	4·9	4·9	4·7	3·9	<0·001	4·4	4·6	4·7	4·7	0·29	4·5	4·7	4·6	4·6	0·95
Current smoking (%)	31·1	26·3	24·7	23·7	<0·001	30·1	26·1	25·4	24·4	<0·001	29·5	26·9	25·8	23·8	0·14
Ethanol intake (g/d)	31·1	27·6	26·9	26·4	<0·001	29·3	28·1	28·2	27·6	<0·001	28·6	28·4	28·4	28·0	<0·001
Walk 30 min or more/ week (%)	68·3	69·9	71·2	73·2	<0·001	65·8	70·6	72·1	74·1	<0·001	68·6	70·2	72·2	71·9	<0·001
Sports 1 h or more/ week (%)	22·1	25·2	29·1	30·5	<0·001	22·5	25·8	27·8	30·7	<0·001	23·9	25·8	27·8	29·4	<0·001
Hours of sleep (h/d)	7·3	7·2	7·2	7·2	0·01	7·2	7·2	7·2	7·3	<0·001	7·2	7·2	7·2	7·3	<0·001
College or higher education (%)	10·4	12·2	14·6	16·3	<0·001	11·4	13·1	13·5	15·4	<0·001	12·3	12·9	14·0	14·3	<0·001
High perceived mental stress (%)	22·3	22·4	22·2	23·1	0·23	23·5	22·4	22·0	22·0	0·004	23·2	22·3	22·4	22·0	0·04
Total energy intake (kJ)	5640	5933	6243	6636	<0·001	5427	5958	6272	6728	<0·001	5498	5929	6314	6669	<0·001
Cholesterol intake (mg)	219	237	250	258	<0·001	208	233	250	271	<0·001	215	235	250	264	<0·001
SFA intake (g)	8·4	9·0	9·5	9·9	<0·001	8·2	9·0	9·4	10·0	<0·001	8·5	9·0	9·5	9·8	<0·001
n-3 Fatty acid intake (g)	1·5	1·6	1·7	1·8	<0·001	1·4	1·6	1·7	1·9	<0·001	1·4	1·6	1·7	1·9	<0·001
Sodium intake (mg)	2065	2110	2141	2167	<0·001	1902	2058	2166	2345	<0·001	1929	2057	2175	2316	<0·001

*Nutrient intakes were adjusted for total energy intake by the residual method.

Table 2. Risk of mortality from stroke, CHD, total CVD and all causes according to quartiles of the frequency of fruit intake (Hazard ratio (HR) values and 95% CI)

	Quartiles of fruit intake							
		Q2		Q3		Q4		
	Q1	HR	95% CI	HR	95% CI	HR	95% CI	*P* for trend
Person-years	187700		178625		223683		166046	
Total stroke								
Number	348		258		284		163	
Age- and sex-adjusted HR	1·00	0·77	0·66, 0·91	0·68	0·58, 0·80	0·57	0·48, 0·69	<0·001
Multivariable HR*	1·00	0·83	0·71, 0·98	0·79	0·67, 0·92	0·67	0·55, 0·81	<0·001
Multivariable HR†	1·00	0·81	0·69, 0·96	0·76	0·64, 0·90	0·65	0·53, 0·80	<0·001
Haemorrhagic stroke								
Number	130		93		108		62	
Age- and sex-adjusted HR	1·00	0·73	0·56, 0·96	0·67	0·52, 0·87	0·55	0·40, 0·74	<0·001
Multivariable HR*	1·00	0·79	0·60, 1·03	0·76	0·59, 0·99	0·63	0·46, 0·87	0·004
Multivariable HR†	1·00	0·76	0·58, 1·00	0·72	0·55, 0·95	0·59	0·42, 0·82	0·002
Ischaemic stroke								
Number	121		82		102		57	
Age- and sex-adjusted HR	1·00	0·71	0·54, 0·95	0·72	0·55, 0·94	0·60	0·44, 0·82	0·002
Multivariable HR*	1·00	0·77	0·58, 1·03	0·85	0·65, 1·12	0·72	0·52, 1·00	0·070
Multivariable HR†	1·00	0·76	0·57, 1·01	0·83	0·63, 1·11	0·71	0·50, 1·00	0·081
CHD								
Number	146		116		117		73	
Age- and sex-adjusted HR	1·00	0·84	0·66, 1·07	0·69	0·54, 0·88	0·63	0·47, 0·83	<0·001
Multivariable HR*	1·00	0·92	0·72, 1·18	0·79	0·62, 1·02	0·74	0·55, 0·99	0·015
Multivariable HR†	1·00	0·97	0·75, 1·24	0·84	0·65, 1·10	0·79	0·58, 1·08	0·061
Other CVD								
Number	205		173		229		131	
Age- and sex-adjusted HR	1·00	0·88	0·72, 1·08	0·94	0·78, 1·14	0·78	0·63, 0·98	0·060
Multivariable HR*	1·00	0·95	0·77, 1·17	1·06	0·87, 1·29	0·89	0·71, 1·12	0·553
Multivariable HR†	1·00	0·99	0·80, 1·22	1·13	0·92, 1·38	0·96	0·76, 1·23	0·988
Total CVD								
Number	699		547		630		367	
Age- and sex-adjusted HR	1·00	0·82	0·73, 0·91	0·76	0·68, 0·85	0·65	0·57, 0·73	<0·001
Multivariable HR*	1·00	0·88	0·79, 0·99	0·87	0·78, 0·97	0·75	0·66, 0·85	<0·001
Multivariable HR†	1·00	0·90	0·80, 1·00	0·89	0·79, 0·99	0·77	0·67, 0·88	<0·001
All causes								
Number	2284		1824		2158		1340	
Age- and sex-adjusted HR	1·00	0·86	0·81, 0·91	0·83	0·79, 0·89	0·76	0·71, 0·81	<0·001
Multivariable HR*	1·00	0·91	0·86, 0·97	0·93	0·87, 0·99	0·86	0·80, 0·92	<0·001
Multivariable HR†	1·00	0·92	0·86, 0·98	0·93	0·87, 0·99	0·86	0·80, 0·93	<0·001

* Adjusted for sex, age, BMI, smoking status, alcohol intake, hours of walking, hours of sleep, education years, perceived mental stress, cholesterol intake, SFA intake, *n*-3 fatty acids intake, sodium intake and histories of hypertension and diabetes.
† Adjusted further for vegetable and bean intakes.

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430–438 and International Classification for Diseases-10 codes I60–I69), CHD (410–414 and I20–I25), other CVD (390–409, 415–429, 439–459, I01–I19, I26–I59 and I70–I99) and total CVD (390–459 and I01–I99). Total stroke was further divided into haemorrhagic stroke (430–431 and I60–61) and ischaemic stroke (433–434 and I63). Total mortality was also examined as a reference. The present study was approved by the Ethical Committee, the Nagoya University School of Medicine and the University of Tsukuba.

Statistical analysis

Statistical analyses were based on sex-specific mortality during the follow-up period from 1989 to 2003. For each participant, the person-year of follow-up was calculated when they died or moved out of his or her community or the end of 2003, whichever was the first. The age- and sex-adjusted risk of mortality from CVD as well as total mortality was defined as the corresponding death rate among the participants according to quartiles of the fruit, vegetable and bean intakes. The means and proportions of selected cardiovascular risk factors were calculated according to quartiles of those food intakes. We calculated the quartile cut-points among the whole study population and used the lowest quartiles as the reference categories for the analyses of relative risk for the second, third and highest quartiles. The HR and their 95% CI were calculated after adjustment for age, sex and potential confounding factors using the Cox proportional hazard model. These confounding variables, which were associated with CVD among Japanese, included BMI (sex-specific quintiles), smoking category (never, ex- and current smokers of ≤19 or ≥20 cigarettes per d), alcohol intake category (never, ex- and current ethanol intake of 1–22, 23–45, 46–68 and ≥69 g/d), hours of walking (rarely, 30, 30–60 and ≥60 min per d), sports (<1 and ≥1 h per week), education (<10, 10–12, 13–15 and ≥16 years), perceived mental stress (low, medium and high), history of hypertension or diabetes and sex-specific quartiles of dietary cholesterol, SFA, *n*-3 PUFA and sodium intake (sex-specific quartiles). Since the plant-based foods have little of those nutrients, the

Table 3. Risk of mortality from stroke, CHD, total CVD and all causes according to quartiles of the frequency of vegetable intake (Hazard ratio (HR) values and 95% CI)

	Quartiles of vegetable intake							
		Q2		Q3		Q4		
	Q1	HR	95% CI	HR	95% CI	HR	95% CI	*P* for trend
Person-years	185 787		193 546		180 543		196 177	
Total stroke								
Number	258		245		254		296	
Age- and sex-adjusted HR	1·00	0·93	0·78, 1·10	0·97	0·81, 1·15	0·91	0·77, 1·08	0·349
Multivariable HR*	1·00	0·97	0·82, 1·16	1·04	0·87, 1·24	0·97	0·81, 1·16	0·790
Multivariable HR†	1·00	1·02	0·85, 1·22	1·11	0·92, 1·34	1·09	0·90, 1·33	0·256
Haemorrhagic stroke								
Number	98		101		76		118	
Age- and sex-adjusted HR	1·00	0·99	0·75, 1·31	0·76	0·56, 1·02	0·98	0·75, 1·28	0·720
Multivariable HR*	1·00	1·06	0·80, 1·40	0·84	0·62, 1·14	1·10	0·82, 1·48	0·638
Multivariable HR†	1·00	1·09	0·82, 1·45	0·88	0·64, 1·21	1·22	0·89, 1·66	0·235
Ischaemic stroke								
Number	92		74		98		98	
Age- and sex-adjusted HR	1·00	0·79	0·58, 1·07	1·05	0·79, 1·39	0·84	0·63, 1·12	0·492
Multivariable HR*	1·00	0·83	0·61, 1·13	1·14	0·85, 1·54	0·91	0·67, 1·24	0·884
Multivariable HR†	1·00	0·87	0·64, 1·20	1·24	0·91, 1·70	1·03	0·74, 1·43	0·591
CHD								
Number	140		105		96		111	
Age- and sex-adjusted HR	1·00	0·74	0·57, 0·95	0·69	0·53, 0·89	0·65	0·51, 0·84	0·002
Multivariable HR*	1·00	0·79	0·61, 1·02	0·78	0·60, 1·02	0·77	0·58, 1·00	0·079
Multivariable HR†	1·00	0·82	0·63, 1·07	0·83	0·63, 1·10	0·85	0·64, 1·14	0·376
Other CVD								
Number	207		176		156		199	
Age- and sex-adjusted HR	1·00	0·83	0·68, 1·01	0·74	0·60, 0·91	0·76	0·62, 0·92	0·067
Multivariable HR*	1·00	0·88	0·72, 1·08	0·81	0·66, 1·01	0·85	0·69, 1·05	0·138
Multivariable HR†	1·00	0·89	0·72, 1·10	0·82	0·66, 1·03	0·87	0·70, 1·10	0·299
Total CVD								
Number	605		526		506		606	
Age- and sex-adjusted HR	1·00	0·85	0·76, 0·95	0·82	0·73, 0·93	0·80	0·71, 0·89	<0·001
Multivariable HR*	1·00	0·90	0·80, 1·01	0·90	0·80, 1·02	0·88	0·78, 0·99	0·069
Multivariable HR†	1·00	0·93	0·82, 1·05	0·95	0·83, 1·08	0·96	0·84, 1·10	0·835
All causes								
Number	1983		1786		1745		2092	
Age- and sex-adjusted HR	1·00	0·89	0·84, 0·95	0·90	0·84, 0·95	0·90	0·85, 0·96	0·007
Multivariable HR*	1·00	0·93	0·87, 0·99	0·96	0·9, 1·02	0·97	0·91, 1·04	0·762
Multivariable HR†	1·00	0·95	0·89, 1·02	0·99	0·93, 1·06	1·03	0·96, 1·10	0·188

* The same variables as shown in the footnote of Table 2.
† Adjusted further for fruit and bean intakes.

adjustment for them is justified. Further adjustment for other plant-based foods was also conducted for another multivariable model. A test for trend was used to assess statistical significance across exposure categories by including ordinal terms for each of the four categories and entering the variable as a continuous term in the model. A test for effect modification by sex was conducted using an interaction term generated by multiplying the fruit, vegetable and bean intakes by sex. A P value of $<0{\cdot}05$ was considered to be significant.

Results

Among the 25 206 men and 34 279 women followed up for an average of 12·7 years, 1207 men and 1036 women died from CVD, and 4514 men and 3029 women died from all causes. The deaths among men included 559 from stroke (128 intraparenchymal haemorrhages, 52 subarachnoid haemorrhages and 214 ischaemic strokes) and 258 from CHD. The respective numbers of deaths among women were 494 (104, 109 and 148) and 194.

Table 1 shows selected cardiovascular risk factors by fruit, vegetable and bean quartile. The participants with higher fruit intake smoked less, had lower mean ethanol intake, walked more and had higher education. The participants with higher vegetable intake were older, smoked less, had lower mean ethanol intake, walked more and had higher education. The participants with higher bean intake were older, smoked less, walked more and had higher education. The participants with higher intakes of fruit, vegetable and bean had higher mean intakes of total energy, cholesterol, *n*-3 fatty acids and sodium.

The associations of the fruit, vegetable and bean intakes with mortality from stroke, CHD, total CVD and all causes did not vary by sex (P for interaction $>0{\cdot}05$). Thus, we combined men and women in the present study. Table 2 shows the sex- and age-adjusted and multivariable HR of mortality from stroke, CHD and total CVD, as well as total mortality according to quartiles of fruit intake. There were inverse associations of fruit intake with age- and sex-adjusted mortality from total stroke, haemorrhagic stroke, total CVD and total mortality. After adjustment for cardiovascular risk

Table 4. Risk of mortality from stroke, CHD, total CVD and all causes according to quartiles of the frequency of bean intake (Hazard ratio (HR) values and 95% CI)

	Quartiles of bean intake							
		Q2		Q3		Q4		
	Q1	HR	95% CI	HR	95% CI	HR	95% CI	*P* for trend
Person-years	191279	198098		156448		210229		
Total stroke								
Number	238	266		261		288		
Age- and sex-adjusted HR	1·00	1·01	0·85, 1·21	1·06	0·89, 1·26	0·86	0·73, 1·02	0·046
Multivariable HR*	1·00	1·00	0·84, 1·19	1·10	0·92, 1·32	0·90	0·75, 1·08	0·188
Multivariable HR†	1·00	1·02	0·85, 1·22	1·14	0·95, 1·38	0·95	0·79, 1·16	0·496
Haemorrhagic stroke								
Number	88	100		99		106		
Age- and sex-adjusted HR	1·00	1·05	0·79, 1·40	1·16	0·87, 1·55	0·90	0·68, 1·20	0·400
Multivariable HR*	1·00	1·06	0·80, 1·42	1·26	0·94, 1·69	1·03	0·77, 1·40	0·857
Multivariable HR†	1·00	1·10	0·82, 1·47	1·34	0·98, 1·82	1·11	0·80, 1·52	0·620
Ischaemic stroke								
Number	85	90		84		103		
Age- and sex-adjusted HR	1·00	0·96	0·71, 1·29	0·92	0·68, 1·25	0·85	0·63, 1·13	0·210
Multivariable HR*	1·00	0·94	0·69, 1·26	0·96	0·70, 1·31	0·88	0·64, 1·19	0·389
Multivariable HR†	1·00	0·95	0·70, 1·29	0·98	0·71, 1·35	0·92	0·66, 1·26	0·554
CHD								
Number	123	115		97		117		
Age- and sex-adjusted HR	1·00	0·85	0·66, 1·10	0·78	0·59, 1·01	0·70	0·54, 0·90	0·006
Multivariable HR*	1·00	0·90	0·69, 1·16	0·85	0·65, 1·12	0·80	0·61, 1·05	0·124
Multivariable HR†	1·00	0·93	0·72, 1·21	0·92	0·69, 1·23	0·88	0·66, 1·18	0·407
Other CVD								
Number	195	169		175		199		
Age- and sex-adjusted HR	1·00	0·78	0·64, 0·96	0·86	0·70, 1·06	0·72	0·59, 0·88	0·020
Multivariable HR*	1·00	0·80	0·65, 0·99	0·93	0·75, 1·15	0·79	0·64, 0·98	0·097
Multivariable HR†	1·00	0·82	0·66, 1·01	0·96	0·77, 1·19	0·82	0·65, 1·02	0·196
Total CVD								
Number	556	550		533		604		
Age- and sex-adjusted HR	1·00	0·90	0·80, 1·01	0·93	0·82, 1·05	0·78	0·69, 0·87	<0·001
Multivariable HR*	1·00	0·91	0·81, 1·02	0·99	0·87, 1·12	0·84	0·74, 0·95	0·010
Multivariable HR†	1·00	0·93	0·82, 1·05	1·03	0·91, 1·17	0·89	0·78, 1·01	0·106
All causes								
Number	1883	1868		1741		2114		
Age- and sex-adjusted HR	1·00	0·92	0·86, 0·98	0·94	0·88, 1·01	0·86	0·80, 0·91	<0·001
Multivariable HR*	1·00	0·94	0·88, 1·00	0·99	0·92, 1·06	0·90	0·84, 0·96	0·006
Multivariable HR†	1·00	0·95	0·89, 1·01	1·00	0·94, 1·08	0·92	0·86, 0·98	0·025

* The same variables as shown in the footnote of Table 2.
† Adjusted further for fruit and vegetable intakes.

factors, these associations were attenuated slightly but remained statistically significant. The multivariable HR (95% CI) of total stroke, haemorrhagic stroke, total CVD and total mortality in the highest *v.* lowest quartiles of fruit intake were 0·67 (0·55, 0·81, *P* for trend < 0·001), 0·63 (0·46, 0·87, *P* for trend = 0·004), 0·75 (0·66, 0·85, *P* for trend < 0·001) and 0·86 (0·80, 0·92, *P* for trend < 0·001). These inverse associations did not alter materially when we adjusted further for vegetable and bean intakes.

Table 3 shows the sex- and age-adjusted and multivariable HR according to quartiles of vegetable intake. Vegetable intake was inversely associated with sex- and age-adjusted mortality from CHD, total CVD and total mortality; after adjustment for cardiovascular risk factors, these associations were weakened but the association with CHD remained statistically significant, that with CVD was borderline statistically significant, but that with total mortality was no longer statistically significant. The multivariable HR (95% CI) of CHD and total CVD in the highest *v.* lowest quartiles of vegetable intake were 0·77 (0·58, 1·00, *P* for trend = 0·08) and 0·88 (0·78, 0·99, *P* for trend = 0·07).

Table 4 shows the sex- and age-adjusted and multivariable HR according to quartiles of bean intake. Bean intake was inversely associated with sex- and age-adjusted mortality from total stroke, CHD, other CVD, total CVD and total mortality. After adjustment for cardiovascular risk factors, these associations were weakened, and were no longer statistically significant except for other CVD, total CVD and total mortality. The respective multivariable HR in the highest *v.* lowest quartiles of bean intake were 0·79 (0·64, 0·98, *P* for trend = 0·10), 0·84 (0·74, 0·95, *P* for trend = 0·01) and 0·90 (0·84, 0·96, *P* for trend = 0·01). After further adjustment for fruit and vegetable intakes, these inverse associations became weak and were of borderline statistical significance.

Discussion

In the present large prospective study of Japanese men and women, we found inverse associations of plant-based food intake with mortality from CVD after adjustment for cardiovascular risk factors. High fruit intake was associated with reduced mortality from haemorrhagic and total stroke, total

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CVD and all causes; vegetable intake tended to be associated with reduced mortality from CHD, total CVD and all causes; bean intake was associated with reduced mortality from total CVD as well as total mortality.

Further adjustment for other plant-based foods did not alter the association of fruit intake with mortality, but attenuated the associations of vegetable and bean intakes with mortality. The weakened associations, however, do not necessarily negate potential protective effects of vegetable and bean intakes, because those intakes were moderately correlated with fruit intake: the Spearman correlation coefficients of vegetable and bean intakes were 0·36 and 0·28, respectively. It is possible that vegetable or bean intake is merely a surrogate for fruit intake in the present study.

The meta-analysis of eight cohort studies showed that vegetable and fruit intakes were associated with a reduced risk of stroke[1], and several Japanese cohort studies also showed that intakes of vegetables, fruits and soya were associated with a reduced risk of stroke[11,12,14]. The present study showed that intakes of fruits, but not vegetables and beans, were associated with a reduced risk of stroke.

The meta-analysis for studies of Western countries showed that vegetable, and fruit intakes were associated with a reduced risk of CHD[2]. The present study added the evidence on fruit intake and reduced mortality from CHD in Japanese.

A recent Japanese study reported that soya intake was associated with a reduced risk of ischaemic stroke and myocardial infarction[14]. The present study extends the evidence that bean intake was associated with reduced mortality from total CVD and all causes.

As for the mechanisms for the inverse association between fruit intake and CVD, vitamin C reduces the lipid oxidation of LDL-cholesterol[20] and enhances the formation of endothelial prostacyclin that decreases vascular tone and inhibits platelet aggregation[21]. Potassium, magnesium, calcium, fibre and folate exert the beneficial effects described previously[22–25].

The protective effects of soyabean intake on CVD are now highlighted in Western countries, based on epidemiological studies that showed a lower incidence of CVD in Asian populations consuming soya foods as a dietary staple compared with those who consumed a typical Western diet[9,10,26,27]. Some clinical trials in Western countries that failed to detect the protective association led to the speculation that only high levels of habitual intake exerted the beneficial effects[10,27], and research into populations with a high level of intake was required. In the Japan Collaborative Cohort Study, we did not ask about the intake of soyabeans specifically, but the present finding of the inverse association between bean intake and mortality from total CVD would suggest a cardio-protective effect of soyabeans, because they are the most common beans eaten in Japan[28].

There are several mechanisms for the inverse association between bean intake and CVD. Potassium, calcium and fibre, which are plentiful in beans, may play a role in lowering blood pressure[23,24]. Potassium also inhibits platelet aggregation[23], and fibre, isoflavones, soya protein and saponins help lower total cholesterol levels[9,24]. Isoflavones also enhance antioxidant activity and improve arterial stiffness[9,25]. Folate, which is also plentiful in beans, lowers serum homocysteine levels, a correlate for arterial endothelial dysfunction[25].

Some limitations warrant discussion. First, the food frequency questionnaire used in the present study had high reproducibility but low-to-moderate validity for the estimation of the fruit, vegetable and bean intakes. Thus, some non-differential misclassification would be to weaken the diet–disease association. Second, a number of subjects were excluded because they did not respond sufficiently to the FFQ. However, a potential selection bias may be small because of no difference in mortality and cardiovascular risk characteristics between persons who responded to the food frequency questionnaire and those who did not.

Healthy behaviours associated with plant-based food intake might confound the association with mortality from CVD. Non-smoking, appropriate alcohol intake, more physical activity and higher education are potential confounders in the present study. However, after adjustment for these confounding variables, the associations with mortality from CVD remained statistically significant, suggesting that independent effects of plant-based foods exist for the prevention of CVD. Residual confounding and the contribution of other unexamined factors, however, were not negated.

In conclusion, fruit intake was inversely associated with mortality from stroke, total CVD and all causes, and bean intake was also inversely associated with mortality from total CVD and all causes among Japanese men and women. The present findings suggest the potential for beneficial effects of plant-based food intake for the prevention of CVD in general populations.

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