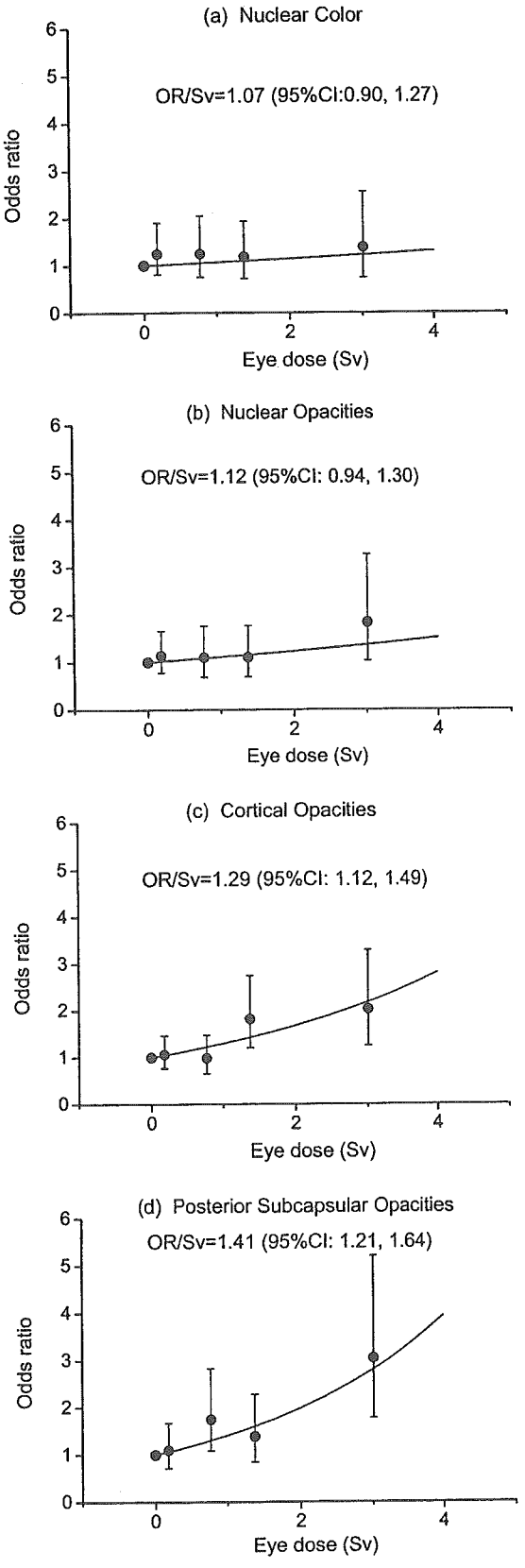

and smoking, and used the lowest group as a reference revealed that ORs at 1 Sv in cortical and posterior subcapsular opacities were 1.34 (95% CI 1.16–1.52) and 1.36 (95% CI 1.17–1.58), respectively. The differences of ORs at 1 Sv with and without adjustment of the intermediate risk factors were 17 % in cortical opacities and 12 % in posterior subcapsular opacities.

4. Discussion

The study revealed that 57 years after radiation exposure, the prevalence of cortical and posterior subcapsular opacities among A-bomb survivors showed a statistically significant correlation with radiation dose after adjusting for city, sex, age at the time of the bombings and smoking. The same was true after excluding the 13 subjects with posterior subcapsular opacities at the previous study (1978–80). The results were consistent with previous reports (Wilde and Sjöstrand 1997) of cortical opacities and demonstrated late onset posterior subcapsular opacities in A-bomb survivors. The ORs of 1.29 in cortical opacity and 1.41 in posterior subcapsular opacity were similar to the 1.35 and 1.50, respectively, reported by Hall (1999). In addition, by introducing the LOCS II system into the present study, interobserver variation in posterior subcapsular opacities was overcome, but not in cortical opacities, as shown by city difference (table 5). The dose–response in cortical opacities, however, was not affected by interobserver variation. The study suggests that the two opacities of cortical and posterior subcapsular regions were significantly associated with each other ($r=0.333$, $p<0.001$), indicating common biological interactions for the two opacities.

The participation rate was low because only a limited number of ophthalmological examinations were offered each week. However, since the examinations were conducted blindly and showed no variation in participation rate with radiation dose, the low sampling rate was unlikely to have caused a bias in the dose–effect besides the low power for detection of radiation effects.

As for significant correlations with radiation dose in diabetic retinopathy, retinal arteriolosclerosis and retinal degeneration, the findings agree with evidence

Figure 1. Odds ratios (OR) of the prevalence for nuclear colour (a), nuclear opacities (b), cortical opacities (c) and posterior subcapsular opacities (d) at 1 Sv (DS86) in 873 A-bomb survivors during 2000–02 using a proportional odds regression model with 'no opacity' as the reference of the LOCS II and adjusting for city, sex and age at the time of the bombings.

Table 5. Odds ratios of city, sex, age the at time of bombings and radiation dose in the prevalence of cortical and posterior subcapsular opacities.

Variable	Odds ratio	95% Confidence interval
Cortical opacity:		
City (Nagasaki/Hiroshima)	3.31	2.56, 4.28
Sex (females/males)	1.62	1.26, 2.08
Age at the time of bombings (/10 years)	3.70	3.09, 4.44
Radiation dose (Sv)	1.29	1.12, 1.49
Posterior subcapsular opacity:		
City (Nagasaki/Hiroshima)	0.92	0.67, 1.26
Sex (females/males)	1.17	0.86, 1.61
Age at the time of bombings (/10 years)	2.10	1.71, 2.58
Radiation dose (Sv)	1.41	1.21, 1.64

previously observed in A-bomb survivors, such as increases of prevalence of diabetes mellitus (Hayashi *et al.* 2003) and findings of fundus photos (unpublished data), although the mechanism(s) is not clear. As a possible mechanism, since inflammation has been persistently observed in A-bomb survivors (Neriishi *et al.* 2001) and since inflammation has been proposed as a risk factor of diabetes mellitus (Pradhan *et al.* 2001) and/or arteriosclerosis (Ross 1999), the present paper is analysing the effect of inflammation on the above findings.

We searched for 'intermediate risk factors' to which radiation causes some alterations, that in turn cause lens opacities and it was found that they comprised retinal arteriolosclerosis and alpha 1 globulin for cortical opacities, and white blood cell count, calcium, and haemoglobin A1C values for posterior subcapsular opacities. Inclusion of the significant intermediate risk factors into the analysis changed the ORs of cortical and posterior subcapsular opacities to 1.34 (17% change) and 1.36 (12% change), respectively. However, it did not affect the statistical significances of the dose–response relationship in either cortical or posterior subcapsular opacities. When inflammatory tests were combined as a primary component and adjusted for, the dose coefficient change was as large as 20% (data not shown). Since elevated levels of inflammation and serum calcium have been significantly associated with A-bomb radiation (Fujiwara *et al.* 1992, Neriishi *et al.* 2001), elevated levels of inflammation and calcium could have played important roles as micro-environmental factors in the development of radiation cataracts. One cannot yet draw conclusions, however, because the study did not show impairment of the blood aqueous barrier, which blocks the influx of blood components into the anterior chamber. To demonstrate that would require further studies, including animal experiments.

There might be other, as yet unknown, mechanisms of lens changes caused by A-bomb exposure, such as a radiation-induced decrease in lens epithelial stem cells. It is also plausible, since inflammation in A-bomb survivors is significantly and negatively associated with CD4 T-cell levels (Neriishi and Nakashima 1999, Hayashi *et al.* 2003) that radiation has an indirect effect via immune impairment (Kusunoki *et al.* 2002). Taking into account the presence of auto-antibodies in those with cataract (Patel *et al.* 1990, Nayak *et al.* 2002), it would be intriguing to investigate lens auto-antibodies in A-bomb survivors.

In conclusion, the present study showed a significant correlation between A-bomb radiation dose and cortical and posterior subcapsular opacities. It also suggested indirect effects of elevated levels of inflammation and serum calcium in the dose–response of posterior subcapsular opacities.

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RESEARCH ARTICLE

Increasing of oxidative stress from mitochondria in type 2 diabetic patients

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Abstract

Background Recent evidence increasingly indicates that oxidative stress may play an important role in the pathogenesis of diabetic vascular complications. Mitochondria has received much attention as an important organ in the generation of oxidative stress. However, the importance of oxidative stress among diabetic patients without vascular complications is unclear.

Methods We compared oxidative stress produced from mitochondria of the mononuclear cells in peripheral blood obtained from 26 diabetic subjects without clinical vascular complications and 52 healthy age-matched subjects using a flow cytometer. Oxidative stress from the mononuclear cells was evaluated by measuring fluorescence of oxidized production from dihydrorhodamine-123, which is a pro-fluorescent compound that selectively accumulates in the mitochondria of living cells. Stimulation of the cells was carried out with phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator. We then calculated the relative fluorescence variation (RFV) that indicated an increasing rate of oxidative stress levels by stimulation with PMA against the levels obtained at baseline. Additionally, we measured the urinary stress markers, 8-hydroxydeoxyguanosine (8OHdG) and 8-epi-prostaglandin F2α (isoprostane).

Results Compared to healthy subjects, diabetic subjects did not exhibit significantly elevated oxidative stress levels at baseline, but did have significantly elevated basal urinary 8OHdG, urinary isoprostane and oxidative stress levels after PMA stimulation as well as RFV.

Conclusions Among diabetic subjects without clinical vascular complications, there was a possibility that mitochondrial oxidative stress balance between generation and scavenging against the additive PKC stimulation was thought to have already been lost. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords oxidative stress; mitochondria; type 2 diabetes mellitus; flow cytometry

Introduction

The relationship between oxidative stress and many diseases has recently received considerable attention. Under a chronic hyperglycemic state, tricarboxylic acid cycle activation in the mitochondria is thought to accelerate the production of reactive oxygen species (ROS), leading to oxidative stress in various organs and cells. For example, it was demonstrated that impaired glycemic control led to increased lysophosphatidylcholine

content [1], which might contribute to atherogenesis [2] and reflect oxidative modification of lipoprotein [1]. Increased oxidative stress is also thought to relate to the injury of various organs, with evidence increasingly indicating that oxidative stress may play an important role in diabetes vascular complications [3]. However, the importance of oxidative stress among diabetic patients without vascular complications is unclear.

In investigating the role of oxidative stress in diabetes, mitochondria are important organelles. A type of diabetes associated with a mutation of mitochondrial DNA has been identified [4]. Mitochondria, which provide energy for adenosine triphosphate through oxidative phosphorylation by the electron transport chain, are also the principal source of ROS resulting from imperfect electron transport. Therefore, many extensive *in vitro* investigations have been carried out about the relationship between oxidative stress and diabetes. However, few clinical indicators exist, because of the difficulty of assessing oxidative stress levels from mitochondria.

Mononuclear cells such as lymphocytes and monocytes are principally inflammatory cells. Once activated, they produce ROS and play an important role not only in the immune response but also in the first stages of atherosclerotic development [5,6]. An increase in oxidative stress is thought to promote atherogenesis [7], and diabetes mellitus is considered to be one of the major risk factors for myocardial infarction [8]. Accordingly, the possibility exists that a functional disorder of the mononuclear cells might affect the production of the cells' oxidative stress because of a hyperglycemic state due to diabetes.

Therefore, we obtained living mononuclear cells from both healthy subjects and diabetic subjects without clinical vascular complications and stained them with dihydrorhodamine-123 (DHR-123), which selectively accumulates in the mitochondria [9]. We subsequently measured the mitochondrial oxidative stress levels of the cells directly before and after addition of phorbol 12-myristate 13-acetate (PMA), which is a protein kinase C (PKC) activator that generates oxidative stress. Comparing these data from the healthy and diabetic subjects, we evaluated the mitochondrial oxidative stress levels of mononuclear cells in diabetes before the development of clinical vascular complications. We also investigated the relationship between these levels and both urinary 8-hydroxydeoxyguanosine (8OHdG) [10] and 8-epi-prostaglandin F2α (isoprostane) [11], which are thought to be putative biomarkers of total systemic oxidative stress *in vivo*.

Materials And Methods

The study group consisted of 52 age-matched apparently healthy volunteers (25 men and 27 women) and 26 subjects with type 2 diabetes (16 men and 10 women, mean duration of diabetes: 8.5 ± 0.5 years), who had consulted an outpatient clinic of diabetes and been diagnosed free from diabetic microangiopathy. All subjects were nonsmokers, free from pain or inflammatory diseases, known myocardial infarction, stroke or arteriosclerosis obliterans, and were aged between 45 and 80 (mean: 62.2 ± 1.0) years. Diabetic subjects and healthy volunteers were treated if they had dyslipidemia (8 and 4 subjects, respectively) or hypertension (11 and 9 subjects, respectively). Eight diabetic subjects were treated by diet and exercise for glycemic control, and 18 subjects by oral hypoglycemic agents. A definition of diabetes was based on the 1998 World Health Organization (WHO) criteria [12]. All diabetic patients were consulted and were classified for diabetic retinopathy by trained ophthalmologists. Nephropathy was defined as the presence of microalbuminuria or proteinuria, and neuropathy was diagnosed if subjective symptoms or a decline in tendon reflexes was present. All subjects provided blood and spot urine samples after an overnight fast for biochemical, hematological parameters and mononuclear cell isolation. We used an automated enzymatic procedure to determine serum triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol level. The concentration of blood glucose was determined with the hexokinase-glucose-6-phosphate dehydrogenase method. HbA_{1c} was measured using ion-exchange high-performance liquid chromatography. Blood pressure (mmHg) was measured by a standard mercury sphygmomanometer with the subjects in the sitting position; standing height (cm) and body weight (kg) were also measured. All subjects were informed about the objectives and methods of this study and written informed consent was obtained from them.

Mononuclear cell isolation was carried out by heparinized blood samples using Cappel lymphocyte separation medium (ICN Biomedicals, Costa Mesa, CA), according to the method previously described [13]. Oxidative stress was analyzed using DHR-123 (Molecular Probe, Eugene, OR) by the previously described procedure [14]. In brief, DHR-123 that accumulates in the mitochondria is a nonfluorescent compound, but when converted to rhodamine-123 by the action of oxidative stress in the mitochondria, it becomes highly fluorescent. This enabled us to directly monitor the mitochondrial oxidative stress levels. The cells were washed twice, counted and resuspended to 1×10^6 cells/mL in RPMI 1640 with 1% bovine serum. They were divided into three tubes. One was expressed for the control without the fluorescent compound (Control: C), one was for baseline mitochondrial oxidative stress levels with DHR-123 (1 μM) (Basal: B), and the last was for stimulated mitochondrial oxidative stress levels with both DHR-123 (1 μM) and PMA (50 nM) (Sigma-Aldrich, St. Louis, MO) (PMA-stimulated: S). The fluorescent levels of tube B were considered to be a reflection of baseline mitochondrial oxidative stress because rhodamine-123 became fluorescent from mitochondrial oxidative stress that already existed at ordinary status without PKC activation. The fluorescent levels of tube S were assumed to be a reflection of the stimulated status of mitochondrial oxidative stress due to respiratory burst with PKC activation. The cells in the three tubes were incubated at 37 °C for 90 min. They were then washed

once rapidly and resuspended in 400 μl of RPMI 1640 with 1% bovine serum and stored on ice until flow cytometry was carried out.

Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA) and analyzed at a level of 10 000 events for each test. The oxidative stress levels of mononuclear cells were measured using a fluorescence histogram through gated flow cytometry of viable cells. The degree of fluorescence was expressed by the mean fluorescence intensity (MFI) calculated for each tube by CELL Quest ver.3.2.1. The relative fluorescence variation (RFV), which is the rate of increase between oxidative stress levels before PMA stimulation, that is, MFI(B)−MFI(C), and after PMA stimulation, that is, MFI(S)−MFI(C), was calculated with minor modifications according to the previously described procedure [15]. Ultimately, it was calculated by the following formula: [(MFI(S)−MFI(C))−(MFI(B)−MFI(C))/(MFI(B)−MFI(C))], namely, (MFI(S)−MFI(B)/MFI(B)−MFI(C)). We also measured both urinary 8OHdG and isoprostane, which could be used to estimate the sum of systemic oxidative stress levels without invasive examinations. Urinary 8OHdG was measured by ELISA kit (NOF Corporation, Tokyo, Japan), as described previously [11,16]. Urinary isoprostane was measured by EIA kit (Oxford Biomedical Research, Inc., Oxford, MI) [17]. These results were expressed as the ratios to the urinary creatinine measured in the same urine samples.

Results were indicated as mean ± SE. The degree of obesity was expressed by the body mass index (BMI) (kg/m^2), calculated as body weight divided by the square of height. Parametric comparisons between healthy and diabetic subjects were performed by analysis of covariance (ANCOVA) with sex as a covariate. In this analysis, because triglycerides, BMI, urinary 8OhdG and isoprostane did not show normal distributions, these data were analyzed after logarithmic transformation. Also, regression analyses were performed by two sets to investigate associations between RFV, as a dependent variable, and each factor, as an independent variable: the first set, adjusted for age and sex (1 = men, 0 = women), and the second set, adjusted for age, sex and diabetes (1 = diabetes, 0 = not diabetes). For all data analysis, SAS package version 8.2 (SAS Institute, Cary, NC) was used.

Results

Clinical characteristics of the study subjects are shown in Table 1. Compared to healthy subjects, diabetic subjects had higher systolic blood pressure, although the difference was not significant ($P = 0.083$). BMI, total cholesterol, HDL-cholesterol and LDL-cholesterol, which were thought to be indicators for the risk of cardiovascular disease, were not significantly different between the two groups. However, diabetic subjects had significantly higher fasting glucose and HbA_{1c} levels ($P < 0.0001$). Thus, these results indicated that the clinical characteristics of these groups did not differ significantly, except in the case of glucose metabolism.

Table 1. Clinical characteristics of the study subjects adjusted for sex

	Healthy subjects	Diabetics	P
Numbers of subjects (men/women)	25/27	16/10	
Age (years)	62.4 ± 1.2	62.1 ± 1.7	0.907
Body mass index (kg/m^2)	24.0 ± 0.4	24.9 ± 0.6	0.213
Systolic blood pressure (mmHg)	129 ± 2	135 ± 3	0.083
Diastolic blood pressure (mmHg)	78 ± 1	82 ± 2	0.161
Total cholesterol (mmol/l)	5.28 ± 0.10	5.09 ± 0.14	0.275
Triglycerides (mmol/l)	1.50 ± 0.16	1.96 ± 0.23	0.176
HDL-cholesterol (mmol/l)	1.43 ± 0.05	1.49 ± 0.07	0.475
LDL-cholesterol (mmol/l)	3.09 ± 0.09	2.85 ± 0.13	0.140
HbA_{1c} (%)	5.2 ± 0.1	7.1 ± 0.1	<0.0001
Fasting glucose (mmol/l)	5.5 ± 0.3	9.5 ± 0.5	<0.0001

Data are expressed as means ± SE.

Each degree of oxidative stress level by calculation of this fluorescence and the data of urinary oxidative stress markers are shown in Table 2. The baseline oxidative stress levels without PMA were higher among diabetic subjects than healthy subjects, but not significantly ($P = 0.549$). On the other hand, diabetic subjects had significantly higher oxidative stress levels stimulated by PMA and RFV ($P < 0.0001$ for each). Urinary 8OHdG and isoprostane were also significantly higher among diabetic subjects ($P = 0.019, P = 0.009$ respectively).

Next, regression analyses were performed to determine the relationships between RFV and each parameter (Table 3). In the first set, adjusted for age and sex, RFV was significantly associated with urinary 8OHdG and isoprostane ($P = 0.039, P = 0.017$ respectively). In the second set, adjusted for age, sex and diabetes, it was not statistically significant, but had a suggestive association with urinary 8OHdG and isoprostane ($P = 0.081, P = 0.091$ respectively).

Discussion

In this study, mitochondrial oxidative stress levels of mononuclear cells from peripheral blood were not

Table 2. Comparisons of basal and PMA-stimulated oxidation levels or the urinary oxidation markers between healthy subjects and diabetic subjects adjusted for sex

	Healthy subjects	Diabetics	P
Control (C) (MFI)	8.7 ± 0.8	10.6 ± 1.1	0.152
Basal (B) (MFI)	281.4 ± 18.8	301.1 ± 26.8	0.549
PMA-stimulated (S) (MFI)	437.4 ± 92.4	1218.0 ± 131.7	<0.0001
RFV	0.70 ± 0.42	4.02 ± 0.60	<0.0001
Urinary 8OHdG (ng/mg · creatinine)	8.8 ± 0.5	11.1 ± 0.8	0.019
Urinary isoprostane (ng/g · creatinine)	0.32 ± 0.13	0.87 ± 0.18	0.009

Data are expressed as means ± SE. PMA, phorbol 12-myristate 13-acetate; MFI, mean fluorescence intensity; RFV, the relative fluorescence variation.

Table 3. Regression analyses between RFV and each parameter

	Adjusted for age and sex		Adjusted for age, sex and diabetes	
	β	P	β	P
Body mass index (kg/m^2)	−0.015	0.914	−0.096	0.432
SBP (mmHg)	−0.020	0.473	−0.037	0.144
DBP (mmHg)	−0.036	0.337	−0.047	0.163
Total cholesterol (mmol/L)	0.422	0.450	0.735	0.141
Triglycerides (mmol/L)	0.154	0.651	−0.105	0.735
HDL-cholesterol (mmol/L)	1.362	0.220	1.001	0.314
LDL-cholesterol (mmol/L)	−0.005	0.993	0.427	0.427
Urinary 8OHdG (ng/mg · creatinine)	0.202	0.039	0.152	0.081
Urinary isoprostane (ng/g · creatinine)	0.092	0.017	0.062	0.091

RFV, the relative fluorescence variation.

significantly higher at baseline, but when stimulated by PKC, oxidative stress levels were significantly higher in diabetic subjects without clinical vascular complications than in healthy subjects. Additionally, it was suggested that whole oxidative stress levels tended to increase in diabetic subjects, because urinary 8OHdG and isoprostane levels were significantly higher, and oxidative stress generation was increased and/or the scavenging mechanisms against oxidative stress under PKC stimulation were diminished in diabetic subjects even before the development of clinical vascular complications.

We should consider that these results have two possible explanations. The first possibility is that diabetes might induce oxidative stress. The second is that, conversely, oxidative stress might induce diabetes. Concerning the first possibility, some activation pathways of oxidative stress in diabetes are theorized: hyperactivity of polyol pathway [18]; increased formation of advanced glycation end products [19] and glucose-induced activation of PKC [20]. PKC is a phospholipid-dependent serine/threonine protein kinase, activated by diacylglycerol that is mainly derived from the *de novo* pathway from the glycolytic intermediates [21,22]. Thus, a hyperglycemic state stimulates ROS production through PKC activation [20]. In addition, it was reported recently that PKC was activated by hyperglycemia-induced oxidative phosphorylation in cultured bovine aortic endothelial cells [23]. However, even in diabetic patients, we did not detect a significant increase in mitochondrial oxidative stress levels at baseline that was supposed to be generated by PKC activation, but we detected an increase in the level upon stimulation by the additive PKC activator (Table 2). Accordingly, it was suggested that PKC was not always activated in diabetic subjects without clinical vascular complications, and that the generation mechanisms of the mitochondrial oxidative stress were accelerated and/or scavenging mechanisms against it were decreased under PKC activation compared to healthy subjects. Actually, a hyperglycemic state is reported to impair radical scavenging activity [24].

We cannot deny the second possibility that oxidative stress itself is related to the development and progression of diabetes; that is, some individuals might be susceptible to the development and progression of diabetes as a result of impairment of sufficient oxidative stress level control, when PKC was activated in various degrees by their own genetic makeup or environmental factors. As a result, diabetic subjects might be at high oxidative stress levels. The possibility exists that oxidative stress of mononuclear cells circulating throughout the body might injure pancreatic β cells, leading to impaired glucose metabolism. In addition, increased oxidative stress could precede the development of endothelial dysfunction and insulin resistance [25]. The imbalance between the generation and scavenging mechanisms of oxidative stress might be associated with the pathophysiology of diabetes. Overproduction of the mitochondrial ROS might induce cell injury, hypothetically leading to a weak but chronic inflammatory state, which is presently believed to relate to the development of diabetes. For example, lysophosphatidylcholine, known to be a major phospholipid compartment of oxidized LDL, was thought to contribute to acute and chronic inflammation [2], and high levels of C-reactive protein, a marker of inflammation, were reported to be one of the risk factors for development of diabetes [26,27]. Thus, there is a possibility that both oxidative stress and its resultant chronic inflammation might be involved in some of the mechanisms of diabetes development.

Because we found in this study that mitochondrial oxidative stress was related to diabetes, a sensitive marker that is suitable for monitoring oxidative stress is anticipated. In general, diabetic patients have high oxidative stress levels for various reasons, for instance, because of obesity [28], NADPH oxidase activation in monocyte [29] and so forth. Urinary 8OHdG [10] and isoprostane [11] are noteworthy markers; they were significantly higher among diabetic subjects than healthy subjects in this study (Table 2). However, they are thought to be a reflection of the sum of systemic oxidative stress levels rather than a reflection of the metabolites of oxidative stress from a specific organ. To the contrary, RFV was thought to be a marker reflecting the mitochondrial oxidative stress levels more directly. In our study, although it had a significant relationship to both urinary 8OHdG and isoprostane adjusted for age and sex, these relationships disappeared after adjustment for age, sex and diabetes (Table 3). Thus, it was suggested that mitochondrial oxidative stress, as well as each urinary oxidative stress marker, might be strongly affected by glucose metabolism.

This study has some limitations. First, it remains a possibility that type 2 diabetes subjects might be inadvertently included in healthy subjects even when they undergo the 75- g oral glucose tolerance test. However, diabetes in this study was diagnosed according to WHO criteria [12], and all healthy subjects were confirmed to have a fasting glucose level less than 7.0 mmol/L and, in addition, an HbA_{1c} level less than 6.5%. Second, the results of this study

might be modified by the diabetes medication. For example, one patient was treated for diabetes by pioglitazone, one of the thiazolidine derivatives, and six patients were treated for hypertension by angiotensin II receptor blockers. These drugs have reported to reduce ROS generation [30–32]. However, the results of our data such as RFV and urinary markers did not differ significantly according to either these drugs or the oral hypoglycemic agents (data not shown), but the problem remains whether the improvement of glycemic control improves the balance of mitochondrial oxidative stress. Third, DHR-123 used to measure oxidative stress is accumulated in mitochondria according to the Nernst equation, due to their high membrane potential [9]. Consequently, the possibility remains that the changes in the mitochondrial membrane potential, not the changes in the mitochondrial oxidative stress level, might alter the uptake of this dye and hence alter the fluorescence measured and the degree of oxidative stress inferred. We must consider that, at least in part, changes in mitochondrial membrane potential could account for the differences in the fluorescence observed following PMA stimulation.

In summary, the mitochondrial oxidative stress levels of mononuclear cells from peripheral blood at baseline among diabetic subjects without clinical vascular complications were not statistically higher than those among healthy subjects, but were significantly higher under PMA stimulation. This result indicated an imbalance of generation and elimination of mitochondrial oxidative stress among diabetic subjects before the development of clinical vascular complications.

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A prospective study of diet and prostate cancer in Japanese men

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Key words: prostate cancer, diet, fish, soya, atomic-bomb survivors, Japan.

Abstract

Background and aims: It has been hypothesized that some aspect of a traditional 'Asian' diet, that is low in animal products and high in soya, may be associated with a reduced risk of prostate cancer. This study aimed to examine the association between dietary intake and prostate cancer risk among 18,115 men in Hiroshima and Nagasaki, Japan, using prospective data from the Life Span Study.

Methods: Subjects completed a food-frequency questionnaire at baseline (1963, 1965 and/or 1979) and were followed for incident prostate cancer until the end of 1996. During this time, 196 incident prostate cancer cases were identified after 252,602 person-years of observation. Poisson regression was used to calculate incidence rates for each dietary factor after adjustment for age, calendar period, city of residence, radiation dose and education level.

Results: Fish intake was significantly associated with an increased risk of prostate cancer; men who consumed fish more than four times per week had a 54% increased risk of developing prostate cancer compared with men who ate fish less than twice per week (RR = 1.54; 95% CI, 1.03–2.31). No other food items, including soya products, were significantly associated with prostate cancer risk.

Conclusions: These data suggest that dietary factors may not be strong determinants of prostate cancer in these Japanese men, although the increased risk associated with a high consumption of fish warrants further study.

Introduction

Prostate cancer incidence rates vary substantially worldwide, being high in Western Europe and the US (age standardized rate; 40–100/100,000 per year) and much lower in China and Japan (2–13/100,000 per year). Indeed, prostate cancer accounts for about 12% of all male cancer deaths in the UK compared with about 4% of all male cancer deaths in Japan [1]. The only established risk factors for prostate cancer are increasing age, being of Africa–American origin and having a family history of the disease. However, the observation that rates of clinical prostate cancer vary more widely between countries than rates of sub-clinical prostate cancer (i.e., latent cancer and incidental cancer) [2, 3], suggests that environmental factors may play an important role in the progression of the disease. The substantial rise in prostate cancer mortality rates over the last 40 years in formerly low-incidence countries, such as Japan [4] strongly suggests that factors associated with increasing 'Westernization' may be involved. In particular, it has been hypothesized that the adoption of a Western-style diet high in animal products (typically meat and dairy foods) may be important in the development of prostate cancer. In contrast to a Western-style diet, a traditional Japanese diet is typically low in meat and dairy products and high in rice, fish and soya products.

Little is known about the role of diet in the aetiology of prostate cancer, although a high consumption of meat, dairy products and saturated fat has frequently

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been associated with an increased risk in studies conducted in Western populations [5]. However, no definite causal or protective associations for specific nutrients or dietary factors have been established [6]. International comparisons also suggest an inverse association between consumption of soya products and prostate cancer, principally due to low mortality rates of prostate cancer in Asian countries with a high soya intake [7]. Soya beans are the main source of the dietary isoflavones, genistein and daidzein, which have been shown to exhibit anti-carcinogenic properties *in vitro* [8] and have been associated with a reduction in prostate cancer growth in some animal studies [9, 10]. However, very few epidemiological studies have reported on the association between soya intake and prostate cancer risk in humans [11–13].

The aim of the present study is to examine the association between diet and prostate cancer risk in the Life Span Study cohort in Hiroshima and Nagasaki, Japan. In particular, we address the hypotheses that a high intake of meat and other animal products is associated with an increased risk of prostate cancer and that a high intake of soya products is associated with a reduced risk.

Materials and methods

Subjects

The Life Span Study cohort was established by the Atomic Bomb Casualty Commission, predecessor of the Radiation Effects Research Foundation, with the aim to follow-up cancer mortality (from 1950) and cancer incidence (from 1958) among atomic-bomb survivors. The cohort includes about 93,000 people who were in Hiroshima or Nagasaki at the time of the bombings and who were residents of one of the cities in the 1950 census. In addition, about 26,500 usual residents who were not present in either city at the time of the bombings were identified in special censuses conducted between 1950 and 1953 and recruited into the cohort [14]. Of the total cohort, approximately 50,000 were men (42%). The Adult Health Study is a 20% sub-sample of the Life Span Study initiated in 1958, designed to focus on individuals who had received a high radiation dose, and which includes biennial clinical examinations and information from interview-based questionnaires. The data used in the present analysis is based on three questionnaires; the first two are derived from the AHS and include 3510 men who completed a questionnaire in 1963 (Survey 1) and 4789 men who completed a questionnaire in 1965 (Survey 2). The third survey was mailed out between 1979 and 1981 to all the 55,650 LSS participants who were alive on September 1, 1978 [15], of whom 40,349 individuals (15,350 men) returned a self-completed questionnaire after up to three mailings (Survey 3). After excluding men who had previously been registered as having a prostate cancer diagnosis, 18,115 men were available for the present analysis. Of these, 2545 men (14%) completed more than one dietary Survey; 1606 completed Surveys 1 and 2; 83 men completed Surveys 1 and 3; 765 men completed Surveys 2 and 3; and 1540 men completed all three Surveys.

Exposure measurement

The three questionnaires were very similar and included questions on socio-demographics, lifestyle factors, past medical history, and a food frequency questionnaire that included the following items: meat, butter/cheese, milk, fish, fruit, green/yellow vegetables, pickled/salted vegetables, confectionery, and bread; Survey 2 (1965) also included rice, pork, eggs, miso soup (fermented soya bean paste), tofu (soya bean curd), seaweed, green tea, black tea, and coffee. Information on intake of chicken, broiled fish and dried fish was only available from Survey 3 (1979).

For rice and bread intake, the frequency of consumption (in Surveys 2 and 3) could be 'do not eat', 'once or less per day', 'twice per day', and 'three or more times per day'. For green tea the categories were 'do not drink', 'once or less per day', 'two to four times per day', and 'five or more times per day' (Surveys 2 and 3). The pre-coded categories for all foods in Survey 1 comprised 'rarely', 'normal' and 'frequently' and the frequency of consumption for Survey 2 for all foods except rice and green tea were 'less than two times per week', 'two to four times per week' and 'almost daily'. Survey 3 also included a category of 'do not eat' in addition to those listed for Survey 2. To ensure comparability across all surveys, 'rarely', 'normal' and 'frequently' were taken as 'less than two times per week', 'two to four times per week' and 'almost daily', respectively, and the 'do not eat' category included in Survey 3 was combined with the next lowest intake category.

Relative risks were calculated for the frequency of intake of individual foods, with the exception of meat intake as this variable referred to different meat products across the three surveys. Relative risks were also calculated for the frequency of intake of composite variables of total meat, total dairy and eggs, total fish and total soya. The analyses on composite foods for total meat and total fish intake were restricted to participants who completed Survey 3 only (n = 15,350) because previous surveys

did not have complete information on all the composite food items. The analysis of total soya and total dairy and eggs intake included participants who completed Survey 2 or Survey 3 (n = 17,834) as the composite food items were available from both surveys. All analyses were based on each individual's reported consumption at each dietary assessment; no attempt was made to combine the dietary surveys to arrive at a cumulative/average exposure.

The frequency of intake of each food product was categorized into three groups: 'missing or less than two times per week' (scored as 1), 'two to four times per week' (scored as 2), and 'almost daily' (scored as 3). For total meat intake, the sum of scores for meat, pork and chicken (3–9) were categorised as 'low' (a score of 3), 'intermediate' (a score of 4–6) and 'high' (a score of 7 or more). Total dairy product and egg intake was defined as the sum of scores for butter/cheese, milk and eggs (3–9) and was categorized in the same way as for total meat intake. Total fish intake was classified as the sum of fish and broiled fish intake (2–6), and re-categorized as 'low' (a score of 2), 'intermediate' (a score of 3–4) and 'high' (a score of 5 or more). Total soya intake was defined as the sum of tofu and miso soup intake and was re-categorized in the same way as for total fish intake.

The validity of the food-frequency questionnaire has been examined and showed a moderate agreement between the frequency of intake of all food items as stated on the FFQ from Survey 3 with that derived from a 24-hour diet diary, completed between 1984 and 1985 [16]. The correlation coefficients between estimated intake from the FFQ and a 24-h diary were highest for coffee (0.51) and milk (0.32), with animal products having an average correlation of 0.14 and the soya products tofu, and miso soup having correlations of 0.14 and 0.25 respectively. Consumption of fish from the FFQ and a 24-h diary was also correlated with a coefficient of 0.14, however, the intake of dried fish from the FFQ was not associated (correlation = −0.03), and was therefore excluded from the present analysis. Participants with missing data were included in the lowest category of intake because a previous analysis found the mean intake of those with missing data to be similar to the mean intake of those in the 'less than two times per week' category [16]. Data were missing for less than 10% of study participants for all foods, with the exception of bread (20%), butter/cheese (12%) and pork (11%). Repeating the analysis to include missing dietary values as a separate category made little difference to the results, and so analyses using missing values combined with the lowest category are presented.

Radiation-dose estimates

Individual radiation-dose estimates were based on the RERF Dosimetry System, 1986 (DS86) [17]. This system provides estimates of individual γ-ray and neutron shielded organ doses based on information about a survivor's location and surrounding shield conditions. The DS86 colon dose (the organ closest to the prostate for which dose information was available) was calculated as γ-ray dose plus ten times the neutron dose in units of Sievert (Sv), to allow for the differences in the radiobiological effectiveness of γ-rays and neutrons.

Follow-up

Follow-up for cancer incidence was by linkage with the population-based cancer registries in Hiroshima and Nagasaki [18]. Incident cases were identified and abstracted through medical records, and were supplemented with death certificate information from local health centres, pathology specimens and reports retrieved through an active tissue registry, and autopsy diagnoses. Matches between the tumour-registry data and the Life Span Study cohort were made through computer linkage and manual searches. For men who responded to either Survey 1 or Survey 2, the start of follow-up was taken as the month and year in which the completed questionnaire was received. For men who responded only to Survey 3, the start of follow-up was taken as January 1, 1980, the date by which all completed questionnaires were received, since the date of receipt of the questionnaire was not recorded for each subject. The end of follow-up was taken as the date of diagnosis of prostate cancer for cases and the date of death, age 90, or the end of follow-up (December 31, 1996), for non-cancer cases, whichever occurred first. Men who were registered with prostate cancer before the start of follow-up were excluded.

Adjustment for migration

Since the cancer registries only cover a specific catchment area, information on cancer diagnoses in subjects who had migrated out of the area during the follow-up period was unavailable. To account for the effect of migration on incidence rates, we applied sex-specific residence probabilities for each city, birth cohort and time period to reduce the person-years at risk, based on migration rates in the Adult Health Study cohort [19]. This resulted in an overall reduction in person-years of 17% (from 304,598 to 252,602).

Statistical analysis

Person-years at risk was calculated for each individual according to dietary intake in Survey 1 until the date of entry to Survey 2, where upon person-years were calculated according to dietary intake as stated in Survey 2 until the date of entry to Survey 3, after which person-years were calculated in relation to dietary intake as recorded in Survey 3.

Case-counts and person-years were cross-tabulated by attained age (<65, 65–69, 70–74, 75–79, 80+ years), calendar period (1963–1969, 1970–1979, 1980–1984, 1985–1989, 1990–1994, 1995–1996), city of residence at the time of the bombing (Hiroshima, Nagasaki), radiation dose in Sv (0, 0.01–0.06, 0.07–0.3, 0.31+, unknown) and education level (none/elementary, secondary or higher, unknown). Relative risks and 95% confidence intervals (CI) were estimated using Poisson regression for grouped survival data and were based on likelihood ratio statistics. All models were assessed for goodness of fit using a series of standard diagnostic plots and by assessment of the likelihood ratio statistic for over-dispersion. Associations between prostate cancer risk and marital status (married, unmarried, unknown), smoking (no, yes, unknown), body mass index (BMI; <20, 20–22, 23+ kg/m^2) and the food exposure of interest were examined after adjustment for age, calendar period, city of residence, radiation dose and education level. For the socio-demographic variables, all missing values were included as a separate category to make full use of the dataset, although this category was not included in the corresponding tests for heterogeneity or linear trend. The test for linear trend in the risk estimates across exposure groups was evaluated using the median value for the lowest, intermediate and highest category of intake, respectively. Reported p values were two-sided, and p values less than 0.05 were regarded as statistically significant. All analyses were carried out using the statistical package R [20].

Results

Prostate cancer was diagnosed in 196 (1.1%) of 18,115 men in the LSS cohort between 1963 and the end of 1996, with a total of 252,602 person-years of observation, after adjusting for migration. The mean follow-up period was 16.9 years. The mean age at entry was 51 years (range: 18–99 years) and the mean age at diagnosis among the cases was 75 years (range: 51–89 years). Table 1 shows the associations of non-dietary variables with prostate cancer risk, after mutual adjustment for attained age, calendar period, city of residence at the time of the bombings, radiation dose and education level. Age was strongly associated with prostate cancer risk, which was 24-fold higher among men aged 80 or older compared with men aged less than 65 years (RR = 24.24; 95% CI, 15.01–39.16; test for linear trend, $p < 0.001$). Calendar period was also associated with a significant increase in incidence rates, which were four-fold higher after 1995 compared with the period between 1963 and 1969 (RR = 3.95; 95% CI, 1.75–8.95; test for linear trend, $p < 0.001$). Prostate cancer risk was not significantly associated with city of residence or radiation dose, although the risk was 51% higher among men who were in the highest category of radiation dose compared with men who had received no radiation (RR = 1.51; 95% CI, 1.00–2.28; test for linear trend, $p = 0.056$). Men with a secondary education or higher had a 30% reduced risk of prostate cancer compared with men with a lower level of education (RR = 0.70; 95% CI, 0.52–0.95; test for heterogeneity, $p = 0.007$). Prostate cancer was not associated with smoking, marital status or BMI after adjustment for age, calendar period, city of residence, radiation dose and education level (Table 1).

At the time of recruitment, fish products were the only animal foods to be consumed relatively frequently, with 62% of participants eating fish (excluding broiled or dried fish) products more than twice per week. In contrast, the proportions of men in Survey 3 who consumed chicken, pork, butter/cheese or milk items this often were 32, 26, 25 and 44%, respectively. In addition, the frequency of intake of soya products was relatively high; the proportions of men who consumed tofu or miso soup more than twice per week in Survey 3 were 62 and 69%, respectively.

The association between animal foods and prostate cancer risk is shown in Table 2 after adjustment for age, calendar period, city of residence, radiation dose and education level. Fish intake was the only food that was significantly associated with prostate cancer risk. Men who consumed fish products (excluding broiled or dried fish) almost daily or more often had a 54% increased risk of developing prostate cancer compared with men who ate fish products less than twice per week (RR = 1.54; 95% CI, 1.03–2.31; test for linear trend; $p = 0.03$). Broiled fish products were also associated with a 25% increased risk in the highest category of intake *versus* the lowest category, but this was not statistically significant (test for linear trend; $p = 0.27$). For total fish intake (the sum of fish and broiled fish intake), men who had a high frequency of fish intake had a 77% increased risk of developing prostate cancer compared with men with a low frequency of intake (RR = 1.77; 95% CI, 1.01–3.11;

Table 1. Associations between sociodemographic variables and risk of prostate cancer, adjusted for age, calendar period, city of residence, radiation dose and education level

Variable	Categories	Cases	Person years[a]	Relative risk	*p*-Value
Age	<65	24	175,484	1.00	
	65–69	29	24,798	6.99 (4.05–12.1)	
	70–74	28	19,638	9.53 (5.50–16.5)	
	75–79	47	16,580	17.44 (10.58–28.75)	
	80+	68	16,103	24.24 (15.01–39.16)	<0.001[b]
Calendar period	1963–1969	7	24,322	1.00	
	1970–1979	15	36,950	0.94 (0.38–2.28)	
	1980–1984	45	65,472	1.64 (0.74–3.66)	
	1985–1989	41	57,661	1.64 (0.73–3.68)	
	1990–1994	52	50,156	2.32 (1.05–5.13)	
	1995–1996	36	18,041	3.95 (1.75–8.95)	<0.001[b]
City of residence	Hiroshima	144	181,180	1.00	
	Nagasaki	52	71,423	0.96 (0.69–1.33)	0.806[c]
Radiation dose (sV)	0	77	97,400	1.00	
	0.01–0.06	33	64,503	0.65 (0.43–0.98)	
	0.07–0.30	26	32,947	1.05 (0.67–1.63)	
	0.31+	32	31,809	1.51 (1.00–2.28)	0.059[b]
	Unknown	28	25,944	1.24 (0.80–1.92)	
Education level	None/elementary	114	95,302	1.00	
	Secondary or higher	74	14,5561	0.70 (0.52–0.95)	0.007[c]
	Unknown	8	11,739	0.54 (0.27–1.10)	
Marital status[d]	Married	166	204,878	1.00	
	Unmarried	15	23,471	0.67 (0.39–1.14)	0.318[c]
	Unknown	15	12,906	0.90 (0.52–1.53)	
Smoking	No	91	75,672	1.00	
	Yes	101	163,014	0.80 (0.60–1.07)	0.178[c]
	Unknown	4	2569	0.93 (0.34–2.52)	
BMI (kg/m^2)	<20	68	69,358	1.00	
	20–22	77	92,726	0.98 (0.70–1.35)	
	23+	43	73,173	0.76 (0.52–1.12)	0.157[b]
	Unknown	8	5997	0.77 (0.37–1.61)	

[a] Person-years are adjusted for migration.
[b] *p*-Value based on a test for linear trend.
[c] *p*-Value based on a test for heterogeneity.
[d] 'Married' includes married and cohabiting men; 'unmarried' includes single, separated, divorced and widowed men.

test for linear trend; $p=0.07$). No associations were found between prostate cancer risk and intake of individual meat or dairy food items. Similarly, no associations were observed for total meat products (meat, pork and chicken) or total dairy products and eggs (butter/cheese, milk and eggs) and prostate cancer risk (Table 2).

The association between the frequency of intake of various plant foods and prostate cancer risk is shown in Table 3. A high frequency of intake of soya foods, either as tofu or miso soup, was not significantly associated with prostate cancer risk. For total soya intake (tofu plus miso soup intake), men who had a high frequency of soya intake had a 21% reduction in prostate cancer risk compared with men with a low frequency of intake, although this was not statistically significant (RR = 0.79; 95% CI, 0.53–1.18; test for linear trend, $p=0.23$). There were no significant associations with prostate cancer risk for other plant foods such as yellow/green vegetables, pickled/salted vegetables, seaweed or fruit (Table 3).

Other food items such as bread, rice and cake were not associated with risk, as shown in Table 4. Drinks including black tea and coffee were also not associated with risk; men who drank green tea five or more times per day had a 29% increased risk of prostate cancer compared with those who drank green tea less than once per day, but this was not statistically significant.

To take into account the possibility that some men may have already had sub-clinical prostate cancer at the time of recruitment, the analyses were repeated after omitting the first two-years of follow-up, which resulted in a loss of ten prostate cancer cases. The results were similar to those when all 196 cases were included (results not shown).

A weakness of many cohort studies is that respondents may have altered their dietary habits after

Table 2. Relative risk of prostate cancer (and 95% confidence interval) for animal foods, adjusted for age, calendar period, city of residence, radiation dose and education level

Food	Frequency (times/week)	Cases	Person-years[a]	Relative risk	Test for trend
Pork	<2	135	161,765	1.00	
	2–4	50	68,944	1.34 (0.96–1.86)	
	Almost daily	8	12,038	1.24 (0.61–2.54)	0.14
Chicken	<2	99	128,233	1.00	
	2–4	52	57,786	1.09 (0.78–1.52)	
	Almost daily	2	2748	0.77 (0.19–3.10)	0.81
Total meat intake[b]	Low	38	40,812	1.00	
	Intermediate	112	140,176	1.27 (0.88–1.84)	
	High	3	6888	0.94 (0.29–3.03)	0.32
Milk	<2	113	129,712	1.00	
	2–4	32	49,463	0.94 (0.64–1.40)	
	Almost daily	51	73,427	0.87 (0.62–1.21)	0.40
Butter/cheese	<2	153	172,772	1.00	
	2–4	24	45,905	0.84 (0.54–1.29)	
	Almost daily	19	33,925	0.84 (0.52–1.37)	0.36
Eggs	<2	48	49,913	1.00	
	2–4	72	98345	1.04 (0.72–1.49)	
	Almost daily	73	94,489	1.14 (0.79–1.65)	0.47
Total dairy and egg[c]	Low	37	31,844	1.00	
	Intermediate	114	148,653	0.94 (0.64–1.37	
	High	42	62,250	0.91 (0.58–1.44)	0.70
Fish	<2	47	67,888	1.00	
	2–4	97	133,610	1.18 (0.83–1.67)	
	Almost daily	52	51,105	1.54 (1.03–2.31)	0.03
Broiled fish	<2	101	131,501	1.00	
	2–4	48	53,390	1.22 (0.87–1.72)	
	Almost daily	4	3849	1.25 (0.48–3.28)	0.27
Total fish intake[d]	Low	38	53234	1.00	
	Intermediate	97	121,024	1.19 (0.82–1.73)	
	High	18	14,050	1.77 (1.01–3.11)	0.07

[a] Person-years are adjusted for migration; person-years vary between composite food items according to the Surveys used.
[b] Total meat intake is the sum of meat, chicken and pork intake from Survey 3 only.
[c] Total dairy and egg intake is the sum of butter/cheese, milk and eggs intake from Surveys 2 and 3.
[d] Total fish intake is the sum of fish and broiled fish intake from Survey 3 only.

recruitment, thus introducing possible misclassification of exposure during the relevant aetiological period. However, because a proportion of participants completed more than one survey, we were able to examine the consistency of reported dietary intake of foods over time. Of the 18,115 men studied, 2388 (13%) had completed a dietary questionnaire in either 1963 (Survey 1) or 1965 (Survey 2) and a dietary questionnaire in 1978 (Survey 3). The proportions of men reporting extremely different consumption at the two time periods (i.e., low intake changed to a high intake or vice versa) was, on average, 9% for fish, 8% for tofu and 16% for miso soup. Conversely, the proportions of men who had a consistent intake of fish, tofu and miso soup over this time period were, on average, 42, 45 and 49%, respectively. This suggests that dietary intake of staple foods such as fish and soya products remained relatively consistent between 1963 or 1965 and 1978 in this population. However, an analysis of the association between dietary intake and prostate cancer risk among men who responded to more than one survey was not conducted due to the low numbers of men with information on dietary intake at two time points.

Discussion

The results from this analysis of prospective data in a Japanese population suggest that men with a high frequency of fish intake have an increased risk of approximately 50% of developing prostate cancer compared with men with a low frequency of fish intake. Previous evidence for an association of fish intake with prostate cancer risk is limited, perhaps because most epidemiological studies have been conducted in Western

Table 3. Relative risk of prostate cancer (and 95% confidence interval) for plant foods, adjusted for age, calendar period, city of residence, radiation dose and education level

Food	Frequency (times/week)	Cases	Person-years[a]	Relative risk	Test for trend
Yellow/green vegetables	<2	82	106,702	1.00	
	2–4	76	103,506	0.95 (0.69–1.29)	
	Almost daily	38	42,394	0.98 (0.66–1.44)	0.87
Pickled/salted vegetables	<2	65	67924	1.00	
	2–4	25	52,327	0.70 (0.44–1.11)	
	Almost daily	106	132,352	1.06 (0.78–1.45)	0.55
Seaweed	<2	76	77,594	1.00	
	2–4	67	104,823	0.74 (0.53–1.03)	
	Almost daily	50	60,331	0.86 (0.60–1.24)	0.38
Fruit	<2	48	71,746	1.00	
	2–4	74	92,851	1.27 (0.88–1.83)	
	Almost daily	74	88,005	1.20 (0.83–1.74)	0.40
Tofu	<2	73	86,557	1.00	
	2–4	89	119,118	0.91 (0.67–1.24)	
	Almost daily	31	37,072	0.88 (0.58–1.35)	0.51
Miso soup	<2	60	63,542	1.00	
	2–4	55	69,758	1.15 (0.80–1.66)	
	Almost daily	78	109,447	0.94 (0.67–1.33)	0.64
Total soya intake[b]	Low	42	39,014	1.00	
	Intermediate	87	114,322	0.92 (0.64–1.33)	
	High	64	89,401	0.79 (0.53–1.18)	0.23

[a] Person-years are adjusted for migration.
[b] Total soya intake is the sum of tofu and miso soup intake from Surveys 2 and 3.

Table 4. Relative risk of prostate cancer (and 95% confidence interval) for other foods and drinks, adjusted for age, calendar period, city of residence, radiation dose and education level

Food	Frequency (times/week)	Cases	Person-years[a]	Relative risk	Test for trend
Bread	<1[b]	105	126,276	1.00	
	2	80	104,229	0.91 (0.68–1.23)	
	3+	11	22,097	0.68 (0.36–1.29)	0.23
Rice	<1[b]	20	19,927	1.00	
	2	71	103,940	0.88 (0.54–1.45)	
	3+	102	118,881	1.11 (0.68–1.80)	0.27
Cake	<2	105	154,113	1.00	
	2–4	59	61,531	1.17 (0.85–1.61)	
	Almost daily	32	36,959	0.87 (0.58–1.31)	0.73
Black tea	<2	165	197,124	1.00	
	2–4	17	28,385	0.88 (0.53–1.45)	
	Almost daily	11	17,239	0.86 (0.47–1.59)	0.52
Green tea	<1[b]	32	37,687	1.00	
	2–4	88	120,145	1.03 (0.69–1.55)	
	5+	73	84,916	1.29 (0.84–1.98)	0.16
Coffee	<2	114	118,689	1.00	
	2–4	33	45,100	1.07 (0.72–1.58)	
	Almost daily	46	78,958	1.02 (0.71–1.46)	0.88
Alcohol	No	53	58,806	1.00	
	Yes	131	189,334	1.03 (0.74–1.42)	0.26
	Missing	12	4462	1.89 (1.00–3.55)	

[a] Person-years are adjusted for migration.
[b] Frequency of intake for rice, bread and green tea is presented in times per day.

populations where fish intake is relatively low. Such studies have reported either no association [21–23] or a reduction in prostate cancer risk associated with a high intake of fatty fish [24, 25]. However, our finding that a high frequency of fish intake was associated with an increase in prostate cancer risk is consistent with a

previous cohort study among Japanese men in Hawaii, which reported a non-significant 22% increased risk among men who consumed fish more than four times per week [13]. It is possible that a high fish intake is a marker for some other aspect of diet that is associated with an increased risk of prostate cancer, such as animal protein, which has frequently been associated with an elevated risk of prostate cancer in Western populations [6]. The absence of an association with meat and dairy products in this population may be due to the relatively low frequency of intake of these foods. Indeed, in many Asian populations, fish, rather than meat or dairy products, is the predominant source of animal protein and per capita fish intake in Japan is one of the highest in the world [26]. The association between fish intake and prostate cancer risk might therefore reflect animal protein consumption, although in the absence of nutrient data, we were unable to estimate animal protein intake in this population. Alternatively, a high frequency of fish intake may be a marker for some other dietary-related factor that is specific to fish but not to other animal products, such as methylmercury compounds, although there is inadequate evidence on the carcinogenicity of organic mercury compounds in humans [27]. Finally, we cannot exclude the possibility that this finding may have been due to chance because of the number of statistical tests performed.

There was little evidence that a high frequency of soya intake, in the form of tofu or miso soup, was associated with a reduction in prostate cancer risk, although men in the highest category of total soya intake had a non-significant 22% reduced risk compared with men in the lowest category of intake. Although soya intake in this study population is likely to be much higher than that in most Western populations, the lack of information on portion size limits our ability to investigate absolute intakes of soya foods and its isoflavone components. Very few epidemiological studies have examined the association between soya intake and prostate cancer risk, despite data from experimental studies that have found dietary isoflavones to be associated with reduced prostate tumour growth [9, 10]. Two case–control studies reported an inverse association between estimated dietary intake of isoflavones and prostate cancer risk in the US [28] and China [29]. To date, two other prospective studies have been conducted among populations with a moderately high consumption of soya intake (largely in the form of tofu or soya milk). These studies included 175 prostate cancer cases identified in a cohort of Japanese men in Hawaii [13] and 225 cases identified in a cohort of Seventh Day Adventists in the US [11]. Both studies found high soya consumption to be associated with a 65–70% reduction in prostate cancer risk, although the results were not statistically significant and were based on small numbers of cases in the highest category of soya intake. The results from the present moderately large prospective study suggest that high soya consumption is not strongly associated with a reduction in prostate cancer risk. The absence of significant associations between other dietary variables and prostate cancer risk is broadly consistent with results from other epidemiological studies. It remains possible that other dietary items and, in particular, specific nutrients that were not assessed in this analysis may be related to prostate cancer risk.

Our data show a strong association of age, calendar period and education level with prostate cancer risk, all of which were adjusted for in the dietary analyses. The increase in prostate cancer incidence rates during the follow-up period is consistent with the 2.5-fold increase in national incidence rates reported between 1970 and 1996 [30]. The cause of this increase is not known, although an increased use of treatment procedures for benign prostatic hyperplasia and more recently of prostate-specific antigen testing may have contributed to an increase in the detection of early or incidental tumours in recent years. Nevertheless, the four-fold increase in prostate cancer mortality rates in Japan over the same time period [31] suggests that the increase in incidence in prostate cancer incidence is real and may reflect changes in exposure to risk factors, such as diet. Consumption of meat and milk products has increased more than four-fold since the Second World War, reflecting the increasing 'Westernization' of dietary habits on a national level, although the intake of fish has only increased by 20% [32]. Unfortunately, we could not examine in detail the changes in the frequency of consumption of meat, fish and dairy products over time in this population due to the low number of men with information on dietary intake in both 1963/1965 and 1979. With the increasing consumption of animal products in many Asian countries, it will be informative to examine whether changes in dietary patterns are related to prostate cancer risk in these populations in the future.

The strengths of this study are that the dietary data were collected prospectively and so are free from recall bias. The questionnaire used in Survey 3 (which is almost identical to the questionnaires used in Surveys 1 and 2) has been validated and shown to produce moderate correlations with a 24-h diary for the main food groups, such as meat, dairy, fish and tofu [16]. Further, previous studies using this food-frequency questionnaire have shown significant associations between fruit intake and cancer mortality [33] and animal

product intakes and stroke mortality [34]. Finally, any misclassification arising from the food-frequency questionnaire is likely to be unbiased and would have led to an under-estimation of the true association between diet and prostate cancer risk, rather than a systematic bias. Information on a wide range of non-dietary factors was also collected at the same time as the dietary questionnaire and was taken into account in the analysis. However, a major disadvantage was that each questionnaire only covered a limited number of foods, and portion size was not included, so that the total consumption of foods or nutrients could not be estimated and adjustment for total energy intake could not be made. Further, despite the long period of follow-up, the relatively low numbers of cases identified limited the power to detect small associations between dietary intake and prostate cancer risk. Some prostate cancers may have been missed among men migrating out of the study catchment areas. The person-years were adjusted accordingly using residence probabilities estimated from the Adult Health Study sub-cohort and although these residence probabilities were calculated according to birth cohort and time period for each city, it is possible that migration rates may differ by other factors, such as education level or social class. However, the overall migration rate was low, which suggests that any differential migration by social class would not have made an appreciable difference to the risk estimates. We were unable to analyse dietary associations by stage or grade of disease, although the low overall numbers would have made such a sub-analysis difficult to interpret. Finally, although the study participants are unique in the fact that they were exposed to the atomic bombings of Hiroshima and Nagasaki in 1945, most individuals had a low exposure to radiation and all analyses here are presented after adjustment for radiation dose.

In summary, data from this relatively large prospective study show that a high frequency of fish intake was associated with an increased risk of prostate cancer. No other food items, including soya products, were significantly associated with prostate cancer risk.

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ORIGINAL ARTICLE

Smoking and fracture risk: a meta-analysis

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Abstract Smoking is widely considered a risk factor for future fracture. The aim of this study was to quantify this risk on an international basis and to explore the relationship of this risk with age, sex and bone mineral density (BMD). We studied 59,232 men and women (74% female) from ten prospective cohorts comprising EVOS/EPOS, DOES, CaMos, Rochester, Sheffield, Rotterdam, Kuopio, Hiroshima and two cohorts from Gothenburg. Cohorts were followed for a total of 250,000 person-years. The effect of current or past smoking, on the risk of any fracture, any osteoporotic fracture and hip fracture alone was examined using a Poisson model for each sex from each cohort. Covariates examined were age, sex and BMD. The results of the different studies were merged using the weighted β-coefficients. Current smoking was associated with a significantly increased risk of any fracture compared to non-smokers (RR = 1.25; 95% Confidence Interval (CI) = 1.15–1.36). Risk ratio (RR) was adjusted marginally downward when account was taken of BMD, but it remained significantly increased (RR = 1.13). For an osteoporotic fracture, the risk was marginally higher (RR = 1.29; 95% CI = 1.13–1.28). The highest risk was observed for hip fracture (RR = 1.84; 95% CI = 1.52–2.22), but this was also somewhat lower after adjustment for BMD (RR = 1.60; 95% CI = 1.27–2.02). Risk ratios were significantly higher in men than in women for all fractures and for osteoporotic fractures, but not for hip fracture. Low BMD accounted for only 23% of the smoking-related risk of hip fracture. Adjustment for body mass index had a small downward effect on risk for all fracture outcomes. For osteoporotic fracture, the risk ratio increased with age, but decreased with age for hip fracture. A smoking history was associated with a sig-

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