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Running title: DM and inflammation associate fracture.

Abstract

Aims/introduction: There are various causes of incident bone fracture. Not only aging, low bone mineral density and history of previous fracture, but also diabetes mellitus (DM) and inflammation are regarded as the risk of fracture. The purpose of this study is to verify the association of glycemic control or one inflammatory marker with incident fracture in a large-scale Japanese cohort.

Materials and Methods: This study was conducted at the Hiroshima Atomic Bomb Casualty Council and included 6,556 participants (2,785 men and 3,771 women, age 55-87) who underwent annual health examination followed for 7.4 years. Information about incident fractures was collected at interviews. Participants were classified into three groups: Normal, Borderline, and DM according to HbA1c levels (treated diabetic patients were included in DM group). Further, participants were classified into four additional groups by glycemic control (DM or non-DM) and C-reactive protein (CRP) levels (low or high). Hazard ratios (HRs) of DM, CRP and their combined risk of incident fracture were evaluated.

Results: After adjusting for age, bone mineral density, and previous fracture, CRP was associated with increased fracture risk (HR in men: 1.04 [95% CI: 1.003-1.06]; in women: 1.07 [1.03-1.13]), and DM predicted fracture risk in men (HR: 1.31 [1.02-1.51]). Fracture risk was significantly higher

among DM with high CRP group compared to non-DM with low CRP (HR in men: 1.47 [1.02-1.98]; in women: 1.41 [1.04-1.92]).

Conclusions: Among a Japanese cohort, CRP measurements were helpful to detect high fracture risk in patients of type 2 DM.

Key words: Incident bone fracture, C-reactive protein, Diabetes mellitus,

Introduction

As the population ages, the number of patients with osteoporosis and bone fracture are increasing. Not only factors like aging, low bone mineral density (BMD), or history of previous fracture, but also those like smoking, over consumption of alcohol, family history of fracture, and insufficient exercise are considered to increase risk of incident bone fracture[1-6].

Moreover, westernization of lifestyle is the cause of increasing number of patients with diabetes mellitus (DM). With aging of society, the number of elderly DM patients is also increasing, and many of them suffer from a complication of DM and fracture. According to meta-analysis conducted in Europe and the U.S., the association between DM and fracture was 6.3 times higher in participants with type 1 DM and 1.7 times higher in type 2 DM than that in non-diabetic participants[7]. In addition, in a large-scale trial of an Asian cohort, patients with DM were at higher risk of proximal humeral

fracture[8], and in a Japanese cohort over 50 years old, the fracture risk in patients with type 2 DM was 4.7 times higher in men and 1.9 times in women[9].

The systemic chronic inflammation affects not only vascular disease but also bone metabolism. One report indicated that high inflammatory markers increased the risk of osteoporosis and frailty fracture in 2,807 normal elderly women in the U.S.[10]. In addition, studies in Japan reported that high levels of inflammatory markers in 751 elderly women[11] or in a general cohort of 7,283 healthy subjects[12] represented an increased risk of fracture.

The involvement of chronic inflammation in adipose tissue was suggested to be a cause of metabolic syndrome, and its basis is insulin resistance[13]. DM and inflammation are considered as fracture risks, and we hypothesized that patients of DM with high level inflammation had a further risk of subsequent incident fracture. The aim of this study was to evaluate the combined effect of DM and one inflammatory marker (high sensitive C-reactive protein [hs-CRP]) on the risk of incident fracture by following a large-scale longitudinal cohort of about 7,200 participants for 7.4 years on average.

Materials and Methods

Participants in this study were 7,205 persons (3,018 men and 4,187 women, age 55-96 years) who visited the Health Management & Promotion Center, Hiroshima Atomic Bomb Casualty Council for the purpose of undergoing health examination during the period April 2003-March 2004. We excluded

participants with CRP value ≥ 3.0 mg/l or White blood cell count ≥ 10,000 /μl, who were potentially supposed to have active inflammatory diseases. We also excluded from the study the participants already under treatment of osteoporosis, rheumatoid arthritis, collagen diseases, other inflammatory disease, all patients with type 1 DM (DM participants in this study were all diagnosed as type 2), those using corticosteroid, lower CRP drug (e.g. NSAIDs), and hormonal replacement therapy for the treatment of other diseases as far as we could grasp in the interview (Figure S1).

The study participants answered a questionnaire about their history of previous fracture and lifestyle, and underwent height and weight measurements, physical examination, blood testing, and BMD measurements. The information about incident fragility fractures (spine, hip, proximal humeral, and forearm) was collected at self-report interview forms once a year by trained nurses. The survey on lifestyle included questions about smoking habit, alcohol intake, exercise habit, family history of fracture, history of ischemic heart disease (IHD) or cardiovascular disease (CVD), and history of previous fracture. BMD was measured by dual energy X-Ray absorptiometry method, (QDR4500A; HOLOGIC, Inc., Bedford, MA) at the lumbar spine (L2-L4). The participants underwent measurements of plasma glucose, glycohemoglobin (HbA1c), serum creatinine, albumin, and CRP. HbA1c values were measured by latex-aggregation immune nephelometry method (Roche Diagnostics K.K., Tokyo, Japan). Values of CRP were measured by the latex-aggregation method (Roche Diagnostics K.K., Tokyo, Japan), which is a high-sensitivity assay technique.

The participants were followed by annual health examinations until the end of March 2014 (mean

observation period was 7.4 years). The information about incident fracture and the period from baseline to the onset were recorded by medical interview. Cases of pathological fracture were excluded. The mortality information was ascertained from death certificates. If a participant in this study had died or had no incident fracture, the observation period was defined as the duration from baseline to the year of death or to the date of participant's last visit for examination.

We classified participants who had not been diagnosed as DM into three groups by their HbA1c levels at baseline (Normal: HbA1c ≤ 5.6%; Borderline: 5.7% ≤ HbA1c ≤ 6.4%; DM: 6.5% ≤ HbA1c) in accordance with American Diabetes Association pre-diabetes recommendation[14]. All participants already diagnosed and under treatment of DM were included in the DM group regardless of their HbA1c values at baseline. Diagnosis of DM was based on medical history of under treatment of DM, or HbA1c ≥ 6.5% and plasma glucose ≥ 126 mg/dl (7.0 mmol/l) after meal time ≥ 10 hours, or plasma glucose ≥ 200 mg/dl (11.1 mmol/l) after meal time < 10 hours. Participants with HbA1c ≥ 6.5% but with low plasma glucose in this criteria (8 persons) were all prevalent patients of DM. Daily amount of alcohol intake was classified into three groups; non-drinker, moderate (less than 29 g/day), and heavy (29 g/day or more)[15]. The participant's smoking habit, exercise habit, family history of fracture, prevalent IHD or CVD, and previous fracture were classified into two groups (yes or no). To evaluate the combined effect of DM and inflammation, we classified participants into four additional groups based on DM status (presence or absence) and CRP level. The cut-off of value of CRP was determined as 0.63 mg/l, the median value of this study participants (low: 0.02-0.62 mg/l, high:

0.63-3.0 mg/l).

Ethics Committee of Hiroshima Atomic Bomb Casualty Council approved the aim and protocol for the study, and all participants filled out informed consent form for the use of their health examination data.

Statistical analysis was performed using R 3.0.0 (R Foundation for Statistical Computing, Vienna, Austria). Data were expressed as mean ± standard deviation or median (interquartile range) after examination of normal distribution with Kolmogolov-Smirnov test. Values of hs-CRP were used after logarithmic transformation in multivariate analysis. BMD comparisons according to HbA1c levels were performed using one-way analysis of variance with Bonferroni method as post hoc multiple comparisons. Trend for values of age, spine BMD, estimated glomerular filtration rate (eGFR), albumin, and CRP according to HbA1c levels were analyzed by Jonckheere-Tarpstra test. The hazard ratios (HRs) of conventional risk factors in multivariate analysis or those of DM and CRP classification were assessed by Cox proportional hazards regression analysis. A *P* value of less than 0.05 was considered as statistically significant.

Results

Characteristics of the study participants are shown in Table 1. Of the total 7,205 persons eligible for the study, 649 were excluded and 6,556 persons (age 55-87 years) were ultimately selected for participation in the study (shown in supplementary material). Female participants numbered 3,771 of

the total (57.5%). Mean age for men was 67.7 years and for women 68.3 years. The average spine BMD for men was 0.993 g/cm^2 and for women 0.805 g/cm^2. The percentages of participants categorized as DM group were 14.8% in men and 10.0% in women. The median value of CRP in men was 0.67 mg/l and in women 0.62 mg/l. The rate of smoking in men was 60.0% and in women 4.1%. The rate of previous fracture in men was 30.4% and in women 25.4%. Over the follow-up period, there were 179 incident fractures in men (6.4%) and 312 in women (8.3%).

As for CRP values, there was no significant difference according to generation in all study participants (Figure S2) and eligibly selected participants (Figure S3).

Characteristics according to HbA1c levels at baseline are shown in Table 2. Spine BMD tended to increase according to bad glycemic control. Values of CRP also increased with bad glycemic control ($P < 0.05$ for trend). As for incidence rates of fracture, there were no significant differences between the three glycemic control groups.

HRs for incident fracture after adjustment for multivariate factors are shown in Table 3. In both men and women, aging, low BMD, previous fracture and high CRP level had a significant association with fracture. Moreover, in men, DM was a significant risk factor after adjusting for multivariate factors (age, BMD, CRP, eGFR, albumin, exercise, smoking, alcohol, family history of fracture, IHD, CVD, and previous fracture). Female smokers had higher risk of fracture.

In the Figure 1, HRs for incident fracture of the four groups categorized by DM (presence or absence) and CRP levels (low or high) are shown. After adjustment was made for multivariate factors

(age, BMD, eGFR, albumin, exercise, smoking, alcohol, family history of fracture, IHD, CVD, and previous fracture), non-DM and with high CRP group had fracture risk of 1.11 times in men and 1.16 times in women compared to non-DM with low CRP group (reference), a marginally significant difference (P = 0.06 for men, and 0.08 for women). In addition, even with DM, fracture incidence in the low CRP group was not statistically significant, but the fracture risk of DM with high CRP group were 1.47 times in men and 1.41 times in women, significantly higher than those of the reference group.

Discussion

To our knowledge, this is the first report showing that combination of type 2 DM and high inflammatory marker (hs-CRP) had significant association with incidence rates of bone fracture among a large-scale Japanese cohort. In this study, in both men and women, fracture risk was associated with aging, low BMD, previous fracture, and CRP. Male participants with type 2 DM had a high risk of incident fracture after adjustment for other confounders. Participants with type 2 DM and high CRP level had a significantly increased risk of incident fracture in both men and women.

CRP was a significant risk factor of incident fracture in both men and women. Some reports shows CRP and other inflammatory markers associate with not only atherosclerotic diseases but also bone fracture[11,12]. In animal studies, inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 were proved to increase bone resorption by activating osteoclasts[16,17]. Kami et

al. investigated the effect of inflammatory markers on hip fracture in a cohort of 1,171 Caucasian elderly women and showed that high value of soluble TNF-α and IL-6 receptors increased fracture risk dependent on their concentration[18].

After adjusting for multivariate risk factors, type 2 DM represents a significant fracture risk in men but not in women. The reason for this result was unclear. To verify the statistical difference by gender, we performed an additional analysis to assess an interaction for sex and DM. As a result, we found there was no significant interaction in these 6,556 participants. Another larger sample size study might be effective to assert that there was truly a difference between men and women.

After dividing the participants into DM and non-DM, the effect of inflammation on bone fracture was not significant in the non-DM group. Even in the DM group, low levels of inflammation did not result in a significant risk for fracture. However, DM and high levels of inflammation group had a significant risk of fracture. In this cohort, CRP values were not different according to generation (Figure S2, S3). These results suggest that, in patients of type 2 DM with high CRP, the combined effects of high blood glucose and high level of inflammation brought about increased bone fracture risk.

In the classification according to HbA1c levels, DM group had a significantly higher BMD level than that of normal HbA1c group. According to meta-analysis, BMD decreases in type 1 DM but increases in type 2 DM[19]. Insulin is a growth factor similar to insulin-like growth factor (IGF)-1, and receptors of both insulin and IGF-1 express in osteoblasts. They promote the proliferation of

osteoblasts[20]. In type 1 DM patients, absolute loss of insulin production and decrease of blood IGF-1 level[21] are considered as the reasons for BMD decrease. Conversely, in type 2 DM, BMD level is maintained due to the obesity and hyperinsulinemia accompanied by insulin resistance[22,23].

BMD explains 70% of bone strength[24] and decreased bone quality is considered as another factor. In DM patients, the accumulation of advanced glycation end products (AGEs) due to constant high blood glucose levels and oxidative stress has been confirmed in bone tissue. Non-physiological and frail cross-linking of pentosidine, one type of AGEs, leads to deteriorated bone quality and exacerbated bone frailty[25,26]. In another study among patients with type 2 DM using high-resolution CT technology, the BMD of the cortical bone surface was low in long bones like the radius and the tibia, contrary to high BMD in cancellous bone[27]. Therefore, despite high BMD levels, patients with type 2 DM tend to have weaker bones due to various risk factors. In addition, vision impairment caused by diabetic retinopathy and gait disorder caused by diabetic neuropathy can lead increased frequency of falls[28,29]. These factors are supposed to be one cause of the increased fracture risk in patients with DM.

The participants of this study included atomic bomb survivors in Hiroshima and Nagasaki, but several reports have suggested that the level of radiation exposure had no significant association with thoracic, lumber spine, or hip fracture[30-33].

A large-scale Japanese cohort participated in our survey and they were followed longitudinally (around a 7.4 years' observation period), which is the strength of this study. However, this study has

several limitations. We used self-reported information of fracture by medical interview at every visit, but not morphological examination using X-ray images. In addition, the association between thiazolidine and fracture risk have been pointed out in Europe, the U.S.[34], and Japan[35]. However, since we did not completely grasp the information about the use of oral hypoglycemic agents (including thiazolidine), we were unable to adjust for this effect. Additionally, the change of CRP values and the onset of inflammatory disease in observation period were unknown. We could not analyze thoroughly whether the onset or condition of inflammatory disease had any effect on incident fracture.

In conclusion, this study of a large-scale Japanese cohort demonstrated that CRP was a predictive factor in incident bone fracture. And independent of BMD, patients of type 2 DM with high CRP were at about a 40% higher risk for fracture compared to non-DM with low CRP. These results suggest that not only BMD but also CRP measurements might detect high fracture risk in patients with type 2 DM.

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Disclosure

Yasuyuki Mitama, Saeko Fujiwara, Masayasu Yoneda, Sakurako Kira, and Nobuoki Kohno declare no conflict of interest associated with this manuscript.

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Figure 1 | Hazard ratio of fracture according to presence of diabetes mellitus (DM) and C-reactive protein (CRP) level in men and women. Hazard ratios were evaluated by Cox proportional hazards regression analysis after adjustment for age, bone mineral density, eGFR, albumin, exercise, smoking, alcohol, family history of fracture, ischemic heart disease, cerebrovascular disease, and previous fracture. White and gray bars represent low CRP and high CRP respectively. Error bars represent 95% confidence interval of hazard ratios compared to reference group. * $P < 0.05$ vs DM(-) with low CRP (reference group).

A list of Supporting Information

Figure S1 | Selection process of study participants for this study

Figure S2 | CRP, WBC levels and inflammatory diseases according to gender or generation in all study participants (N = 7205).

Figure S3 | CRP levels and inflammatory diseases according to gender or generation in all eligible participants (N = 6556).

Table 1 | Characteristics of study participants at baseline

	Men	**Women**
N	2785	3771
Age (yr)	67.7 ± 6.7	68.3 ± 7.5
Age at menopause (yr)	—	49.2 ± 4.8
Observation period (yr)	7.3 ± 0.8	7.5 ± 0.7
Spine BMD† (g/cm^2)	0.993 ± 0.182	0.805 ± 0.159
BMI‡ (kg/m^2)	23.2 ± 2.9	22.7 ± 3.3
HbA1c (%)	5.73 ± 0.79	5.74 ± 0.64
(Normal / Borderline / DM [DM %])	2110 / 262 / 413 [14.8%]	3005 / 387 / 379 [10.0%]
eGFR§ ($ml/min/1.73m^2$)	54.4 ± 9.2	51.7 ± 8.8
Albumin (g/dl)	4.43 ± 0.21	4.48 ± 0.23
CRP¶ (mg/l)	0.67 (0.38 - 1.32)	0.62 (0.38 - 1.28)
Exercise (yes) [%]	1680 [60.3%]	2033 [53.9%]
Smoking (yes) [%]	1670 [60.0%]	154 [4.1%]
Alcohol (non-drinker / moderate / heavy)	810 / 583 / 1392	2623 / 756 / 392
Family history of fracture (yes) [%]	563 [20.2%]	1060 [28.1%]
IHD†† (yes) [%]	78 [2.8%]	37 [1.0%]
CVD‡‡ (yes) [%]	207 [7.4%]	176 [4.7%]
Previous fracture (yes) [%]	846 [30.4%]	958 [25.4%]
Incident fracture (yes) [%]	179 [6.4%]	312 [8.3%]

Data are expressed as mean ± standard deviation or median (interquartile range). Exercise, Smoking, Family history of fractures, IHD, CVD, and prevalent fracture are expressed as number and percentages of yes. Alcohol intake according daily to amount are expressed as number.
† bone mineral density, ‡ body mass index, § estimated glomerular filtration rate, ¶ C-reactive protein, †† ischemic heart disease, ‡‡ cardiovascular disease.

Table 2 | Classification according to HbA1c levels

	Men			Women		
	Normal	Borderline	DM	Normal	Borderline	DM
HbA1c (%)	5.4 ± 0.2	6.1 ± 0.1	7.2 ± 1.3	5.5 ± 0.2	6.1 ± 0.1	7.3 ± 1.2
N	2110	262	413	3005	387	379
Age (yr)	67.6 ± 6.7	68.4 ± 7.2	68.2 ± 6.4	68.0 ± 7.6	69.0 ± 7.4	70.1 ± 7.6
Spine BMD† (g/cm^2) *	0.981 ± 0.180	1.014 ± 0.183	1.038 ± 0.182**	0.803 ± 0.126	0.808 ± 0.154	0.824 ± 0.152**
BMI‡ (kg/m^2) *	23.1 ± 2.8	23.4 ± 3.0	23.9 ± 2.9	22.5 ± 3.2	23.6 ± 3.2	23.7 ± 3.5
eGFR§ ($ml/min/1.73m^2$)	54.5 ± 9.5	53.5 ± 9.0	55.1 ± 10.1	51.9 ± 8.5	51.2 ± 8.2	51.3 ± 10.1
Albumin (g/dl) *	4.43 ± 0.25	4.44 ± 0.19	4.46 ± 0.21	4.47 ± 0.20	4.50 ± 0.18	4.51 ± 0.24
CRP¶ (mg/l) *	0.66 (0.47-1.45)	0.78 (0.55-1.55)	0.89 (0.38-2.29)	0.62 (0.42-1.65)	0.77 (0.55-1.54)	0.90 (0.37-2.19)
Previous fracture (%)	30.9	23.9	32.0	25.9	21.0	25.1
Incident fracture (%)	6.6	4.6	6.9	8.8	5.7	6.8

Data are expressed as mean ± standard deviation or median (interquartile range) unless otherwise indicated.
† bone mineral density, ‡ body mass index, § estimated glomerular filtration rate, ¶ C-reactive protein.
* $P < 0.05$ for trend for glycemic control in both men and women.
** $P < 0.05$ vs Normal by using one way analysis of variance with Bonferroni method.

Table 3 | Hazard ratios for incident fracture

Variables		Men		Women	
Age (+1 yr)		**1.03**	**(1.01-1.05)** *	**1.05**	**(1.01-1.07)** *
Spine BMD† (-0.1 g/cm^2)		**1.14**	**(1.07 - 1.23)** *	**1.14**	**(1.06 - 1.18)** *
HbA1c	Normal vs Borderline	1.05	(0.75 - 1.41)	1.01	(0.54 - 1.28)
	Normal vs DM	**1.31**	**(1.02 - 1.51)** *	1.14	(0.82 - 1.25)
eGFR‡ (+1.0 ml/min/1.73m^2)		0.99	(0.97 - 1.03)	0.97	(0.94 - 1.04)
Albumin (+1.0 g/dl)		0.63	(0.33 - 1.42)	0.76	(0.40 - 1.40)
CRP§ (+1.0 mg/l)		**1.04**	**(1.003 - 1.06)** *	**1.07**	**(1.03 - 1.13)** *
Exercise (yes / no)		0.70	(0.59 - 1.25)	0.99	(0.85 - 1.46)
Smoking (yes / no)		1.34	(0.81 - 1.54)	**1.80**	**(1.15 - 2.43)** **
Alcohol	non-drinker vs moderate	0.76	(0.54 - 1.37)	0.97	(0.80 - 1.60)
	non-drinker vs heavy	1.02	(0.63 - 1.44)	1.02	(0.43 - 1.43)
Family history of fracture (yes / no)		1.14	(0.80 - 1.68)	1.53	(0.94 - 1.70)
IHD¶ (yes / no)		0.94	(0.49 - 1.62)	0.99	(0.55 - 1.47)
CVD†† (yes / no)		1.10	(0.82 - 1.52)	1.33	(0.82 - 1.51)
Previous fracture (yes / no)		**1.72**	**(1.51 - 3.82)** **	**2.90**	**(2.20 - 5.65)** **

Data are expressed as hazard ratio (95% CI) evaluated by Cox proportional hazards regression analysis after adjustment for multivariate factors shown above. CRP value was normalized by logarithmic conversion before analysis.

† bone mineral density, ‡ estimated glomerular filtration rate, § C-reactive protein, ¶ ischemic heart disease, †† cardiovascular disease.

* $P < 0.05$, ** $P < 0.01$