

ORIGINAL ARTICLE

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Decreased activities of daily living and associations with bone loss among aged residents in a rural Japanese community: the Miyama Study

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Abstract The present study aimed to clarify frequencies of decreased activities of daily living (ADL) and associations with rate of bone loss among inhabitants more than 60 years old in Miyama, a rural community in Japan. A cohort of 1543 inhabitants aged 40–79 years was established according to Miyama resident registrations in 1989. Men ($n = 50$) and women ($n = 50$) from each of two age strata between 60 and 79 years ($N = 200$) were selected from this cohort, and bone mineral density (BMD) of the lumbar spine and proximal femur was measured using dual-energy X-ray absorptiometry in 1990 (initial survey) and again in 1993, 1997, and 2000. Difficulties involving ADL were surveyed at every follow-up study. Of the 200 initial participants, 124 (57 men, 67 women; 62.0%) completed all BMD measurements and answered all items about ADL in the follow-up survey. The following items were investigated as a general indication of changes to ADL: reaching objects on a high shelf or cupboard (reaching); washing and drying the body (washing body); washing hair over a washbasin (washing hair); sitting for 1 h on a hard chair (sitting); raising the torso from a lying position in bed (raising); standing continuously for 30 min (standing); taking socks on and off the feet (taking socks); bending down from a seated position and picking up a small object at the side of the chair (bending); lifting heavy objects (lifting); and running 100 m without stopping (running). Among ADL items, the most frequent difficulties in men involved running (50.0%), followed by

raising (30.6%), standing (27.1%), sitting (24.7%), and reaching (16.5%). In women, difficulties involved running (67.0%), followed by lifting (36.3%), standing (33.1%), reaching (30.8%), and sitting (23.6%). To evaluate relationships between decreased ADL and changes in BMD, annual rates of change for BMD at the lumbar spine and femoral neck were compared to changes for each ADL item (2 grade decrease; 1 grade decrease; or no change). Analysis of covariance (ANCOVA) was then performed on decreased ADL and annual bone changes after adjustment for age, concomitant disease (previous fractures, gastrectomy, diabetes mellitus, and renal dialysis at initial survey). In men, annual rates of change in BMD at the femoral neck over 10 years were significantly correlated with decreased abilities in bending ($P = 0.046$; $R^2 = 0.10$). In women, annual rates of change in BMD at the lumbar spine over 10 years were significantly correlated with decreased abilities in reaching ($P = 0.007$; $R^2 = 0.25$), and lifting ($P = 0.014$, $R^2 = 0.27$), and those at the femoral neck were significantly correlated with decreased abilities in lifting ($P = 0.001$, $R^2 = 0.33$).

Key words activities of daily living (ADL) · bone loss · cohort study · epidemiology · Japanese

Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mass, microarchitectural deterioration of bone tissue, and increased incidence of bone fragility and fractures. As one of the fractures associated with osteoporosis, fracture of the femoral neck results in confinement to bed and marked impairment of activities of daily living (ADL) in aged individuals. The number of patients with femoral neck fracture has nearly doubled over the 15 years from 1987 to 2002 [1,2]. Because the elderly population in Japan is rapidly increasing, prevention of osteoporosis represents an urgent issue. However, as longitudinal observation of bone mass in a general population is indispensable to evalu-

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ation of asymptomatic conditions such as bone loss, few studies have reported risk factors for bone loss in the general Japanese population [3,4].

Conversely, various indices of ADL have been some of the more intensively investigated lifestyle risk factors examined for associations with osteoporosis. The importance of weight-bearing activity for maintaining bone mineral density (BMD) has been suggested by observations such as bone loss in astronauts who have experienced weightlessness [5]. Few studies have investigated the influence of ADL on bone mass. Wickham et al. [6] reported a lower incidence of femoral neck fracture in subjects who often walk, climb stairs, and are active outdoors. However, another study showed no clear differences in bone mass between nonclerical female workers who often walk around at work and those who do office work and often sit at work [7]. Ulrich et al. [8] undertook an interview survey of daily activity in 25 premenopausal females and suggested a relationship between lifetime physical activity including occupation and housework and total BMD. However, no studies have shown an association between changes in ADL and bone loss.

We previously measured BMD and observed age-related changes in a cohort established in Miyama village, Wakayama Prefecture, Japan. Follow-up surveys were performed after 3, 7, and 10 years, with BMDs measured in the same sites of the same participants [3,9]. Herein we report a population-based longitudinal study clarifying the frequencies of observed difficulties in ADL and changes over 10 years and associations between BMD or rate of bone loss and decreases in ADL in aged inhabitants of a rural Japanese community.

Methods

Design

The Miyama study is a population-based longitudinal study for the ascertainment of rates of bone loss and clarification of associations between factors in daily life and bone loss.

Participants

Details of the study conducted in Miyama, a village located in a mountainous area of Wakayama Prefecture, Japan, have been described elsewhere [10,11]. In brief, a cohort comprising all 1543 inhabitants aged 40–79 years (716 men, 827 women) was compiled from the register of residents as of December 31, 1989. All members of the cohort completed a self-administered questionnaire (125 items) covering daily activities such as dietary habits, smoking habits, alcohol consumption, and physical exercise.

From the complete cohort, a BMD cohort was recruited, comprising 400 inhabitants aged 40–79 years (50 men and 50 women from each decade of age). The BMD subcohort did not differ from the total cohort in the distribution of lifestyle items such as smoking, alcohol consumption, sleep-

ing hours, exercise, walking, dietary habits, or stress as measured by the initial questionnaire [10]. An interviewer administered a second questionnaire covering items of past history, including previous fractures, gastrectomy, diabetes mellitus and renal dialysis, family history, calcium intake, dietary habits, physical exercise, occupational activities, sun exposure, ADL variables, and reproductive variables in women, to the 400 participants. All subjects provided informed consent for participation in the study. In the present study, to assess decreases in ADL and relationships with changes in BMD, subjects 60 years of age or older were investigated, as ADL decreases were predominantly observed in elderly people. The total number of subjects in the present ADL study was therefore 200 (100 men, 100 women).

ADL assessment

Difficulties concerning ADL were surveyed by the interviewer-administered second questionnaire in the initial BMD survey and in the follow-up study in 2000. To select ADL items, the questionnaire used in the European Vertebral Osteoporosis Study [12–14] was translated into Japanese and the expressions modified slightly to suit Japanese customs. The following items were investigated as a general indication of changes to ADL: reaching objects on a high shelf or cupboard (reaching); washing and drying the body (washing body); washing hair over a washbasin (washing hair); sitting for 1 h on a hard chair (sitting); standing continuously for 30 min (standing); raising the torso from a lying position in bed (raising); taking socks on and off the feet (taking socks); bending down from a seated position and picking up a small object at the side of the chair (bending); lifting heavy objects (lifting); and running 100 m without stopping (running). Three answers for these questions were utilized: "Can do without difficulty"; "Can do, but with some difficulty"; and "Either unable to do, or able to do only with help." According to each item, BMDs were compared among the three answer categories. Significant items were selected, and multiple regression analysis was performed with adjustment for age.

BMD measurement

Baseline measurements of BMD were made in 1990 using dual-energy X-ray absorptiometry (DXA) (Lunar DPX; GE Lunar, Madison, WI, USA), from anteroposterior images at lumbar vertebrae L2–L4 and the proximal femur (femoral neck, Ward's triangle, trochanter, and total hip). In addition to BMD measurements, physical parameters of height and body weight were taken, and body mass index (BMI) was calculated. BMD measurements were repeated in all follow-up examinations (1993, 1997, and 2000).

To control for precision of the DXA apparatus, the equipment was checked during every examination (1990, 1993, 1997, and 2000) using the same phantom, with BMD of the phantom regulated to $1.270 \pm 0.025 \text{ g/cm}^2$ (2%). In addition, to control for observer variability, all participants

were examined by the same medical doctor. Intraobserver variability of DXA in vitro and in vivo had been measured for a prior study [15] using the same doctor. Coefficient of variation (CV%) for L2-L4 in vitro was determined as 0.35%, whereas CV% for L2-L4, proximal femur, Ward's triangle, and trochanter, examined in vivo in five male volunteers, was 0.61%–0.90%, 1.02%–2.57%, 1.97%–5.45% and 1.77%–4.17%, respectively.

In the present study, changes in BMD over 10-year periods, classified by sex and categories of ADL items, were calculated.

Statistical analysis

Statistical analyses were performed using statistical software packages SPSS (SPSS, Chicago, IL, USA) and STATA (STATA, College Station, TX, USA). Differences were tested for statistical significance using one-way analysis of variance (ANOVA) for comparisons among multiple groups and Scheffe's least squares difference (LSD) test for pairs of groups. Analysis of covariance (ANCOVA) was then performed with adjustment of suitable variables.

Results

From the 200 participants in the initial survey, 124 subjects (57 men, 67 women; 62.0%) completed all follow-up BMD examinations in 1993, 1997, and 2000 and the ADL questionnaire survey in 2000. A total of 76 participants were not included for evaluation of relationships between ADL and BMD: 43 had died (28 men, 15 women); 15 had moved (6 men, 9 women); 8 were ill (2 men, 6 women); 4 were not present during a follow-up (1 man, 3 women); 3 declined to participate (3 men); and the remaining 3 (3 men) did not complete all items for ADL in the questionnaire at the first survey in 1990.

Table 1 shows the characteristics of all participants aged 60–79 years at the initial survey. Mean age between men and women was very similar. Frequencies of coexisting diseases that might influence ADL, such as fractures, gastrec-

tomy, diabetes mellitus, and renal dialysis, are also shown in Table 1. Frequency of previous fractures was the highest both in men and women.

Table 2 compares physical characteristics between participants and dropouts at the time of initial survey. Mean age of the remaining participants in their seventies was significantly younger than that of dropouts ($P < 0.001$), whereas mean ages in other age groups of women and in all age groups of men did not differ significantly.

Table 3 shows the frequencies of observed difficulties in ADL for participants at the initial survey. Among ADL items, the most frequent difficulties in men involved running (50.0%), followed by raising (30.6%), standing (27.1%), sitting (24.7%), and reaching (16.5%). In women, difficulties involved running (67.0%), followed by lifting (36.3%), standing (33.1%), reaching (30.8%), and sitting (23.6%).

Changes in ADL between baseline and follow-up after 10 years were calculated. A decrease of two grades in each ADL item means ADL decreased from "Can do without difficulty" to "Either unable to do, or able to do only with help," and a 1-grade decrease means an ADL decrease from

Table 1. Characteristics and frequencies of coexisting disorders among all participants aged 60–79 years old at the initial study

	Men	Women
Number of participants	100	100
Age (years)	68.7 (6.0)	68.8 (5.3)
Height (cm)	158.4 (6.4)	145.5 (5.6)
Weight (kg)	53.6 (8.0)	45.8 (7.5)
BMI (kg/m ²)	21.3 (2.6)	21.6 (3.0)
BMD (g/cm ²)		
L2–L4	1.05 (0.22)	0.81 (0.19)
Femoral neck	0.81 (0.11)	0.65 (0.11)
Past history ^a		
Previous fractures	33 (38.8)	23 (25.0)
Gastrectomy	7 (8.3)	2 (2.2)
Diabetes mellitus	5 (6.0)	4 (4.4)
Renal dialysis	0 (0.0)	0 (0.0)

Data are mean (SD)

BMI, body Mass Index; BMD, bone mineral density

^a Percent in parentheses; the number of participants with no answer or "unknown" is excluded from the denominator

Table 2. Comparisons of physical characteristics and BMD at the initial survey between participants and dropouts because of death

Sex	Age-stratum	Category	n	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m ²)	BMD (g/cm ²)	
								L2–L4	Femoral neck
Men	60–69	Alive	38	63.3 (2.8)	159.4 (5.4)	56.1 (7.5)	22.0 (2.4)	1.03 (0.19)	0.82 (0.12)
		Dead	8	64.5 (1.9)	162.0 (6.5)	55.5 (9.7)	21.0 (2.5)	1.05 (0.15)	0.86 (0.08)
	70–79	Alive	22	73.2 (2.7)	155.3 (6.5)	50.0 (8.4)	20.6 (2.6)	1.03 (0.20)	0.79 (0.11)
		Dead	20	75.0 (3.4)	157.2 (7.3)	51.1 (7.3)	20.8 (3.0)	1.07 (0.25)	0.76 (0.09)
Women	60–69	Alive	40	64.4 (7.8)	147.4 (5.1)	47.4 (6.8)	21.8 (3.0)	0.86 (0.20)	0.69 (0.11)
		Dead	5	63.6 (2.3)	144.2 (5.1)	46.0 (13.1)	21.9 (5.0)	0.81 (0.23)	0.60 (0.11)
	70–79	Alive	27	71.7 (1.8)	143.1 (5.5)	45.4 (7.7)	22.1 (3.0)	0.79 (0.16)	0.65 (0.10)
		Dead	10	74.8 (3.1)***	143.9 (4.1)	41.7 (4.6)	20.1 (2.2)	0.75 (0.19)	0.58 (0.06)*

Data are mean (SD)

^a Alive means participants of all surveys performed in 1990, 1993, 1997, and 2000; dead means participants at the initial survey who died by the last follow-up performed in 2000

***, *Significantly different from values of the participants of the same age group at $p < 0.001$, * $p < 0.05$, respectively

Table 3. Frequencies of observed difficulties in activities of daily living (ADL) among participants 60–79 years old at the initial survey

Questions/categories	Men	Women
<i>Can you reach, for example, a book from a high shelf or cupboard?</i>		
Can do without difficulty	71 (83.5)	63 (69.2)
Can do but with some difficulty	12 (4.1)	21 (23.1)
Either unable to do, or able only with help	2 (2.4)	7 (7.7)
<i>Can you wash and dry yourself all over?</i>		
Can do without difficulty	81 (96.4)	86 (94.5)
Can do but with some difficulty	3 (3.6)	5 (5.5)
Either unable to do, or able only with help	0 (0.0)	0 (0.0)
<i>Can you wash your hair over a washbasin?</i>		
Can do without difficulty	75 (89.3)	86 (93.5)
Can do but with some difficulty	9 (10.7)	6 (6.5)
Either unable to do, or able only with help	0 (0.0)	0 (0.0)
<i>Can you sit for a hour on a hard chair?</i>		
Can do without difficulty	64 (75.3)	68 (76.4)
Can do but with some difficulty	17 (20.0)	17 (19.1)
Either unable to do, or able only with help	4 (4.7)	4 (4.5)
<i>Can you stand continuously for 30 minutes?</i>		
Can do without difficulty	62 (72.9)	60 (65.9)
Can do but with some difficulty	17 (20.0)	20 (22.0)
Either unable to do, or able only with help	6 (7.1)	11 (12.1)
<i>Can you raise yourself in bed from a lying position?</i>		
Can do without difficulty	59 (69.4)	74 (81.3)
Can do but with some difficulty	23 (27.1)	12 (13.2)
Either unable to do, or able only with help	3 (3.5)	5 (5.5)
<i>Can you take socks on and off your feet?</i>		
Can do without difficulty	82 (96.5)	85 (92.4)
Can do but with some difficulty	3 (3.5)	7 (7.6)
Either unable to do, or able only with help	0 (0.0)	0 (0.0)
<i>Can you bend down from a seated position and pick up a small object at the side of your chair?</i>		
Can do without difficulty	78 (91.8)	81 (88.0)
Can do but with some difficulty	7 (8.2)	9 (9.8)
Either unable to do, or able only with help	0 (0.0)	2 (2.2)
<i>Can you lift a plastic bag containing, for example, three 1.5-liter bottles?</i>		
Can do without difficulty	75 (88.2)	58 (63.7)
Can do but with some difficulty	6 (7.1)	21 (23.1)
Either unable to do, or able only with help	4 (4.7)	12 (13.2)
<i>Can you run 100 meters without stopping?</i>		
Can do without difficulty	42 (50.0)	31 (33.7)
Can do but with some difficulty	28 (33.3)	33 (35.9)
Either unable to do, or able only with help	14 (16.7)	28 (30.4)

Percent in parentheses; the number of participants with no answer or "unknown" is excluded from the denominator

"Can do without difficulty" to "Can do, but with some difficulty" or from "Can do, but with some difficulty" to "Either unable to do, or able to do only with help." Table 4 shows the distribution of frequencies for ADL changes over 10 years for participants at the initial survey (Table 4). Among ADL items, the most frequent decreases in men involved sitting (14.0%), followed by standing (12.3%), running (7.1%), reaching (7.0%), lifting (5.3%), and raising (5.3%). In women, the most frequent decreases involved standing (23.9%), followed by sitting (19.7%), running (19.4%), reaching (17.9%), and lifting (14.9%). These results show that decreases in ADL items over 10 years were similar for both men and women, but frequencies of decreased ADL items were higher in women than in men.

To evaluate relationships between decreased ADL and changes in BMD, changes to BMD at the lumbar spine and femoral neck were compared to changes for each ADL item (2 grade decrease; 1 grade decrease; or no change) (Table 5). At the crude analysis using one-way ANOVA, annual

changes in BMD at the lumbar spine in men were not associated with changes in any ADL items men whereas those at the femoral neck were significantly associated with decreased bending and running. In contrast, annual changes in BMD at the lumbar spine in women were significantly associated with decreased reaching and lifting. Furthermore, decreased BMD at the femoral neck was significantly associated with decreased standing and lifting.

ANCOVA was performed in each sex for decreased ADL and annual bone changes after adjustment for age, past history (previous fractures, gastrectomy, diabetes mellitus, and renal dialysis at initial survey). In men, annual change rates in BMD at the femoral neck over 10 years were no longer correlated with decreased running, but were significantly correlated with decreased bending ($P = 0.046$; $R^2 = 0.10$). In women, annual change rates in BMD at the lumbar spine over 10 years were significantly correlated with decreased reaching ($P = 0.007$; $R^2 = 0.25$) and lifting ($P = 0.014$, $R^2 = 0.27$), and changes at the femoral neck

Table 4. Frequencies of observed decrease in ADL among participants over 10 years among those 60–79 years old

Change of ADL ^a	Men	Women
<i>Can you reach, for example, a book from a high shelf or cupboard?</i>		
No change	53 (93.0)	55 (82.1)
1 Grade decrease	1 (1.8)	10 (14.9)
2 Grade decrease	3 (5.3)	2 (3.0)
<i>Can you wash and dry yourself all over?</i>		
No change	57 (100.0)	65 (97.0)
1 Grade decrease	0 (0.0)	1 (1.5)
2 Grade decrease	0 (0.0)	1 (1.5)
<i>Can you wash your hair over a washbasin?</i>		
No change	55 (96.5)	63 (94.0)
1 Grade decrease	2 (3.5)	3 (4.5)
2 Grade decrease	0 (0.0)	1 (1.5)
<i>Can you sit for a hour on a hard chair?</i>		
No change	49 (86.0)	53 (80.3)
1 Grade decrease	5 (8.8)	11 (16.7)
2 Grade decrease	3 (5.3)	2 (3.0)
<i>Can you stand continuously for 30 minutes?</i>		
No change	50 (87.7)	51 (76.1)
1 Grade decrease	5 (8.8)	8 (11.9)
2 Grade decrease	2 (3.5)	8 (11.9)
<i>Can you raise yourself in bed from a lying position?</i>		
No change	54 (94.7)	64 (97.0)
1 Grade decrease	3 (5.3)	1 (1.5)
2 Grade decrease	0 (0.0)	1 (1.5)
<i>Can you take socks on and off your feet?</i>		
No change	55 (96.5)	64 (95.5)
1 Grade decrease	2 (3.5)	3 (4.5)
2 Grade decrease	0 (0.0)	0 (0.0)
<i>Can you bend down from a seated position and pick up a small object at the side of your chair?</i>		
No change	56 (98.3)	66 (98.5)
1 Grade decrease	1 (1.8)	1 (1.5)
2 Grade decrease	0 (0.0)	0 (0.0)
<i>Can you lift a plastic bag containing, for example, three 1.5-liter bottles?</i>		
No change	54 (94.7)	57 (85.1)
1 Grade decrease	1 (1.8)	4 (6.0)
2 Grade decrease	2 (3.5)	6 (9.0)
<i>Can you run 100 meters without stopping?</i>		
No change	52 (92.9)	54 (80.6)
1 Grade decrease	1 (1.8)	11 (16.4)
2 Grade decrease	3 (5.4)	2 (3.0)

Percent in parentheses

^aCategories of change of ADL are as follows: no change, no change or improvement; 1 grade decrease, from "Can do without difficulty" to "Can do but with some difficulty" or from "Can do but with some difficulty" to "Either unable to do, or able only with help"; 2 grade decrease, from "Can do without difficulty" to "Either unable to do, or able only with help"

were significantly correlated with decreased lifting ($P = 0.001$, $R^2 = 0.33$), although no associations remained between BMD changes at the femoral neck and standing.

Discussion

In the present study, inhabitants of a rural community were established as a cohort and followed for a long period with a high participation rate. Frequencies of ADL difficulties for 60- to 79-year-old subjects among general inhabitants were clarified, and change rates were also shown. BMD was followed for 10 years in both men and women to clarify decreases in ADL abilities among aged residents and to investigate associations between ADL change and bone loss.

Some limitations to the present study must be noted. First, attention should be paid to survival bias. Because BMD was measured using DXA in the clinic, all participants presented to the place of examination, and inhabitants who were bedridden because of disease or advanced age were thus unable to participate in the survey. Among the 101 participants who could not participate in follow-ups, 55 had died (54.5%) and 13 were too ill to attend the clinic (12.9%). A total of 67% of nonparticipants were thus dead or too ill to attend BMD measurements, and loss of ADL abilities in these subjects would presumably have been greater than in the participants. Survival bias in the present survey would thus have led to an underestimation of associations between ADL and BMD. In addition, analysis of changes in ADL included subjects in the lowest category at the initial survey. Subjects in the lowest category were automatically classified as showing no change at final follow-up.

Table 5. Change of BMD over 10 years classified by categories of change of ADL

Questions/categories ^a	Men			Women		
	n	L2-L4	Femoral neck	n	L2-L4	Femoral neck
<i>Can you reach, for example, a book from a high shelf or cupboard?</i>						
No change	53	-0.10 (0.82)	-0.88 (0.99)	55	-0.36 (0.73)**	-0.74 (1.14)
1 Grade decrease	1	0.92 (-)	-0.19 (-)	10	-0.61 (1.41)	-0.54 (0.67)
2 Grade decrease	3	0.23 (1.10)	-1.89 (1.92)	2	-3.11 (3.85)	-2.29 (0.42)
<i>Can you wash and dry yourself all over?</i>						
No change	57	-0.07 (0.83)	-0.92 (1.05)	65	-0.47 (1.06)	-0.77 (1.12)
1 Grade decrease	0			1	-2.21 (-)	-0.95 (-)
2 Grade decrease	0			1	0.63 (-)	0.13 (-)
<i>Can you wash your hair over a washbasin?</i>						
No change	55	-0.03 (0.81)	-0.85 (0.97)	63	-0.46 (1.07)	-0.75 (1.13)
1 Grade decrease	2	-1.13 (0.88)	-2.86 (1.67)	3	-1.28 (0.93)	-1.22 (0.25)
2 Grade decrease	0			1	0.63 (-)	0.13 (-)
<i>Can you sit for a hour on a hard chair?</i>						
No change	49	-0.05 (0.83)	-0.83 (0.99)	53	-0.52 (1.15)	-0.72 (1.17)
1 Grade decrease	5	-0.15 (1.21)	-1.00 (0.69)	11	-0.31 (0.82)	-0.70 (0.79)
2 Grade decrease	3	-0.26 (0.22)	-2.14 (1.98)	2	-0.58 (0.28)	-1.97 (0.88)
<i>Can you stand continuously for 30 minutes?</i>						
No change	50	-0.00 (0.83)	-0.88 (0.10)	51	-0.46 (1.05)	-0.58 (0.87)
1 Grade decrease	5	-0.68 (0.83)	-1.60 (1.53)	8	-0.64 (0.72)	-1.53 (2.07)
2 Grade decrease	2	-0.18 (0.16)	-0.23 (0.20)	8	-0.44 (1.56)	-1.11 (0.85)
<i>Can you raise yourself in bed from a lying position?</i>						
No change	54	-0.07 (0.85)	-0.94 (1.05)	64	-0.45 (1.08)	-0.73 (1.11)
1 Grade decrease	3	-0.00 (0.47)	-0.54 (1.01)	1	-2.21 (-)	-0.95 (-)
2 Grade decrease	0			1	-0.39 (-)	-2.59 (-)
<i>Can you take socks on and off your feet?</i>						
No change	55	-0.07 (0.79)	-0.90 (1.06)	64	-0.44 (1.07)	-0.73 (1.11)
1 Grade decrease	2	-0.12 (2.30)	-1.47 (0.30)	3	-1.32 (0.91)	-1.31 (1.15)
2 Grade decrease	0			0		
<i>Can you bend down from a seated position and pick up a small object at the side of your chair?</i>						
No change	56	-0.06 (0.84)	-0.86 (0.97)	66	-0.48 (1.08)	-0.75 (1.11)
1 Grade decrease	1	-0.50 (-)	-4.05 (-)	1	-0.35 (-)	-1.29 (-)
2 Grade decrease	0			0		
<i>Can you lift a plastic bag containing, for example, three 1.5-litre bottles?</i>						
No change	54	-0.06 (0.85)	-0.88 (0.98)	57	-0.37 (0.86)*	-0.58 (0.84)**
1 Grade decrease	1	-0.17 (-)	-0.80 (-)	4	-0.42 (0.82)	-0.76 (0.82)
2 Grade decrease	2	-0.28 (0.31)	-2.07 (2.80)	6	-1.58 (2.21)	-2.45 (2.03)
<i>Can you run 100 meters without stopping?</i>						
No change	52	-0.01 (0.81)	-0.85 (0.98)	54	-0.47 (0.83)	-0.61 (0.84)
1 Grade decrease	1	-0.50 (-)	-4.05 (-)	11	-0.66 (1.94)	-1.33 (1.87)
2 Grade decrease	3	-0.40 (1.08)	-0.80 (0.83)	2	0.25 (0.91)	-1.57 (1.45)

Data are mean (SD)

Minus (-), bone loss

^aCategories of change of ADL are as follows: no change, no change or improvement; 1 grade decrease, from "Can do without difficulty" to "Can do but with some difficulty" or from "Can do but with some difficulty" to "Either unable to do, or able only with help"; 2 grade decrease, from "Can do without difficulty" to "Either unable to do, or able only with help"

** * P significantly different among categories after adjustment for age, coexisting diseases (previous fractures, gastrectomy, diabetes mellitus, and renal dialysis at the initial survey): (** P < 0.01, * P < 0.05)

A total of 8 men and 19 women were classified in the lowest category (either enable to do, or able to do only with help) for various ADL items at the initial survey. However, similar results to the main analysis were obtained after separating these 27 participants from ANCOVA.

The present study clarified the frequencies of difficulties in ADL for aged residents in a rural community in Japan. Among the ADL items, frequent difficulties in men involved running, raising, standing, sitting, and reaching. In women, difficulties involved running, lifting, standing, reaching, and sitting. Clearly distinguishing characteristics for these items using the ADL questionnaire might be difficult, but our study demonstrated that the ability to maintain endurance (e.g., running, sitting, standing, lifting) and per-

form complicated actions (e.g., raising, reaching) becomes difficult more rapidly for aged residents than other items.

In the present study, frequencies of ADL change between initial and follow-up after 10 years were also clarified. Among ADL items, frequent decreases involved sitting, standing, running, reaching, and lifting for both men and women, while frequencies of decreased ADL items were higher in women than in men. This study shows that decreases in ADL items over a 10-year period were similar for both men and women. Likewise, our study demonstrates that ADL items requiring endurance (e.g., running, sitting, standing, lifting) or complicated actions (e.g., reaching) decreased more rapidly among aged residents than other items.

Concerning associations between physical activity and osteoporosis and/or osteoporotic fracture, two competing consequences may be relevant. Some investigators have reported that physical activity might exert local or generalized positive influences on BMD, thereby reducing the risk of fracture. Some cross-sectional [16–20] and prospective studies [21–23] have shown that high levels of physical activity and physical fitness are associated with increased BMD in both men and women. Other studies have suggested that increased levels of physical activity might be associated with adverse consequences [24,25]. Another report demonstrated that regular walking in middle-aged and elderly women is associated with reduced risk of vertebral deformity, but high levels of physical activity in early and middle adult life are associated with increased risk of this condition in men [26].

The present study revealed associations between bone loss and decreased ADL items of bending in men and reaching, lifting, and running in women. This association remained after adjustment for age and prevalence of coexisting diseases. In the present study, distinguishing causation between bone loss and ADL decreases might be very difficult. The possibility that decreases in ADL could influence bone loss should be considered, and vice versa. Further observation is therefore necessary to clarify the direction of influence between ADL and BMD.

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Suppression of Adjuvant-Induced Arthritic Bone Destruction by Cyclooxygenase-2 Selective Agents With and Without Inhibitory Potency Against Carbonic Anhydrase II

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ABSTRACT: *In vitro* assays revealed that COX-2 inhibitors with CA II inhibitory potency suppressed both differentiation and activity of osteoclasts, whereas that without the potency reduced only osteoclast differentiation. However, all COX-2 inhibitors similarly suppressed bone destruction in adjuvant-induced arthritic rats, indicating that suppression of osteoclast differentiation is more effective than that of osteoclast activity for the treatment.

Introduction: Cyclooxygenase (COX)-2 and carbonic anhydrase II (CA II) are known to play important roles in the differentiation of osteoclasts and the activity of mature osteoclasts, respectively. Because several COX-2 selective agents were recently found to possess an inhibitory potency against CA II, this study compared the bone sparing effects of COX-2 selective agents with and without the CA II inhibitory potency.

Materials and Methods: Osteoclast differentiation was determined by the mouse co-culture system of osteoblasts and bone marrow cells, and mature osteoclast activity was measured by the pit area on a dentine slice resorbed by osteoclasts generated and isolated from bone marrow cells. *In vivo* effects on arthritic bone destruction were determined by radiological and histological analyses of hind-paws of adjuvant-induced arthritic (AIA) rats.

Results: CA II was expressed predominantly in mature osteoclasts, but not in the precursors. CA II activity was inhibited by sulfonamide-type COX-2 selective agents celecoxib and JTE-522 similarly to a CA II inhibitor acetazolamide, but not by a methylsulfone-type COX-2 inhibitor rofecoxib. *In vitro* assays clearly revealed that celecoxib and JTE-522 suppressed both differentiation and activity of osteoclasts, whereas rofecoxib and acetazolamide suppressed only osteoclast differentiation and activation, respectively. However, bone destruction in AIA rats was potently and similarly suppressed by all COX-2 selective agents whether with or without CA II inhibitory potency, although only moderately by acetazolamide.

Conclusions: Suppression of osteoclast differentiation by COX-2 inhibition is more effective than suppression of mature osteoclast activity by CA II inhibition for the treatment of arthritic bone destruction.

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Key words: cyclooxygenase, prostaglandin, carbonic anhydrase, osteoclast, bone resorption, arthritis

INTRODUCTION

BONE DESTRUCTION CAUSING joint deformities is one of the most serious problems in patients with rheumatoid arthritis (RA). Histological analyses have shown that osteoclastic bone resorption at the bone-pannus interface plays a pivotal role in the RA bone destruction.⁽¹⁾ Among pro-inflammatory cytokines that play pivotal roles in the RA pathogenesis, fibroblast growth factor 2 (FGF-2) is reported to be strongly associated with osteoclastic bone re-

sorption by RA in animal models and clinical settings.^(2–4)

Osteoclastic bone resorption is regulated mainly by two consecutive steps: the differentiation of osteoclasts from hemopoietic precursors and the activity of mature osteoclasts to resorb bone.

The differentiation of osteoclasts is stimulated by a variety of cytokines and hormones including FGF-2, a large part of which is mediated by the production of prostaglandins (PGs).⁽⁵⁾ PGs stimulate osteoclast differentiation through the induction of RANKL in osteoblastic cells,⁽⁶⁾ whereas they inhibit the bone resorptive activity of mature osteoclasts.⁽⁷⁾ Among the synthetic enzymes of PGs, we

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and others have reported that cyclooxygenase-2 (COX-2) plays a central role in the biosynthesis of PGs in response to bone resorptive stimuli by cytokines and hormones,⁽⁸⁻¹²⁾ so that COX-2 induction is involved in bone resorptive disorders including RA by inducing osteoclast differentiation.^(2,13,14)

Although little is known about the direct signaling to activate mature osteoclasts, a study of random sequence analysis of PCR-amplified cDNA clones detected 14 distinct kinase-related genes in purified mature osteoclasts, and 8 of them were identified as receptor tyrosine kinases (RTKs).⁽¹⁵⁾ RTKs expressed on mature osteoclasts include FGF receptor type 1 (FGFR1) and Tyro 3, whose ligands FGF-2 and the growth arrest-specific gene 6 (Gas6), respectively, are known to be potent bone resorptive cytokines. We previously reported that FGF-2 at physiological concentrations (≥ 10 pM) acts directly on mature osteoclasts to resorb bone by activating FGFR1,⁽¹⁶⁾ whereas at higher concentrations (≥ 10 nM), it stimulates osteoclast differentiation by inducing RANKL mainly through COX-2 expression in osteoblasts.^(10,11) In the meantime, Gas6, although ubiquitously expressed in bone cells, did not affect the osteoclast differentiation, but potentially stimulated the mature osteoclast activity because of the restricted localization of its receptor Tyro 3 on mature osteoclasts.^(15,17) Under these stimuli, osteoclasts resorb bone by attaching to the surface and secreting protons in an extracellular compartment formed between osteoclast and the bone surface.⁽¹⁸⁾ This secretion is necessary for bone mineral solubilization and the digestion of organic bone matrix by acid proteases. For this process, carbonic anhydrase II (CA II), which is abundant in the osteoclast cytoplasm and on the inner surface of its ruffled border, catalyzes the hydration of CO₂ to bicarbonate and a proton, thereby contributing to the H⁺ secretion acidifying the bone resorption compartment and to HCO₃⁻ secretion by the HCO₃⁻/Cl⁻ exchanger, which maintains pH homeostasis.⁽¹⁹⁾ The blockage of CA II with antisense oligonucleotides or acetazolamide, a potent CA II inhibitor, has been reported to suppress bone resorption by osteoclasts,^(20,21) and a CA II deficiency syndrome has been shown to exhibit nonfunctional osteoclasts and osteopetrosis.⁽²²⁾ In addition, CA II expression is stronger in actively resorbing mature osteoclasts than in nonresorbing osteoclasts.⁽²³⁾ All these findings strongly suggest that CA II plays an important role in the bone resorptive activity of mature osteoclasts.

Interestingly, a recent report revealed that sulfonamide-type COX-2 inhibitors that contain an aryl sulfonamide moiety, such as celecoxib, show an unexpected nanomolar affinity to and inhibition of the totally unrelated carbonic anhydrase family including CA II, whereas methylsulfonamide-type COX-2 inhibitors without the sulfonamide moiety, such as rofecoxib, do not.⁽²⁴⁾ Hence, to investigate the importance of the regulation of osteoclast differentiation by COX-2 and osteoclast activity by CA II in arthritic bone destruction, this study examined the suppressive effects of COX-2 selective agents with and without inhibitory potency against CA II both in vivo and in vitro. For the in vivo analyses, we initially examined the stimulatory effects of FGF-2 and Gas6 on the formation of osteoclasts in the

co-culture of mouse osteoblasts and bone marrow cells and on the activity of mature osteoclasts generated and isolated from osteoclast precursor macrophage colony-stimulating factor (M-CSF)-dependent bone marrow macrophages (M-BMM ϕ s). Suppressions of osteoclast differentiation and activation in these cultures by celecoxib, rofecoxib, JTE-522, and acetazolamide, which show distinct inhibitions on COX-2 and CA II, were examined. For the in vivo analyses, suppressions of inflammation and bone destruction of hind-paws of adjuvant-induced arthritic (AIA) rats as a model of RA by administration of these inhibitors were studied.

MATERIALS AND METHODS

Animals and materials

Eight-week-old ddY mice and 6-week-old male Lewis rats were purchased from Charles River Japan Laboratories (Kanagawa, Japan). All animal experiments were performed according to the guidelines of the International Association for the Study of Pain.⁽²⁵⁾ In addition, the experimental work was reviewed by the committee of Tokyo University charged with confirming ethics.

Celecoxib and a COX-1 selective inhibitor SC-560 were generously provided by Pfizer (New York, NY, USA). Rofecoxib was provided by Merck Sharp & Dohme (Rahway, NJ, USA) and JTE-522, a novel sulfonamide-type COX-2 selective agent,⁽²⁶⁾ was synthesized and provided by Japan Tobacco (Osaka, Japan). Rat recombinant Gas6 and human recombinant FGF-2 were generously provided by Shionogi Research Laboratory (Osaka, Japan) and Kaken Pharmaceutical Co. (Kyoto, Japan), respectively. α -MEM was purchased from Life Technologies (Grand Island, NY, USA), and FBS was from the Cell Culture Laboratory (Cleveland, OH, USA). Recombinant human M-CSF was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human soluble RANKL was purchased from PeproTech (London, UK). Bacterial collagenase and ISOGEN were purchased from Wako Pure Chemicals Co. (Osaka, Japan), and dispase from Nitta Gelatin Co. (Osaka, Japan). Other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise specified.

RT-PCR for COX-2 and CA II expression

For the osteoclast precursor cells, we used the M-BMM ϕ culture described previously.⁽²⁷⁾ Briefly, bone marrow cells from tibias of 8-week-old mice were seeded at a density of 2×10^5 cells/well in 6-well dishes and cultured in α -MEM containing 10% FBS with M-CSF (100 ng/ml). After culturing for 3 days, nonadherent cells were washed with PBS, and adherent cells (M-BMM ϕ s) were further cultured with soluble RANKL (30 ng/ml) and M-CSF (10 ng/ml). Total RNA was extracted from the cells cultured for 1–5 days, using ISOGEN following the manufacturer's instructions, and 2 μ g of RNA was reverse transcribed and amplified by PCR using the RT-PCR kit (Takara Biomedicals, Shiga, Japan). The primers were as follows: sense, 5'-TCAGCCAGGCAGCAAATCCTTG-3' and antisense, 5'-TAGTCTCCTATGAGTATGAGTC-3' for COX-2; sense, 5'-CTCTCAGGACAATGCAGTGC-3' and anti-

sense, 5'-ATCCAGGTCACACATTCCAGC-3' for CA II; and 5'-CATGTAGGCCATGAGGTCCACCAC-3' and 5'-TGAAGGTCGGTGTGAACGGATTGGC-3' G3PDH. The cycling parameters were 45 s at 94°C, 30 s at 56°C, and 90 s at 72°C for 26 cycles.

CA II activity

The inhibitory effects of COX-2 inhibitors and acetazolamide, a positive control, on CA II activity was determined by the method reported by Wilbur et al.⁽²⁸⁾ Briefly, the Universal buffer (25 mM; lot 01020; Helena Laboratories, Beaumont, TX, USA) with or without CA II (100 Wilbur-Anderson units/ml; Sigma) at pH 8.6 was incubated with the above agents or the vehicle alone for 30 s on ice. Fifteen milliliters of distilled water saturated with CO₂ by bubbling >1 h was added to the mixture, and the times until the pH of the mixture decreased from 8.6 to 6.3 were measured under the saturated condition. CA activity was expressed as $(T_0 - T)/T_0$ (T_0 = time without CA II, T = time with CA II), the effect of each agent was expressed as the percentage of CA activity over that of the vehicle alone (control), and the ED₅₀ values were determined by linear regression.

Assay for osteoclast differentiation in the mouse co-culture system

To isolate osteoblasts, calvariae dissected from 1- to 4-day-old mice were washed in PBS and digested with 1 ml of trypsin/EDTA (Life Technologies) containing 10 mg collagenase (Sigma type 7) for 10 minutes × 5 times, and cells from fractions 3–5 were pooled. Cells were plated in 6-multiwell dishes at a density of 5000 cells/cm² and grown to confluence in α -MEM containing 10% FBS. Osteoblasts (3×10^6 cells/well) prepared as described above and bone marrow cells (1×10^6 cells/well) from tibias of 8-week-old mice were co-cultured in 24-multiwell dishes containing α -MEM/10% FBS with or without FGF-2 (1 nM), Gas6 (1 nM), and soluble RANKL (30 ng/ml), and/or celecoxib, rofecoxib, JTE-522, acetazolamide, and SC-560 (all 1 μ M) for 6 days with a medium change at 3 days. After 6 days of culture, cells were fixed with 3.7% (vol/vol) formaldehyde in PBS and ethanol-acetone (50:50, vol/vol) and stained at pH 5.0 in the presence of 1(+)-tartaric acid using naphthol AS-MX phosphate in *N,N*-dimethyl formamide as the substrate. TRACP⁺ multinucleated cells containing more than three nuclei were counted as osteoclasts.

Assay for mature osteoclast activity from M-BMM ϕ culture

The activity of mature osteoclasts was measured using the M-BMM ϕ culture above. After culture of bone marrow cells for 3 days, adherent M-BMM ϕ s were isolated and cultured as described above. Just before the fusion of cells, adherent cells were stripped by trypsin/EDTA, and further cultured on a dentine slice placed in each well of 96-well dishes containing α -MEM/10% FBS with or without FGF-2 (10 pM), Gas6 (1 nM), and soluble RANKL (30 ng/ml), and/or the agents above (all 1 μ M). After 48 h of culture, cells were removed with 1N NH₄OH solution, and the dentine surface was stained with 0.5% toluidine blue. Total

area was estimated under a light microscope with a micrometer to assess osteoclastic bone resorption using an image analyzer (System Supply Co., Nagano, Japan). At the same time, cells on a dentine slice in the independent culture were fixed and stained with TRACP as described above, and TRACP⁺ multinucleated cells containing more than three nuclei were counted as mature osteoclasts.

To determine survival, osteoclasts generated as described above were cultured on a plastic dish for an additional 48 h. At 3, 6, 12, 24, and 48 h, the number of TRACP⁺ and trypan blue-negative osteoclasts were counted.

Induction of AIA and drug administration in rats

AIA was induced in 6-week-old rats with subcutaneous injection of Freund's complete adjuvant containing 2% (wt/vol) *Mycobacterium butyricum* into the left hind-paw (day 0). From days 15 to 24, the indicated dosages (0.3–10 mg/kg/day) of celecoxib, rofecoxib, JTE-522, and acetazolamide suspended in 0.5% methylcellulose and 0.025% Tween-20 solution or the vehicle alone were administered orally twice a day (at 9:00 a.m. and 5:00 p.m.) at a volume of 5 ml/kg body weight.

Measurement of hind-paw swelling

The right hind-paw volume was measured with an instrument for plethysmography (MK-310; Muromachi Kikai Co., Tokyo, Japan) by vertically immersing the paw to the level of the proximal end of the lateral malleolus in a water-filled tub on day 25. The effect of each agent was expressed as the percent inhibition: the volume difference between normal and the drug-treated AIA divided by that between normal and the vehicle-treated AIA, and the dose of 50% inhibition determined by linear regression was expressed as the ED₅₀ for each drug. After the measurement, the animals were killed, and the right hindlimbs were excised for the following radiological and histological examinations. The evaluations were performed by the same observer without knowledge of treatment.

Measurement of BMD

BMD (mg/cm²) of the excised right hind-paws was measured by DXA using a bone mineral analyzer (QDR-2000; Hologic, Bedford, MA, USA). On the monitoring image of hindlimbs by lateral projection, each paw image was divided into five parts, and data from the first and second parts from the posterior heel side were represented as data for paw BMD. The effect of each agent was expressed as the percent inhibition: the BMD difference between normal and the drug-treated AIA divided by that between normal and the vehicle-treated AIA, and the dose of 50% inhibition determined by linear regression was expressed as the ED₅₀ for each agent.

Measurement of serum concentration of type I collagen C-telopeptide

Immediately after the rats were killed, ~5 ml of blood was collected from the femoral vein. The blood was allowed to clot at room temperature for ~30 minutes after collection. The serum was separated by centrifugation at 1500g for 15

minutes at 4°C and stored at -80°C. Type 1 collagen C-telopeptide (CTX) concentration in the serum was measured using Rat Laps ELISA (Osteometer Bio Tech A/S, Herlev, Denmark), according to the manufacturer's instruction. Briefly, 20 µl of each sample was mixed with 100 µl of primary antibody in a preincubated ELISA plate and incubated overnight at 4°C. After 100 µl of peroxidase-conjugated goat anti-rabbit IgG antibody was added into each well, the mixture was incubated with 100 µl of the substrate solution. To stop the color reaction, 100 µl of stopping solution was added to each well, and the absorbance was measured at 450 nm with the reference at 650 nm. The effect of each agent was expressed as the percent inhibition: the difference between normal and the drug-treated AIA divided by that between normal and the vehicle-treated AIA, and the dose of 50% inhibition determined by linear regression was expressed as the ED₅₀ for each agent.

Radiological scoring

Radiographs of the right hindlimbs were taken using a soft X-ray apparatus (CMB-2; Softex Co., Kanagawa, Japan). A 0–5 subjective grading system (0 = normal, 5 = the most severe) was used to evaluate bone destruction and periostitis, respectively, in the hind-paw, and the sum of the scores for the parameters was expressed as the radiological score (maximum 10). Radiographs of the adjuvant noninjected hindlimb were taken with a Softex-CMB X-ray unit (Softex Co.). The severity of bone damage was assessed blindly from the radiographs by grading the bone destruction and periostitis.

Histological scoring

After radiography, specimens were placed in 3.7% formaldehyde for 24 h and decalcified in 10% EDTA for 14 days. They were dehydrated with an increasing concentration of ethanol, embedded in paraffin, longitudinally sectioned into 4-µm-thick sections, and stained with H&E. A 0–5 subjective grading system (0 = normal, 5 = the most severe) was used to evaluate the overall change including synovial thickening (pannus formation) and joint destruction in the talo-tibial joint (maximum 5).

Statistical analysis

Statistical analysis was performed using Student's *t*-test or ANOVA. *p* < 0.05 was considered significant. Values are expressed as the mean ± SE.

RESULTS

Expressions of COX-2 and CA II during osteoclast differentiation

To investigate the expressions of COX-2 and CA II during osteoclast differentiation, these mRNA levels were examined in osteoclast precursor M-BMMφs cultured in the presence of soluble RANKL and M-CSF without support of osteoblasts/stromal cells (Fig. 1). Because we extracted mRNA from cultured M-BMMφs every day, various differentiation stages of osteoclastic cells were assumed to be

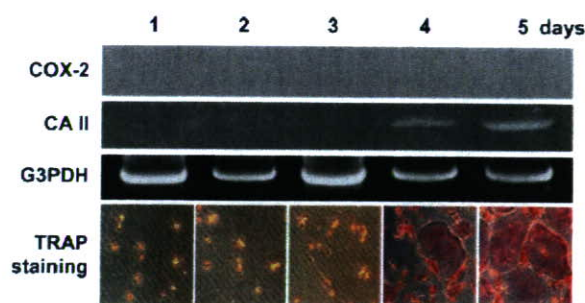


FIG. 1. Expressions of COX-2 and CA II during osteoclast differentiation. For osteoclast precursor cells, we used M-BMMφs that were isolated from mouse bone marrow cells cultured in the presence of M-CSF. M-BMMφs were further cultured with soluble RANKL and M-CSF for 1–5 days, and RNA extraction or TRAP staining was performed. The mRNA levels of COX-2 and CA II were determined by RT-PCR with that of G3PDH as a loading control. The PCR products for COX-2, CA II, and G3PDH were 939, 411, and 984 bp, respectively. TRAP⁺ multinucleated osteoclasts stained in red were visible after 4 days of culture with the expression of CA II.

included. COX-2 expression was not detected in any differentiation stage of osteoclasts as we previously reported in other osteoclastic cell lines,^(10,11) indicating that COX-2 in osteoblasts/stromal cells, but not in cells of osteoclastic lineage, plays an important role in osteoclastic bone resorption. In the meantime, the CA II expression was detected at 4 days and increased at 5 days of culture, in accordance with the appearance of TRAP⁺ multinucleated osteoclasts. This confirms that CA II is expressed predominantly in mature osteoclasts, but not in the precursors.

Inhibition of CA II activity by COX-2 selective agents

To study the inhibition of CA II activity by COX-2 selective agents and acetazolamide, a CA II inhibitor as a positive control, these drugs were incubated with CA II in the CO₂-saturated water (Fig. 2). The pH measurement revealed that the sulfonamide-type COX-2 inhibitors celecoxib and JTE-522 as well as acetazolamide exhibited inhibitory potency against CA II, whereas the methylsulfone-type rofecoxib did not, as previously reported.⁽²⁴⁾ The ED₅₀ values of celecoxib, JTE-522, and acetazolamide were 97.7, 3.2, and 6.6 nM, respectively.

Effects of COX-2, COX-1, and CA II inhibitors on the differentiation of osteoclasts

We initially examined the actions of FGF-2, Gas6, and soluble RANKL on TRAP⁺ multinucleated cell formation in the co-culture of mouse osteoblasts and bone marrow cells. As we reported previously, FGF-2 and soluble RANKL, but not Gas6, exhibited the stimulation of osteoclast formation^(10,11,17) (Fig. 3A). When COX-2 inhibitors celecoxib, rofecoxib, and JTE-522, a CA II inhibitor acetazolamide, and a COX-1 selective inhibitor SC-560 were added to the co-cultures, only the COX-2 inhibitors with or without the CA II inhibitory potency clearly inhibited the FGF-2 action on osteoclast formation. Neither acetazol-

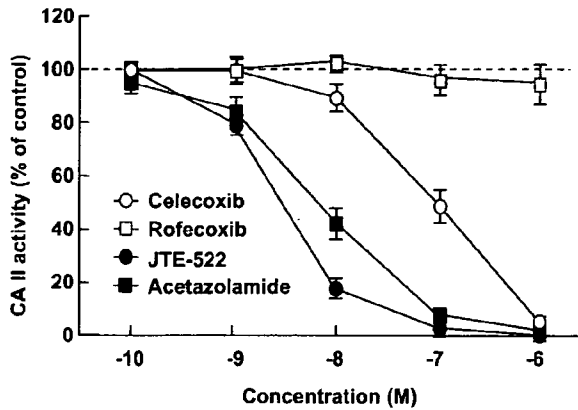


FIG. 2. Inhibition of CA II activity The inhibition of CA II activity by COX-2 selective agents celecoxib, rofecoxib, JTE-522, and a potent CA II inhibitor acetazolamide were determined by pH measurement in the mixture of each agent and CA II in the CO₂ saturated water. Data are expressed as means (symbols) ± SE (error bars) of the percentage of that of the vehicle alone (control) for three assays per group.

amide nor SC-560 altered the osteoclast formation. This indicates that COX-2, but not CA II or COX-1, is involved in the osteoclast differentiation of by FGF-2. In the meantime, none of the inhibitors reduced the soluble RANKL-stimulated osteoclast differentiation, confirming that RANKL lies downstream of COX-2 in the bone stimulatory signaling as previously reported.^(6,10,11)

Effects of COX-2, COX-1, and CA II inhibitors on the activity of mature osteoclasts

To evaluate the effects of the inhibitors on the activity of mature osteoclasts, the pit area on a dentine slice resorbed by osteoclasts formed in the M-BMMφ culture without osteoblasts/stromal cells was measured. We first confirmed our previous findings that FGF-2, Gas6, and soluble RANKL stimulated the resorbed pit area.^(10,16,17) Among the inhibitors, celecoxib, JTE-522, and acetazolamide, which have the potency to inhibit CA II, abrogated the stimulations by the cytokines, whereas neither rofecoxib nor SC-560 without the CA II inhibitory potency altered them (Fig. 3B, top). These regulations by the cytokines and the CA II inhibitors were not caused by the change of the number of osteoclasts but by the regulation of each osteoclast activity, because none of the agents affected the number of TRACP⁺ multinucleated osteoclasts on a dentine slice (Fig. 3B, middle). In fact, the effects of the agents on the pit area per osteoclast (resorbed pit area/osteoclast number on a dentine slice) showed a similar pattern to those on the resorbed pit area (Fig. 3B, bottom), indicating that CA II, but not COX-2 or COX-1, is involved in the activity of mature osteoclasts.

The lines of the in vitro results revealed the distinct effects of COX-2 inhibitors and CA II inhibitors on the differentiation of osteoclasts and the activity of mature osteoclasts, respectively.

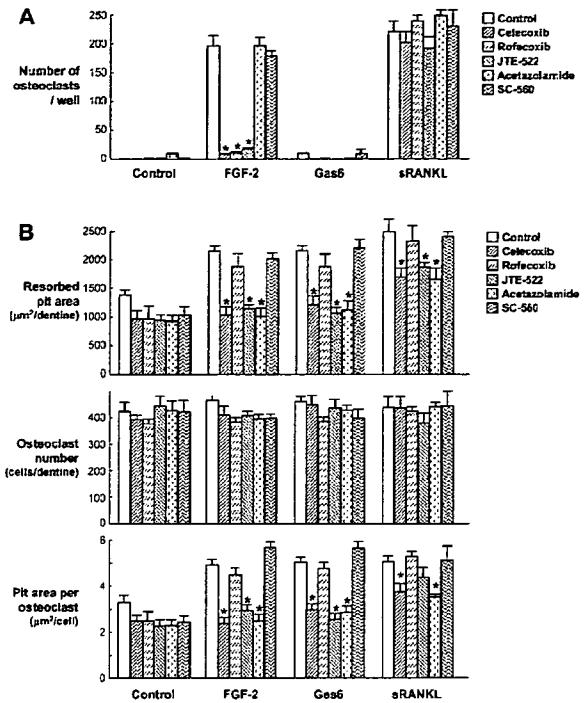


FIG. 3. Effects of COX-2, COX-1, and CA II inhibitors on (A) the differentiation and (B) activity of osteoclasts. (A) Osteoblasts from neonatal mouse calvariae and bone marrow cells from 8-week-old mice were co-cultured with or without FGF-2 (1 nM), Gas6 (1 nM), and soluble RANKL (30 ng/ml), and/or celecoxib, rofecoxib, JTE-522, acetazolamide, and SC-560 (all 1 μM) for 6 days. TRACP⁺ multinucleated cells containing more than three nuclei were counted as osteoclasts. (B) Activity of mature osteoclasts was determined by the pit area on a dentine slice resorbed by osteoclasts generated in the M-BMMφ culture without osteoblasts/stromal cells. The isolated osteoclasts were further cultured on a dentine slice with or without FGF-2 (1 nM), Gas6 (1 nM), and soluble RANKL (30 ng/ml), and/or the agents above (all 1 μM) for 48 h. The resorbed pit area was measured by toluidine blue staining on the dentine slice (top). At the same time, cells on a dentine slice in independent cultures were stained with TRACP, and the number of TRACP⁺ multinucleated osteoclasts was counted (middle). As the bone resorptive activity of an individual osteoclast, the pit area per osteoclast (resorbed pit area/osteoclast number on a dentine slice) was also calculated (bottom). Data are expressed as means (bars) ± SE (error bars) for eight cultures per group. **p* < 0.01, significant inhibition by the agents.

Effects of COX-2 and CA II inhibitors on AIA bone destruction in vivo

Based on the in vitro results above, we examined the suppression of arthritic bone destruction by celecoxib, rofecoxib, JTE-522, and acetazolamide in AIA rats. Inflammation by AIA determined by the hind-paw swelling was dose-dependently inhibited by the COX-2 selective agents whether with or without the CA II inhibitory action (Fig. 4A). The ED₅₀ values of celecoxib, rofecoxib, and JTE-522 were 0.26, 0.72, and 0.81 mg/kg/day, respectively. Bone destruction by AIA determined by the BMD loss of the hind-paw was also dose-dependently inhibited by the COX-2 selective agents with and without the CA II inhibitory ac-

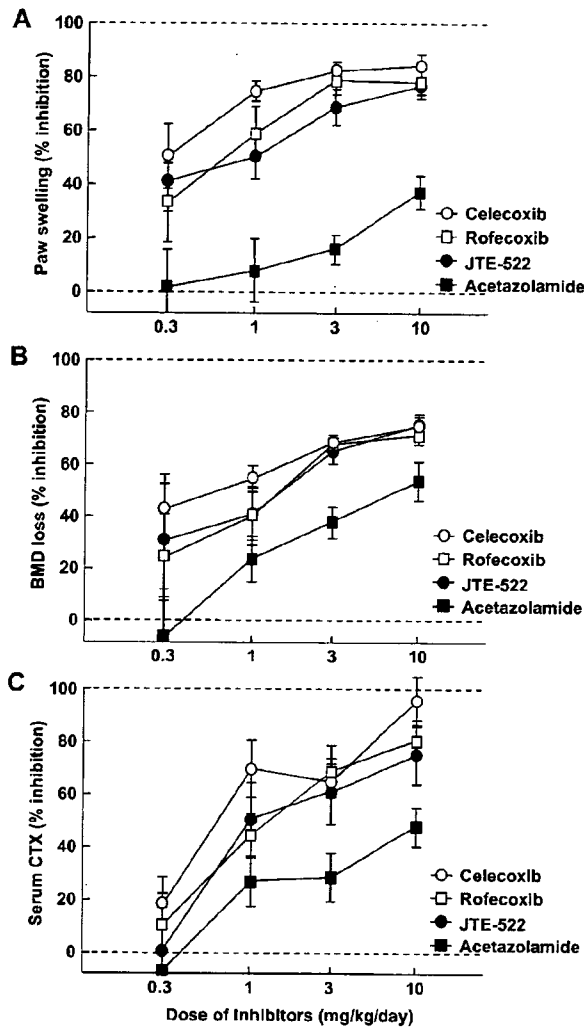


FIG. 4. Inhibitory effects of COX-2 and CA II-selective agents on (A) paw swelling, (B) BMD loss, and (C) a bone resorption marker CTx in AIA rats. AIA was induced in 6-week-old rats with subcutaneous injection of Freund's complete adjuvant containing *Mycobacterium butyricum* into the left hind-paw (day 0), and the indicated dosages (0.3–10 mg/kg/day) of celecoxib, rofecoxib, JTE-522, and acetazolamide were orally administered from day 15 to day 24. The right hind-paw volume was measured by a plethysmometer before killing the animals, and the serum CTx level by ELISA immediately after death on day 25. BMD of the excised right hind-paws was measured by DXA. Effects of the drugs on the parameters were expressed as the percent inhibition: the difference between normal and the drug-treated AIA divided by that between normal and the vehicle-treated AIA. Data are expressed as means (symbols) \pm SE (error bars) for eight animals per group.

tion, and the ED_{50} values of celecoxib, rofecoxib, and JTE-522 were 0.69, 1.40, and 1.50 mg/kg/day, respectively (Fig. 4B). Similar results were obtained when BMC was used as a parameter for bone destruction (data not shown). In addition, serum level of CTx, a specific bone resorption marker, was also decreased by the COX-2 inhibitors: ED_{50} values of the above agents were 0.62, 1.14, and 0.86 mg/kg/day,

respectively (Fig. 4C). Acetazolamide caused moderate inhibitions of paw swelling, BMD loss, and CTx level, although all were weaker than those of the COX-2 selective agents.

Figure 5A shows representative radiographs of hindlimbs and scorings of bone destruction and periostitis of normal and AIA rats with and without application of the COX-2 selective agents and acetazolamide. Bone destruction around the ankle joint and periostitis in the tibia and talus were markedly induced by AIA induction and were significantly reduced by all COX-2 selective agents with or without CA II inhibitory potency. Figure 5B shows the representative histological features of the talo-tibial joints and scorings of synovial thickening and joint destruction of normal and AIA rats with and without application of the agents above. Substantial trabecular bone loss in the tibia and talus, synovial thickening with cell proliferation, and decrease of the joint space were distinguished in the AIA vehicle-treated rats compared with normal rats. Here again, all COX-2 inhibitors significantly suppressed these disorders, and the effects were comparable between those with and without CA II inhibitory potency. Although acetazolamide tended to decrease both radiological and histological scores, the effects were weaker than those of COX-2 selective agents and were not significant as compared with that of the vehicle alone.

DISCUSSION

Based on a recent finding that COX-2 selective agents containing a sulfonamide moiety show an unexpected inhibition of CA II,⁽²⁴⁾ this study initially examined the CA II inhibitory potency of representative COX-2 selective agents and confirmed that the sulfonamide-type agents celecoxib and JTE-522 exhibited CA II inhibition, whereas the methylsulfone-type rofecoxib did not. In vitro assays for the differentiation of osteoclasts and the activity of mature osteoclasts clearly revealed that COX-2 selective agents with CA II inhibitory potency suppressed both differentiation and activity of osteoclasts, whereas those without potency reduced only osteoclast differentiation. Although this implies a superiority of COX-2 selective agents with CA II inhibitory potency over those without it as bone-sparing drugs, the present in vivo studies on AIA rats showed that all COX-2 selective agents similarly suppressed arthritic bone destruction with or without CA II inhibitory potency. Although inhibition of CA II alone by acetazolamide caused a moderate suppression of bone destruction, the effect was weaker than that by COX-2 inhibition. The lines of these results indicate the importance of suppression of osteoclast differentiation by COX-2 inhibition rather than suppression of mature osteoclast activity by CA II inhibition, at least for the treatment of AIA bone destruction.

The predominance of osteoclast differentiation over osteoclast activation in the mechanism of arthritic bone destruction is in accordance with accumulated evidence showing the pivotal role in RA bone destruction of bone resorptive cytokines TNF- α , IL-1, and IL-6, which stimulate osteoclast differentiation through induction of RANKL with little effect on mature osteoclast activity.⁽²⁹⁾ Hence,

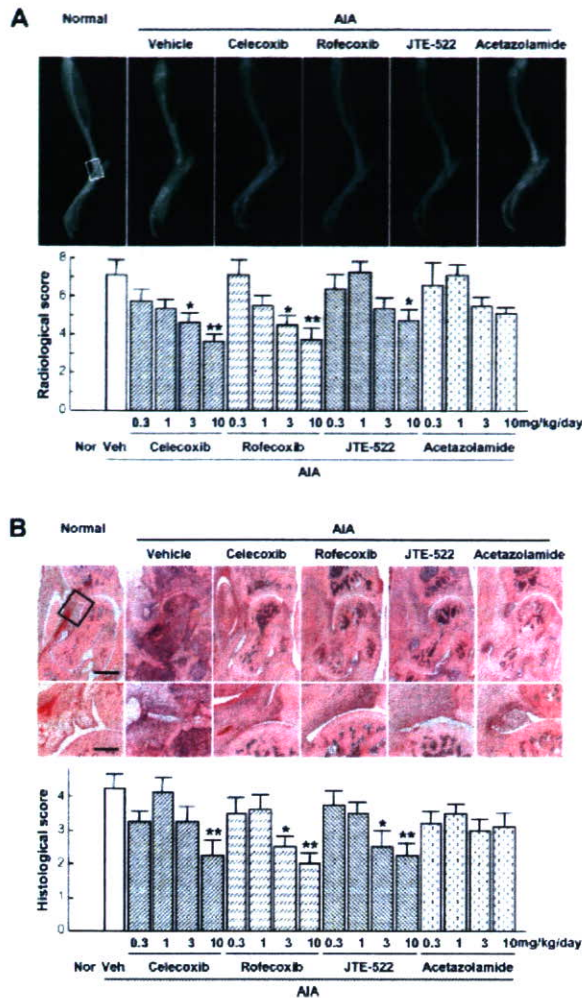


FIG. 5. Inhibitory effects of COX-2 and CA II-selective agents on joint destruction in AIA rats. (A) Representative radiographs of right hindlimbs of normal and AIA rats with and without application of celecoxib, rofecoxib, JTE-522, and acetazolamide. The graph shows the dose response of inhibitory effects of the agents on radiological scoring. A 0–5 grading system (0 = normal, 5 = the most severe) was used to evaluate bone destruction and periostitis, respectively, and the sum of the scores for the parameters was calculated (maximum 10). (B) Representative histological features (H&E staining) with low (upper) and high (lower) magnifications of the talo-tibial joints of normal and AIA rats with and without application of celecoxib, rofecoxib, JTE-522, and acetazolamide. The inset box in A indicates the region of the upper figure in B, and that in the upper figure indicates the region of the lower figure. Bar, 200 and 50 μ m. The graph shows the dose response of inhibitory effects of the agents on histological scoring. A 0–5 subjective grading system (0 = normal, 5 = the most severe) was used to evaluate the overall change including synovial thickening (pannus formation) and joint destruction (maximum 5). Data are expressed as means (bars) \pm SE (error bars) for eight animals per group. * p < 0.05, ** p < 0.01; significant inhibition by the agents.

biological therapies targeting these cytokines are known to be the most effective treatment for RA bone destruction.⁽³⁰⁾ Regarding the cytokines used in the in vitro studies,

FGF-2 stimulates both differentiation and activity of osteoclasts and has been reported to play a role in RA bone destruction in the animal model and clinical settings.^(2–4) On the other hand, Gas6 that stimulates only osteoclast activity is reported to be involved in bone loss by estrogen deficiency but not in RA bone destruction.⁽¹⁷⁾ In addition to the differentiation and activity of osteoclasts, their survival might be another therapeutic target for RA bone destruction. Although these studies showed that none of the COX-2 selective agents or acetazolamide affected the survival of mature osteoclasts generated and isolated from the M-BMM ϕ culture (data not shown), bisphosphonates that inhibit the osteoclast survival are now considered to be a possible candidate to treat this disorder.^(31,32)

In this study, the same range of dosage (0.3–10 mg/kg/day) was applied to AIA rats for all drugs, which were decided according to previous reports on their anti-inflammatory potencies in the same model.^(33–35) This range of dosage was also determined to cover their clinical daily dosages: 4 mg/kg for celecoxib, 0.5 mg/kg for rofecoxib, 2–5 mg/kg for JTE-522, and 5 mg/kg for acetazolamide. Although the dosage of rofecoxib seems about 1/10 of those of other drugs, gastrointestinal absorption of this drug in rats is reported to be about 1/10 that in humans,^(36,37) suggesting that these drugs of the same dosage have similar biological potencies in AIA rats. Indeed, this does not lead to the conclusion that the efficacy of the agents can be compared simply by the ED₅₀ values calculated by the dose-response experiments (Fig. 4); however, the fact that the ED₅₀ value of JTE-522 with the strongest potency of CA II inhibition (Fig. 2) was higher than other COX-2 inhibitors also implies that CA II inhibition is less important than COX-2 inhibition for the suppression of AIA bone destruction.

The representative COX-2 selective agents celecoxib and rofecoxib have been reported to yield similar efficacy comparable with conventional nonsteroidal anti-inflammatory drugs (NSAIDs) with less incidence of gastrointestinal side effects.^(38–40) This study, however, revealed a discrepancy of their biological effects: celecoxib inhibited mature osteoclast activity, whereas rofecoxib did not. This may be because of the sulfonamide moiety that has the potency to inhibit CA II, because JTE-522 and acetazolamide with the moiety also inhibited the mature osteoclast activity, whereas SC-560 without it⁽²⁴⁾ did not. Recent studies also have provided evidence that the use of celecoxib and rofecoxib can lead to several differences in response patterns in animal models as well as in the clinic.⁽⁴⁰⁾ In glaucomatous rabbits, celecoxib, but not rofecoxib, lowered intraocular pressure similarly to acetazolamide, a conventional drug for glaucoma.⁽²⁴⁾ The sulfonamides are known to constitute an important class of drugs possessing not only anticarbonic anhydrase, but also antibacterial, diuretic, hypoglycemic, antithyroid, protease inhibitory, and anticancer activities,⁽⁴¹⁾ suggesting that the sulfonamide-type COX-2 inhibitors such as celecoxib and JTE-522 may have use in the treatment of these disorders. More importantly, a recent social topic is that rofecoxib significantly increased the risk of cardiovascular events in clinical trials, which finally led to the voluntary worldwide withdrawal of this drug, although

it is controversial whether this serious adverse event is common to COX-2 selective agents including celecoxib or, more generally, to NSAIDs.^(42,43)

This study used mouse cell cultures and a rat arthritic model for *in vitro* and *in vivo* analyses, respectively, and suggested the significant effect of COX-2 selective agents not only on inflammation but also on arthritic bone resorption. Accumulated evidence has also shown the bone-sparing effects of COX-2 inhibitors using mouse and rat models *in vitro* and *in vivo*.^(10,34,44-47) Because both COX-2 and CAII show 80-90% homologies in nucleotides and amino acids among mice, rats, and humans,⁽⁴⁸⁻⁵⁰⁾ these results may be applicable to humans. For the clinical application of COX-2 selective agents as a treatment of bone resorptive disorders including RA, however, we should be cautious not only with cardiovascular events above, but also with possible inhibition of bone repair and ingrowth that has recently been reported in animal models when administered for a long period of time.⁽⁵¹⁻⁵³⁾ A large-scale clinical trial will clarify the possibility and limitation of these drugs as a novel treatment of the skeletal disorders.

ACKNOWLEDGMENTS

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Regulation of Bone Formation by Adiponectin Through Autocrine/Paracrine and Endocrine Pathways

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Abstract Since interaction between bone and lipid metabolism has been suggested, this study investigated the regulation of bone metabolism by adiponectin, a representative adipokine, by analyzing deficient and overexpressing transgenic mice. We initially confirmed that adiponectin and its receptors were expressed in osteoblastic and osteoclastic cells, indicating that adiponectin can act on bone not only through an endocrine pathway as a hormone secreted from fat tissue, but also through an autocrine/paracrine pathway. There was no abnormality in bone mass or turnover of adiponectin-deficient (Ad^{-/-}) mice, possibly due to an equivalent balance of the two pathways. In the culture of bone marrow cells from the Ad^{-/-} mice, osteogenesis was decreased compared to the wild-type (WT) cell culture, indicating a positive effect of endogenous adiponectin through the autocrine/paracrine pathway. To examine the endocrine action of adiponectin, we analyzed transgenic mice overexpressing adiponectin in the liver, and found no abnormality in the bone. Addition of recombinant adiponectin in cultured osteoprogenitor cells suppressed osteogenesis, suggesting that the direct action of circulating adiponectin was negative for bone formation. In the presence of insulin, however, this suppression was blunted, and adiponectin enhanced the insulin-induced phosphorylations of the main downstream molecule insulin receptor substrate-1 and Akt. These lines of results suggest three distinct adiponectin actions on bone formation: a positive action through the autocrine/paracrine pathway by locally produced adiponectin, a negative action through the direct pathway by circulating adiponectin, and a positive action through the indirect pathway by circulating adiponectin via enhancement of the insulin signaling. *J. Cell. Biochem.* 99: 196–208, 2006. © 2006 Wiley-Liss, Inc.

Key words: adiponectin; adipokine; fat; osteoblast; bone

Adiponectin, also called Acrp30, apM1, and adipoQ, is a recently discovered adipokine that is synthesized and secreted mainly by fat tissue [Scherer et al., 1995; Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996]. It is a 244-

amino acid protein structurally similar to tumor necrosis factor- α (TNF- α) with an N-terminal collagenous repeat and a C-terminal globular domain [Hu et al., 1996]. Although it is abundant in plasma, the level is reduced in association with obesity and obesity-linked diseases including type 2 diabetes, unlike most other adipokines including leptin, resistin, TNF- α , and interleukin-6 (IL-6) [Arita et al., 1999; Hotta et al., 2000; Weyer et al., 2001; Matsubara et al., 2002; Ukkola and Santaniemi, 2002]. Accumulated evidence has shown that adiponectin plays important roles in the regulation of insulin sensitivity, energy homeostasis, atherogenic changes of vessels, and inflammatory responses [Berg et al., 2001; Combs et al.,

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2001; Fruebis et al., 2001; Diez and Iglesias, 2003], indicating that adiponectin possesses potent functions in various tissues. Two adiponectin receptors, AdipoR1 and AdipoR2, were recently identified: the former is predominantly expressed in muscle whereas the latter in the liver [Yamauchi et al., 2003a]. Expression of both receptors has also been reported at high levels in human and rat pancreatic β cells, and their presence is suggested to be one mechanism for modulating the effects of circulating adiponectin [Kharroubi et al., 2003].

The low incidence of osteoporosis in obese people [Felson et al., 1993; Tremollieres et al., 1993], suggested a hypothesis whereby bone and adipose tissues would be controlled by the same hormone(s). Testing this hypothesis revealed that leptin, another representative adipokine regulating the fat mass, is a powerful inhibitor of bone formation by way of a sympathetic nerve system [Ducy et al., 2000; Takeda et al., 2002]. The adiponectin signal is also suggested to be involved in bone homeostasis since expressions of adiponectin, AdipoR1, and AdipoR2 were detected in human primary osteoblasts [Berner et al., 2004] and exogenous adiponectin has been reported to regulate osteoblast functions [Luo et al., 2005; Oshima et al., 2005]. A clinical study also showed that serum adiponectin was inversely associated with bone density [Lenchik et al., 2003]. Despite accumulation of evidence for adiponectin being a possible signal linking fat mass to bone mass, its physiological function on bone metabolism remains unclarified. Hence, the present study investigated the effects of gain and loss of functions of adiponectin on bone metabolism by analyzing adiponectin deficient and over-expressing transgenic mice.

MATERIALS AND METHODS

Animals

The adiponectin-deficient (Ad $-/-$) mice were generated and maintained as reported previously [Kubota et al., 2002]. In each analysis of Ad $-/-$ mice, homozygous wild-type (WT) and Ad $-/-$ male mice that were littermates generated from the intercross between heterozygous mice were compared. The transgenic (Ad-Tg) mice that overexpress the mouse globular adiponectin driven by the human serum amyloid P component (SAP) promoter, so that the adiponectin expression is limited to liver, were

generated as described previously [Yamauchi et al., 2003b]. In each analysis of Ad-Tg mice, male Ad-Tg and WT littermates that were generated from the intercross between heterozygous mice were compared. All mice were kept in plastic cages under standard laboratory conditions with a 12-h dark, 12-h light cycle, a constant temperature of 23°C, and humidity of 48%. The mice were fed a standard rodent diet (CE-2; CLEA Japan, Inc.) containing 25.2% protein, 4.6% fat, 4.4% fiber, 6.5% ash, 3.44 kcal/g, 2.5 IU vitamin D₃/g, 1.09% calcium, and 0.93% phosphorus with water ad libitum. All experiments were performed on male mice at 8 weeks of age and were reviewed and approved by the Medical Animal Care and Use Committee of the University of Tokyo.

Expression Patterns of Adiponectin and its Receptors

Bone marrow cells were collected from long bones of 8-week-old WT mice. For isolation of osteoblasts, calvariae of neonatal WT and Ad $-/-$ mice were digested for 10 min at 37°C in an enzyme solution containing 0.1% collagenase and 0.2% dispase five times, and cells isolated by the last four digestions were combined. For cells of osteoclastic lineage, we used the culture of macrophage colony-stimulating factor (M-CSF)-dependent bone marrow macrophages (M-BMM Φ) as reported previously [Kobayashi et al., 2000]. Briefly, bone marrow cells from WT and Ad $-/-$ mice were seeded at a density of 3×10^5 cells/well in a 24-multi-well plate and cultured in α MEM (Invitrogen, Carlsbad, CA) containing 10% FBS (HyClone Laboratories, Inc., Logan, UT), with macrophage colony-stimulating factor (M-CSF; 100 ng/ml). After culturing for 3 days, adherent cells were isolated as M-BMM Φ and used as osteoclast precursors. For mature osteoclasts, M-BMM Φ were further cultured with M-CSF (100 ng/ml) and soluble receptor activator of nuclear factor κ B ligand (RANKL; 100 ng/ml) for 3 days, and multi-nucleated cells were isolated. In addition to these primary cells, mouse bone marrow-derived stromal cell line ST2 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in α MEM/10% FBS to subconfluency, and harvested. To investigate the expression of adiponectin and its receptors AdipoR1 and AdipoR2 in the bone cells above, RT-PCR was performed within an exponential phase of the amplification. An aliquot (1 μ g) of total RNA extracted

using an ISOGEN kit (Wako Pure Chemical Industries, Ltd.) was reverse transcribed using Super Script reverse transcriptase (Takara Shuzo Co., Ltd., Shiga, Japan) with random hexamer (Takara Shuzo), and 5% of the reaction mixture was amplified with LA-Taq DNA polymerase (Takara Shuzo) using specific primer pairs: 5'-TGTTGCTGGGAGCTGTTCTACTG-3' and 5'-ATGTCTCCCTTAGGACCAATAAG-3' for adiponectin, 5'-GAAGACAGTGGGTA-CATGCGAATG-3' and 5'-CCTCATGGAGGAA-GGCACTGCTG-3' for AdipoR1, 5'-GCACCG-CCGAGATGGACTGCTGAA-3' and 5'-GG-C-GGAAAGAGGATGGAGGTGACG-3' for AdipoR2, and 5'-CATGTAGGCCATGAGGTCCAC-CAC-3' and 5'-TGAAGGTCGGTGTGAACGGA-TTTGGC-3' for G3PDH. Up to 25 cycles of amplification were performed with a Perkin Elmer PCR Thermal Cycler (PE-2400; Perkin-Elmer Corp., Norwalk, CT) at 94°C for 30 sec, at 52–60°C for 60 sec, and at 72°C for 90 sec. As negative controls to exclude genomic amplification, total RNA without reverse transcription was directly used for PCR.

Analysis of Skeletal Morphology

A bone radiograph of whole bodies, femurs, and tibiae was taken with a soft X-ray apparatus (SOFTEX, CMB-2, Tokyo, Japan). Bone mineral density (BMD; mg/cm²) of the right femurs, tibiae, and L2-L5 vertebral bodies were determined using dual-energy X-ray absorptiometry (DEXA) (PIXImusTM Mouse Densitometer; Lunar Corp., Madison, WI), according to the manufacturer's instructions. All histological analyses were carried out using left tibiae of 8-week-old mice. For the assessment of dynamic histomorphometric indices, mice were injected subcutaneously with 8 mg/kg BW of calcein at 10 days and 3 days before sacrifice. After the sacrifice, the left tibiae were excised, fixed with ethanol, and the undecalcified bones were embedded in glycolmethacrylate. Three micrometers sagittal sections from the proximal parts of the tibiae were stained with Villanueva-Goldner and were visualized under fluorescent light microscopy for calcein labeling. The specimens were subjected to histomorphometric analyses using a semiautomated system (Osteoplan II; Carl Zeiss). Parameters for the trabecular bone were measured in an area 1.2 mm in length from 250 μm below the growth plate at the proximal metaphysis of the tibiae. Nomenclature, symbols, and units are

those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research [Parfitt et al., 1987].

Bone Marrow Cell Cultures

Bone marrow cells were collected from long bones of 8-week-old WT and Ad-/- male littermates. Cells were plated at a density of 10⁶ cells on a six-multi-well plate in αMEM containing 10% FBS, with 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate for osteogenesis assay, and with 1 μM troglitazone for the adipogenesis assay. For the alkaline phosphatase (ALP) staining, cultured plates were rinsed with PBS, fixed in 100% ethanol at 10 days of culture, and stained with Tris-HCl-buffered solution (pH 9.0) containing naphthol AS-MX phosphate as a substrate and Fast Blue BB salt (Sigma-Aldrich, St. Louis, MS) as a coupler. For the Alizarin red staining, cultured plates were rinsed with PBS at 21 days of culture, fixed in 10% buffered formalin, and stained with 2% Alizarin red S (pH 4.0) (Sigma-Aldrich). For the analysis of adipogenesis, the medium was supplemented with 1 μM troglitazone (Sankyo Pharmaceutical Co., Tokyo) for 10 days, fixed in 10 mM sodium periodate, 2% paraformaldehyde, 75 mM L-lysine dihydrochloride, and 37.5 mM sodium phosphate, and then stained in a filtered solution of 0.3% oil red O in 60% isopropanol for 15 min. The red-stained, lipid vacuole-containing cells in a well were counted.

Osteoclast Formation Assay

Tartrate resistant acid phosphatase (TRAP)-positive multi-nucleated osteoclasts were generated by coculturing bone marrow cells (5 × 10⁵ cells/well) and calvarial osteoblasts (1 × 10⁴ cells/well), derived from either WT or Ad-/- littermates, in a 24-multi-well plate for 6 days in αMEM containing 10% FBS and 1,25(OH)₂D₃ (10 nM). The staining was performed at pH 5.0 in the presence of L(+)-tartaric acid using naphthol AS-MX phosphate (Sigma-Aldrich) in N, N-dimethyl formamide as the substrate. Cells positively stained for TRAP containing more than three nuclei were counted as osteoclasts.

Effects of Recombinant Adiponectin on Bone Marrow Cells and ST2 Cells

Recombinant mouse full-length adiponectin expressed in *Escherichia coli* was isolated and

purified as previously described [Yamauchi et al., 2003a]. Bone marrow cells collected from long bones of WT mice were plated at a density of 10^6 cells/well in a six-multi-well plate, cultured with 0, 3, and 10 $\mu\text{g}/\text{ml}$ of recombinant adiponectin in $\alpha\text{MEM}/10\%$ FBS/ascorbic acid/ β -glycerophosphate in the presence and absence of insulin (10 nM), and stained with ALP and Alizarin red after 10 and 21 days, respectively, as described above. ST2 cells were inoculated at a density of 1×10^4 cells/well in a 24-multi-well plate, cultured with 0, 1, 3 and 10 $\mu\text{g}/\text{ml}$ of recombinant adiponectin in $\alpha\text{MEM}/10\%$ FBS/ascorbic acid with insulin-like growth factor-I (IGF-I, 100 nM) or bone morphogenetic protein-2 (BMP-2, 10 nM). At 7 days of culture, cells were sonicated in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 and 0.5% Triton X-100. ALP activity in the lysate was measured using a Wako ALP kit (Wako Pure Chemical) and the protein content was determined using BCA protein assay reagent (Pierce Biotechnology, Rockford, IL).

Immunoprecipitation and Immunoblotting

For immunoprecipitation and immunoblotting, bone marrow cells collected from long bones of WT mice were plated at a density of 2×10^6 cells/well in a 6 cm dish and cultured for 10 days as described above. After 6 h of serum starvation, cells were cultured with or without 10 $\mu\text{g}/\text{ml}$ of recombinant adiponectin for 24 h. Cells were then stimulated with insulin (100 nM) or the vehicle for 10 min, and lysed with TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4 , 1 mM aminoethyl-benzenesulfonyl fluoride, and aprotinin). A part of the cell lysates

(100 μg) were immunoprecipitated with an anti-insulin receptor substrate (IRS)-1 antibody (Upstate, Waltham, MA) conjugated to protein G-Sepharose (Invitrogen) overnight at 4°C. The cell lysates with or without the immunoprecipitation that contained an equivalent amount of protein (20 μg) were electrophoresed by 8% SDS-PAGE, and transferred to PVDF membrane. After blocking with 5% BSA solution, they were incubated with either anti-phosphotyrosine (clone 4G10), anti-mouse phospho-Akt (Ser 472), anti-mouse Akt, or anti-mouse IRS-1 antibody (all from Upstate). Immunoreactive bands were stained using the ECL chemiluminescence reaction (Amersham Co., Arlington Heights, IL).

Statistical Analysis

Means of groups were compared by ANOVA and significance of differences was determined by post hoc testing using Bonferroni's method.

RESULTS

Expressions of Adiponectin and its Receptors in Bone Cells

Because adiponectin and its two receptors AdipoR1 and AdipoR2 were reported to be differentially expressed in a variety of cells and tissues [Yamauchi et al., 2003a; Berner et al., 2004], we first investigated their expressions in cells of osteoblastic and osteoclastic lineages by RT-PCR (Fig. 1). All mRNA expressions were detected in bone marrow cells, calvarial osteoblasts, osteoclast precursor M-BMM Φ , and isolated mature osteoclasts that were derived from WT mice, suggesting that they are ubiquitously expressed in various

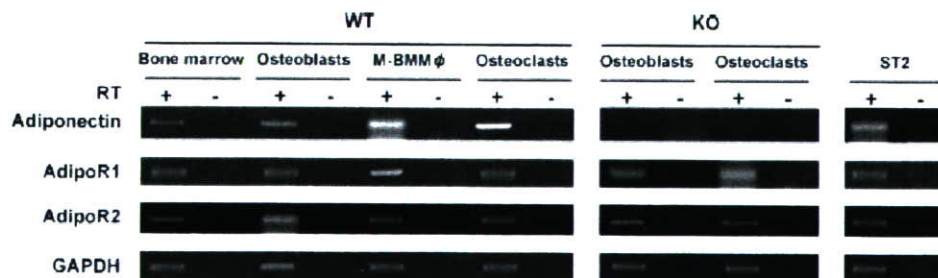


Fig. 1. Expressions of adiponectin and its receptors AdipoR1 and AdipoR2 in cells of osteoblastic and osteoclastic lineages derived from WT and Ad $^{-/-}$ mice by RT-PCR. Total RNA was extracted from bone marrow cells collected from adult mouse long bones, neonatal mouse calvarial osteoblasts, osteoclast precursor M-CSF-dependent bone marrow macrophages (M-

BMM Φ), mature osteoclasts formed and isolated from the culture of M-BMM Φ with M-CSF and RANKL, and mouse bone marrow-derived stromal cell line ST2 cells. An aliquot of total RNA with (+) or without (-) reverse transcription (RT) was amplified using specific primers within an exponential phase of the amplification.