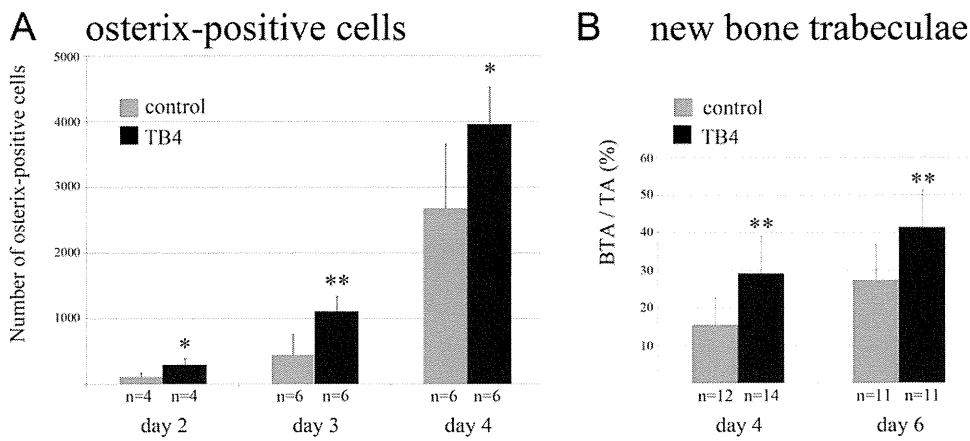

Fig. 5. Number of osterix-positive cells (**A**) and percentage of newly formed bone trabeculae area (TBA) relative to the tooth extraction socket area (TA) (**B**). Data are expressed as mean and SD. Significant differences between control and TB4-treated groups are indicated by asterisks (*$P < .05$; **$P < .01$).

chemical staining was performed using anti-Sp7/osterix rabbit polyclonal antibody (ab22552, 1:200; Abcam, Cambridge, U.K.) and antiperiostin rabbit polyclonal antibody (ab14041, 1:150; Abcam) as the first antibodies. Histofine Simple Stain Rat Max-PO (Multi) (Nichirei Biosciences, Tokyo, Japan) was used as the second antibody. Detection was performed with the Immpact DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Nuclear counterstaining was carried out with the use of either methyl green or hematoxylin.

The digital images of the histologic sections were analyzed by Adobe Photoshop CS4 Extended (Adobe Systems, San Jose, CA) and ImageJ, version 1.44a (National Institutes of Health, Bethesda, MD) software. To simplify the calculation of the area, the tooth extraction socket area (TA) was defined as the area bounded by a curve along the medial border of the alveolar bone and a straight line (upper limit of TA) connecting the 2 ends of the curve. The granulation tissue area (GA) was distinguished from the blood clot area (BA) and periodontal ligament area (PA). BA was defined as the area stained deep blue by PTAH. PA was confirmed by immunohistochemical staining for periostin on the serial sections. The percentage of GA relative to TA was calculated for each PTAH-stained section. When we assessed granulation tissue formation, we did not exclude areas of newly formed bone trabeculae or osteoid tissue from GA. For the assessment of new bone and osteoid formation in the tooth extraction socket, the percentage of newly formed bone trabecular area (BTA) relative to TA was calculated from the digital images of the H-E sections. The BTA, including osteoid, was confirmed by immunostaining for osterix on serial sections. For further assessment of new bone and osteoid formation, all cells positively immunostaining for osterix were counted on the digital images.

Apoptosis assay based on terminal deoxynucleotide transferase–mediated dUTP nick-end labeling method

Serial unstained sections from the paraffin-embedded tissues were used for apoptosis assay with the ApoMark Apoptosis Detection Kit (Exalpha Biologicals, Maynard, MA) according to the manufacturer's protocol. Apoptotic cells were counted on the digital images.

Preparation of total RNA and reverse-transcription polymerase chain reaction

Immediately after the rats were killed, at either 9 hours or 3 days after tooth extraction, granulation tissue was isolated from the socket with a 27-gauge needle, and then total RNAs were prepared from this with the use of the SV total RNA isolation system (Promega, Madison, WI).

Reverse-transcription polymerase chain reaction (RT-PCR) was performed using the Titanium One-Step RT-PCR kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The primers used in our study are summarized in Table I. The RT-PCR products after 25-35 PCR cycles were electrophoresed on 1%-2% agarose gel, visualized with ethidium bromide staining under ultraviolet light exposure, photographed using a digital camera (PowerShot A640; Canon, Tokyo, Japan), and stored as digital images. The integrated density (IntDen) of each band was calculated with the use of ImageJ. The relative mRNA levels of target genes were expressed as a percentage of

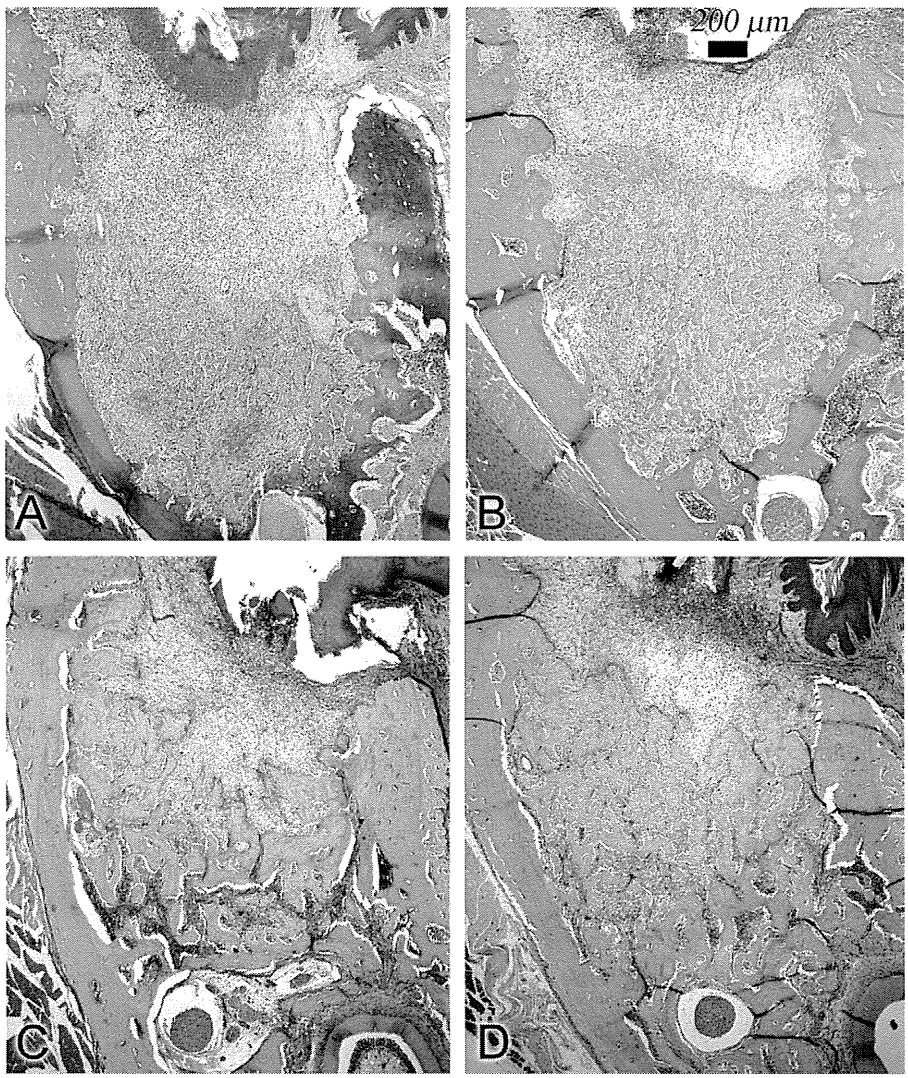

Fig. 6. Representative hematoxylin-eosin sections from days 4 (**A** and **B**) and 6 (**C** and **D**) in the control (**A** and **C**) and TB4-treated (**B** and **D**) groups. A greater amount of newly formed bone trabeculae is noted in the TB4-treated group on day 4 (B) than in the control group (**A**). On day 6, the socket in the TB4-treated group (**D**) still shows a greater amount of bone trabeculae than in the control group (**C**).

the ratio of target gene IntDen relative to glyceraldehyde-3-phosphate dehydrogenase IntDen. Analysis was performed 4 times using different batches of total RNA samples. The results are shown as mean values with standard deviations (n = 4).

Data analysis

The statistical significance of differences in the mean values between the 2 groups was assessed by Student *t* test if variances were equal as determined by an *F* test, otherwise by Welch *t* test. All tests were 2 tailed, with differences reported as significant when $P < .05$.

RESULTS

Comparative histologic findings in the healing process after tooth extraction

The extraction sockets on day 1 in the control group were filled mostly with blood clots, although remaining periodontal tissue and only a very small amount of granulation tissue were observed at the bottom. In contrast, there was a slightly greater amount of granulation tissue in the bottom of sockets in the TB4-treated group. In addition, the TB4-treated group had fewer apoptotic cells than the control group (Figs. 2 and 3). On day 2, the sockets in the control group still contained only a small amount of granulation tissue at the

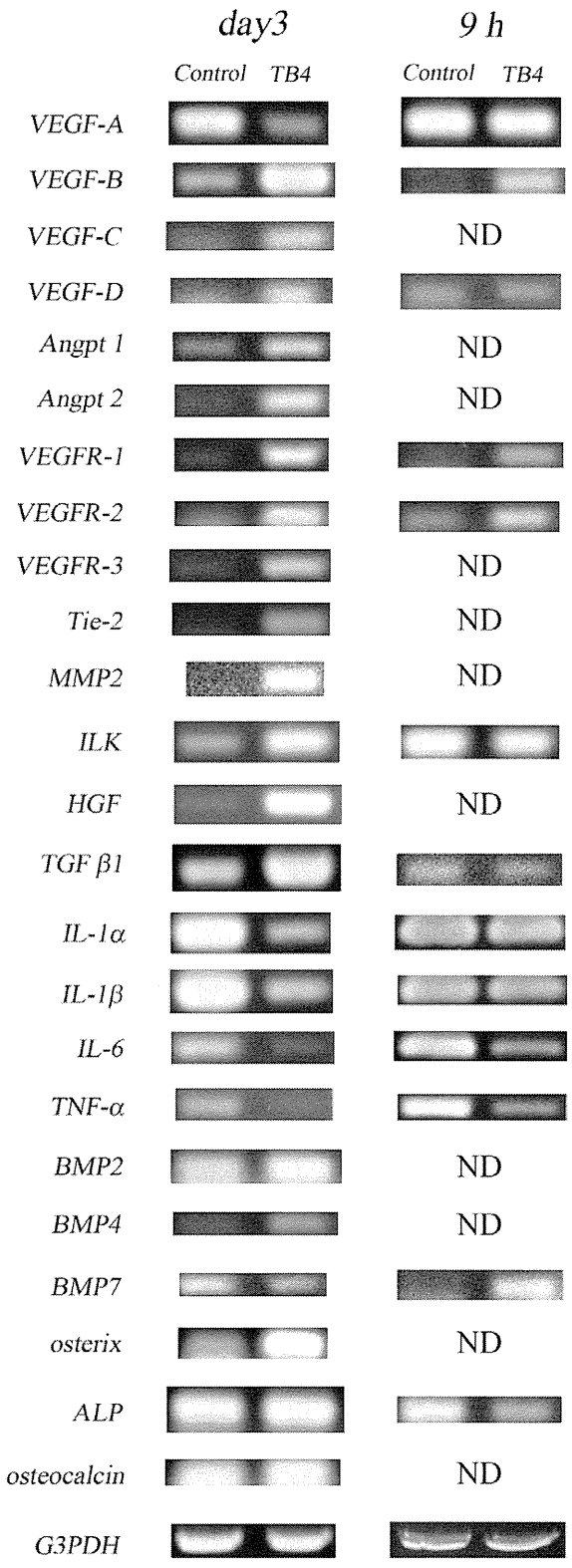

Fig. 7. Representative data from reverse-transcription polymerase chain reaction using total RNAs from granulation tissue in the tooth sockets after 9 hours and 3 days. *ND*, Not detected by amplification of up to 35 cycles. For abbreviations of target genes, see Table I.

bottom, while a greater amount of granulation tissue was present in the TB4-treated group, with or without a small amount of bone matrix–like tissue. Although the granulation tissue grew rapidly in both groups from days 3 to 4, the TB4-treated group always showed a greater amount of granulation tissue than the control group (Figs. 3 and 4). Furthermore, a greater amount of osteoid tissue and new bone trabeculae appeared in the TB4-treated group on days 3 and 4 than in the control group (Figs. 4-6). Immunohistochemical staining with osterix revealed more osterix-positive granulation tissue in the TB4-treated animals than in control animals from days 2 to 4 (Figs. 4 and 5). The TB4-treated group had a greater amount of bone trabeculae on day 6 than the control group (Figs. 5 and 6).

Differential expression of mRNA between thymosin β4–treated and control groups

Compared with the control group, the TB4-treated group showed increased expression of the following genes associated with angiogenesis, cell proliferation, and migration on day 3: vascular endothelial growth factor B (*Vegf-B*), *Vegf-C*, *Vegf-D*, *angiopoietin-1 (Angpt1)*, *Angpt2*, *VEGF receptor 1 (Vegfr-1)*, *Vegfr-2*, *Vegfr-3*, *tyrosine kinase with Ig-like loops and epidermal growth factor homology domains 2 (Tie2)*, *matrix metalloproteinase 2 (Mmp2)*, *integrin-linked kinase (Ilk)*, *hepatocyte growth factor (Hgf)*, and *transforming growth factor β1 (Tgf-β1)* (Fig. 7; Table II). However, the expression level of *Vegf-A* in the TB4-treated group was lower than in the control group. The expression levels of representative genes related to inflammation, such as *interleukin-1α (Il-1α)*, *Il-1β*, *Il-6*, and *tumor necrosis factor α (Tnf-α)*, were significantly decreased in the TB4-treated group than in the control group. With regard to genes related to osteogenesis, significantly higher expression levels of *osterix* and *bone morphogenetic protein 4* (*Bmp-4*) were observed on day 3 in the TB4-treated group than in the control group.

There were significant differences between the 2 groups regarding gene expression levels at 9 hours after tooth extraction (Fig. 7; Table II). In terms of angiogenesis, only 3 genes, *Vegf-B*, *Vegfr-1*, and *Vegfr-2*, were significantly expressed more in the TB4-treated group than in the control group. Regarding inflammatory cytokines, *Il-6* and *Tnf-α* were significantly decreased in the TB4-treated group than in the control group. Most osteogenesis-related genes were not detected by amplification of up to 35 cycles. Interestingly, *Bmp-7* was significantly expressed more in the TB4-treated group 9 hours after extraction than in the control group, although no significant difference for samples on day 3 was noted between the 2 groups.

Table II. Summary of reverse-transcription polymerase chain reaction (RT-PCR) results

	Day 3 RNAs, ratio to G3PDH (SD)			*9-h RNAs, ratio to G3PDH (SD)*		
Target gene	*Control (n = 4)*		*TB4 (n = 4)*	*Control (n = 4)*		*TB4 (n = 4)*
VEGF-A	92.4 (20.2)	>**	43.7 (8.8)	89.0 (20.2)	NS	87.4 (14.3)
VEGF-B	52.4 (8.0)	<***	150.1 (12.7)	16.5 (4.1)	<**	54.0 (12.6)
VEGF-C	9.6 (2.2)	<*	40.2 (15.9)	ND	—	ND
VEGF-D	13.4 (3.6)	<*	41.6 (16.6)	25.7 (4.3)	NS	28.6 (4.1)
Angpt1	17.8 (6.5)	<*	41.3 (13.4)	ND	—	ND
Angpt2	13.9 (3.2)	<*	48.9 (19.4)	ND	—	ND
VEGFR-1	14.3 (3.3)	<*	58.3 (23.1)	18.1 (1.0)	<*	50.5 (15.6)
VEGFR-2	20.6 (3.6)	<**	93.1 (24.4)	24.2 (7.7)	<*	53.8 (17.5)
VEGFR-3	17.9 (3.5)	<***	66.5 (10.6)	ND	—	ND
Tie2	13.5 (5.8)	<*	75.3 (24.9)	ND	—	ND
MMP2	88.7 (46.1)	<**	287.2 (68.9)	ND	—	ND
ILK	73.5 (20.5)	<**	187.3 (36.6)	66.1 (14.3)	NS	64.3 (11.6)
HGF	29.7 (4.3)	<***	189.2 (4.7)	ND	—	ND
TGF-β1	106.0 (49.4)	<**	331.4 (80.3)	23.9 (5.4)	NS	27.4 (7.2)
IL-1α	294.6 (22.7)	>***	115.7 (6.9)	282.5 (9.2)	NS	268.4 (14.4)
IL-1β	302.7 (16.3)	>***	152.6 (16.7)	207.2 (12.4)	NS	190.2 (9.9)
IL-6	87.5 (24.5)	>*	28.6 (5.6)	182.6 (22.0)	>**	111.5 (18.3)
TNF-α	74.5 (22.1)	>*	29.7 (5.3)	118.8 (18.9)	>**	65.1 (10.3)
BMP-2	25.4 (3.8)	NS	35.5 (7.2)	ND	—	ND
BMP-4	18.7 (4.2)	<*	56.5 (14.3)	ND	—	ND
BMP-7	26.4 (8.9)	NS	25.3 (13.2)	18.0 (4.2)	<*	49.2 (15.9)
Osterix	30.9 (3.9)	<**	53.6 (8.7)	ND	—	ND
ALP	58.1 (12.3)	NS	67.3 (13.4)	41.1 (5.2)	NS	39.4 (4.3)
Osteocalcin	41.4 (8.4)	NS	45.8 (11.1)	ND	—	ND

Ratios of target gene to G3PDH expressed as mean percentage values.
NS, No significant difference; *ND*, not detected by RT-PCR (up to 35 cycles). For abbreviations of target genes, see Table I.
Significant differences between control and TB4-treated groups are indicated by asterisks: $*P < .05$; $**P < .01$; $***P < .001$.

DISCUSSION

The full-length TB4 is composed of 43 AA residues. Its functional sequence motif and certain biologic activities of TB4-derived partial peptides have been reported[19]; the main functions are shown in Fig. 1. In this study, we used a 20-AA synthetic peptide that included the 9-AA G-actin–binding motif (LKKTETQEK)[5-8] and 7-AA essential sites for angiogenic activity (LKKTETQ),[25] because it is not easy to synthesize a full-length TB4 polypeptide owing to the distribution of proline throughout the TB4 sequence. Our 20-AA synthetic peptide included 2 glutamines corresponding to the sites where TB4 is cross-linked to fibrin by factor XIIIa, a transglutaminase.[26,27] We anticipated that these 2 glutamine residues might increase the local concentration of the TB4 partial peptide near the site of the blood clot in the tooth extraction socket after intraperitoneal injection of the peptide.

Histologic analysis showed that TB4 treatment promoted granulation tissue formation in the tooth socket after extraction (Figs. 2-4). This finding supports the previously reported promoting effects of TB4 on angiogenesis, cell proliferation, and migration,[9-16,18] because the characteristic histologic feature of granulation tissue is the presence of new small blood vessels (angiogenesis); cell proliferation and migration are also essential for granulation tissue formation. Meanwhile, gene expression analysis revealed increased expression of the following genes associated with angiogenesis, cell proliferation, and migration on day 3 in the TB4-treated group: *Vegf-B*, *-C*, and *-D*, *Angpt1* and *2*, *Vegfr1*, *-2*, and *-3*, *Tie2*, *Mmp2*, *Ilk*, *Hgf*, and *Tgf-β1* (Fig. 7; Table II). This finding from gene expression analysis corroborates the promoting effect of TB4 on granulation tissue formation shown by histologic analysis (Figs. 3 and 4). Among those genes listed above, TB4 has been reported to up-regulate the expression levels of mRNA and/or proteins of *Mmp2*, *Angpt 2*, *Hgf*, *Ilk*, and *Tgf-β1* in various in vitro and/or in vivo experiments.[10,28-33] Regarding all other genes under study (*Vegf-B*, *-C*, and *-D*, *Angpt1*, *Vegfr1*, *-2*, and *-3*, and *Tie2*), which are angiogenic factors and their receptors, for the first time the up-regulation of these genes by TB4 is suggested from the findings of our in vivo experiment, although it is unclear whether TB4 directly up-regulated the expression levels of the genes in the granulation tissue on day 3.

On the other hand, only 3 genes from those described above (*Vegf-B* and *Vegfr-1* and *-2*) were up-regulated by TB4 treatment in the extraction socket at 9 hours

after tooth extraction (Fig. 7; Table II). Because even the extraction socket at 9 hours in the TB4-treated group should contain only remaining periodontal tissue and, if at all, a very small amount of granulation tissue, many genes may not be up-regulated by TB4 treatment. Nevertheless, up-regulation of these 3 genes may be convincing evidence for the augmentation of angiogenesis by TB4 administration during the early stage of granulation tissue formation. Recent studies have revealed that VEGF-B acts as a survival factor for many types of cells, such as vascular endothelial cells, pericytes, smooth muscle cells, neurons, and cardiac myocytes, by inhibition of apoptosis via both VEGFR-1 and neuropilin-1.[34-37] TB4-induced expression of VEGF-B not only may have contributed to angiogenesis but also may have decreased the number of apoptotic cells in the TB4-treated group on day 1 (Figs. 2 and 3).

The full-length TB4 polypeptide has been shown to be effective in reducing inflammation.[13,15] It is also reported that only the 4-AA, amino-terminal peptide of TB4, known as Ac-SDKP, can block inflammation.[38] Other than that, however, there has been no information reported on the active sites of TB4 regarding antiinflammatory activity.[19] We have showed in the present study that the 20-AA TB4 partial peptide suppressed the expression levels of the representative inflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α mRNA (Fig. 7; Table II). This finding suggests that AA residues 17-36 may include other functional sites for antiinflammatory activity. In addition, the down-regulation of *Tnf*-α mRNA 9 hours after extraction when treated by the peptide may have suppressed TNF-α–induced apoptosis and resulted in a decreased number of apoptotic cells in the experimental group (Figs. 2 and 3).

TB4 promotes wound healing in various tissues, such as skin, cornea, and heart.[12-16,18] There has been, however, no report to date on the effects of TB4 regarding bone regeneration or osteogenesis. Our results show that a greater quantity of osterix-positive cells, osteoid, and bone trabeculae appeared in the TB4-treated group than in the control group (Figs. 4-6). mRNA expression of the osteogenesis-promoting factors BMP-4 and TGF-β1 was significantly higher in the TB4-treated group on day 3 than in the control group, although there were no significant differences between the 2 groups regarding the expression levels of BMP-2 and BMP-7 mRNA on day 3 (Fig. 7; Table II). Regarding the osteogenic differentiation markers alkaline phosphatase (ALP) and osteocalcin mRNA, there were no significant differences between the 2 groups. Although regarding the comparison of expression levels of osteogenic differentiation markers, it would be reasonable to compare those between days 4 and 6, it was hard to obtain sufficient RNA, especially from the TB4-treated group, where newly formed bone trabeculae occupied the socket from day 4 onward.

Expression of osteogenesis-related genes, apart from *Bmp7*, at 9 hours showed no significant differences between the 2 groups and was undetectable after 35 PCR cycles (Fig. 7; Table II). Although the up-regulation of *Bmp7* mRNA after 9 hours in the TB4-treated group is an interesting finding, it is hard to speculate what role BMP-7 plays in the early healing process after tooth extraction. From our in vivo experiments, it remains unclear whether TB4 affects osteogenic progenitor cells directly or indirectly during osteogenesis. In any case, our findings raise the possibility that TB4 accelerates bone regeneration and osteogenesis during the healing of bone and certain bone-associated tissues.

Taken together, we have shown that the 20-AA partial peptide (AA residues 17-36) has effects on angiogenesis, cell migration, granulation tissue formation, inflammation, and apoptosis similar to the full-length 43-AA TB4 polypeptide. Because the shorter peptide is easier and less expensive to synthesize, the 20-AA partial peptide of TB4 has an advantage regarding cost. Furthermore, our findings implicate a potential therapeutic value for TB4 in treating problems of bone and bone-associated tissues.

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Physical fitness and 6.5-year mortality in an 85-year-old community-dwelling population

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Participation

ABSTRACT

Although poor physical fitness is known to be associated with increased mortality in adult and elderly populations, this association is not conclusive in very elderly. The purpose of the present study was to evaluate the association for a very old community-dwelling population. The participants (90 males, 117 females) were 85-year-old individuals residing in Fukuoka, Japan. Baseline examinations including muscle strength of the handgrip and leg extension, one-leg standing, leg stepping rate, and walking were performed in 2003 and these subjects were followed for 6.5 years. During the follow-up period, 81 individuals (49 males and 32 females) died. Handgrip strength and leg extension strength at age 85 were stronger in surviving men than in non-survivors. Total mortality adjusted for both gender and serum level of total cholesterol fell 5–6% with a 1-kg increase in the handgrip strength of a single hand or both hands. Total mortality also decreased 2% with a 1 kg increase in the leg extension strength of both legs. With adjustment for gender and total cholesterol, mortality fell by 57% in participants of the walking test and fell by 45% in participants of the stepping-rate test compared to mortality in nonparticipants. No association was found between mortality and participation in the handgrip strength test, leg extension strength test, or one-leg standing time test. In conclusion, not only poor muscle strength in handgrip or leg extension, but also nonparticipation in walking test or leg-stepping test were independent predictors of total mortality in a very elderly population.

1. Introduction

An association between mortality and physical activity or physical fitness is known in patients with diseases such as diabetes (Kokkinos et al., 2009c; Lyerly et al., 2009; McAuley et al., 2009a), hypertension (Kokkinos et al., 2009a,b; McAuley et al., 2009b), and cardiovascular diseases (Al-Khalili et al., 2007; Carlisle and Swart, 2007; Holtermann et al., 2010; Mandic et al., 2010) and in community-dwelling adults (Rantanen, 2003; Miller et al., 2005; Kokkinos et al., 2008; Park et al., 2009) and old (Mitnitski et al., 2005; Spencer et al., 2005; Newman et al., 2006b; Sui et al., 2007; Cesari et al., 2009b; Ling et al., 2010) persons. Strength of the handgrip (Newman et al., 2006a; Gale et al., 2007; Ling et al., 2010) and lower extremities (Newman et al., 2006a) was a predictor of mortality in elderly individuals. Walking speed (Cesari et al., 2009a; Bandinelli et al., 2009) and standing balance (Bandinelli et al., 2009; Cesari et al., 2009a) were also associated with mortality in the elderly.

Although there are several studies that examined the relations between physical fitness and mortality among the elderly as mentioned above, there have been very few investigations of very old community-dwelling persons of age 80 years and over. The ages of the subjects in these investigations were 60 years and over (Sui et al., 2007), 65 years and over (Mitnitski et al., 2005; Gale et al., 2007; Bandinelli et al., 2009; Cesari et al., 2009b), 65–83 years (Spencer et al., 2005), 70–79 years (Cesari et al., 2009a; Newman et al., 2006a,b), and 85 or 89 years (Ling et al., 2010), respectively. In the very old population of 85-year-old or 89-year-old community-dwelling individuals (Ling et al., 2010), the mortality of the subjects in the lowest tertile for handgrip strength

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at age 85 years was 1.35 times higher than that of the subjects in the highest tertile, and the mortality of the lowest tertile at age 89 years was 2.04 times higher. We also examined the association between mortality and physical fitness in a Japanese 80-year-old community-dwelling population, and found that all-cause mortality fell with increasing stepping rate of the lower extremities, and mortality due to pneumonia fell with increasing leg extension strength (Takata et al., 2007). However, in this population, no association was found between mortality and physical fitness measurements such as handgrip strength or one-leg standing balance (Takata et al., 2007).

Non-participating men aged 40–59 who stopped bicycle exercising tests because of impaired breathing had a higher mortality during follow-up for 26 years (Bodegard et al., 2005). Nonparticipation for regular exercise was associated with higher mortality among women aged 40–70 years (Nechuta et al., 2010). Thus, although mortality seems higher in non-participants than in participants for exercise program, little is known about an association in mortality with nonparticipation for fitness test in a very elderly population.

Although it is likely that very old community residents also have a similar association between poor physical fitness and increased mortality, this finding is not conclusive. Therefore, the purpose of the present study was to evaluate this association for another very old community-dwelling population. In addition, an association between mortality and nonparticipation in fitness test was evaluated.

2. Materials and methods

2.1. Participants

The data were from 5 years of follow-up in a population-based study of age-related general and oral health in Fukuoka Prefecture, Japan. The subjects in this study were 827 persons who were 85 years of age, who were born in 1917 and lived in 1 of 9 districts (Bunzen City, Munakata City, Yukuhashi City, Tobata Ward of Kitakyushu City, Kanda Town, Katsuyama Town, Toyotsu Town, Tsuiki Town, or Shinyoshitomi Village) in Fukuoka Prefecture, Japan. Of the 827 persons, 410 refused, 210 had died in the previous 5 years, and 207 participated in the present study, when physical fitness measurements were made and questionnaires were obtained. Of 207, 140 subjects were independent and was able to go out using public transportation by an own effort, and 53 subjects were independent, but went out only to the neighborhood by an own effort. Some subjects ($n = 12$) were living in indoor and almost independent, but did not go out without assistance, and 2 subjects were living in indoor and needed some assistance. No subjects took part in some fitness program. There were no exclusion criteria. The study was approved by the Human Investigations Committee of Kyushu Dental College, and was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki, as revised in 2002. Informed written consent was obtained from all participants according to the principles mentioned above.

2.2. Physical fitness

The 85-year-old subjects completed five types of neuromuscular tests in 2003: two tests of muscle strength (hand-grip, isometric leg extension), one test of balance (one-leg standing time), one test of neuromuscular endurance (stepping rate), and one test of walking (walking speed and step). Handgrip strength test, leg extension strength test, one-leg standing test, stepping rate test, and 10 m walking test were selected, because these were safe and easy for very elderly individual. Handgrip strength test and leg extension strength test have been used in previous studies for elderly. One-leg standing test for assessing balance ability was done with eyes open to avoid falling. Stepping rates test was an index of agility of legs. Waling test 10 m was adopted for assessing lower leg functional capacity. Physical fitness tests were started from mild exercise test, and the rest time between assessments was taken approximately 10 min not to leave fatigue for the next test. The numbers of subjects participating in the tests were 198, 159, 169, 168, and 166, respectively. The hand-grip strength (Rantanen et al., 1998) on each side was measured by a Smedley hand dynamometer (DM-100S; Yagami, Nagoya, Japan). Handgrip strength test was done carefully in standing position so that dynamometer or an arm did not touch lower extremities when they measured it. The width of the dynamometer was adjusted for each participant so that the proximal interphalangeal joint becomes a right angle. Two values for each hand–one for each hand when used alone (single hand on left side, single hand on right side) and one for each hand when used in combination with the other hand (both hands mainly on right side, both hands mainly on left side)—were averaged together to determine the score for the test. Leg extension strength (Sonn et al., 1995) was measured by a portable chair incorporating a strain gage connected to a load cell. The material for isometric leg extension test was checked and calibrated before starting the daily test. The subject sat upright with a single leg or both legs hanging vertically and the knee initially bent at 90°. The trial was performed once for each single leg. The values for the two sides were averaged to find the subject's single leg extension strength score ((right leg + left leg)/2). The leg extension strength test with both legs was performed once. The stepping rate (Shindo et al., 1987) was measured once for each side using an industrial stepping rate counter (Stepping Counter GF-300; Yagami, Nagoya, Japan); while sitting, the subject was instructed to step with each leg as rapidly as possible for 10 s. The stepping rates obtained for each side were also averaged to determine the subject's score. The one-leg standing time (Haga et al., 1986) was defined as the number of seconds the subject was able to stand on one leg (with eyes open) without hopping or putting down the raised foot, or until 2 min had elapsed. Each subject took instructions from a study investigator, who observed the test from beginning to end. One trial was performed on the right leg and one trial on the left leg, and the average value of the two side measurements was considered to be the individual's score. Walking speed (Shinkai et al., 2000) was defined as the number of seconds required for individuals to walk 10 m. Walking steps were defined as the number of steps required for subjects to walk 10 m. The walking trial was performed twice, with the average value taken as the score for the subject. The baseline examinations were completed in October and November of 2003; these included medical examinations, blood sampling, and a questionnaire filled out in various public buildings. We recorded medical complication, medication, smoking, drinking, and education period from questionnaire. Blood pressure, total cholesterol, HbA1c, and body mass index (BMI) were taken from medical examination. The subjects were kept in a sitting position, and the sitting blood pressure was measured via an oscillometric method using an automatic device. BMI was defined as weight (kg) divided by square of the height (m^2). Most of the information is shown in Table 3. Percent of subjects who took medicine with prescription drug were 80.5%. Blood sampling was done to measure the serum concentrations of total cholesterol and blood concentration of HbA1c.

2.3. Follow-up for death

The 207 participants were followed up for 6 years and 5 months after the physical fitness tests and baseline examination.

Confirmation of whether the patient was living or had died was obtained by asking the family by calling or visiting the home. The cause of death was classified according to the 10th version of the International Classification of Diseases (ICD-10). Seven subjects were lost to follow-up over the 6 years and 5 months.

2.4. Statistical analysis

All data are reported as means ± SDmg/dL. The differences in mean values between groups were determined using analysis of variance. Categorical variables were compared using the chi-squared test. Associations of physical fitness measurements and time with 6.5-year mortality were assessed using the multivariate Cox proportional hazards regression analysis, in which only gender was adjusted for (Model 1), or both gender and serum level of total cholesterol were adjusted for (Model 2) as confounding factors. Results were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Physical fitness and basal characteristics

Physical fitness measurements, such as single-handgrip strength (right side), single-handgrip strength (left side), double-handgrip strength (right side), double-handgrip strength (left side), one-leg standing time, stepping rate of legs, leg extension strength (single leg), leg extension strength (both legs), walking speed, and walking steps are shown in Table 1. The scores on the muscle strength tests were much higher in males than in females, while that for standing time was much longer in males than in females, that for stepping rate or walking speed was much faster in males than in females, and that for walking steps was lower in males than in females. Scores on the tests of handgrip strength and leg extension strength were significantly higher in males who survived the 6.5-year follow-up period than in those who died. On the other hand, one-leg standing time, stepping rate, walking speed, and walking steps did not differ between those who survived the follow-up period and those who did not (Table 2A). In females, none of the scores on any fitness measurements differed between survivors and non-survivors (Table 2B). Basal characteristics other than physical fitness measurements are shown in Table 3. Males died at a much higher rate than females, and serum levels of total cholesterol were much higher in individuals who survived than in those who died during the follow-up years. The prevalence of smokers, alcohol drinkers, and patients with complications did not differ between survivors and non-survivors, and no difference was found in BMI, systolic blood pressure (SBP), HbA1c, or education period between the groups.

Table 1
Physical fitness measurements in 85-year-old males and females at the start of the study.

Physical fitness tests	Gender (*n*)	Mean ± SDmg/dL	*p* value
Handgrip strength, single hand, right (kg)	Male (84) Female (114)	26.4 ± 6.4 17.1 ± 3.8	0.001
Handgrip strength, single hand, left (kg)	Male (81) Female (114)	24.9 ± 6.0 16.2 ± 4.0	0.001
Handgrip strength, both hands, right (kg)	Male (82) Female (115)	26.4 ± 6.7 16.9 ± 3.7	0.001
Handgrip strength, both hands, left (kg)	Male (82) Female (115)	24.2 ± 6.0 15.5 ± 3.9	0.001
One-leg standing time (s)	Male (77) Female (92)	14.2 ± 1.6 5.3 ± 0.6	0.001
Stepping rate (steps/10 s)	Male (76) Female (92)	8.2 ± 0.9 6.5 ± 0.7	0.001
Leg extension strength, single leg (kg)	Male (71) Female (88)	27.8 ± 8.3 17.3 ± 6.0	0.001
Leg extension strength, both legs (kg)	Male (71) Female (88)	49.2 ± 15.8 29.9 ± 10.3	0.001
Walking speed (s)	Male (75) Female (91)	6.1 ± 1.6 7.4 ± 2.2	0.001
Waking step number (steps)/10 m	Male (75) Female (91)	13.6 ± 2.6 16.4 ± 4.8	0.001

3.2. Causes of death

During the 6.5-year follow-up period from October 2003 to March 2010, 81 individuals (49 males and 32 females) out of 207

Table 2
Physical fitness measurements in 85-year-old males and females at the start of the study who died and did not die during the follow-up period.

Physical fitness tests	Alive or Dead (*n*)	Mean ± SDmg/dL	*p* value
A. Males			
Handgrip strength, single hand, right (kg)	Alive (36) Dead (45)	28.7 ± 6.9 24.5 ± 5.6	≤0.004
Handgrip strength, single hand, left (kg)	Alive (36) Dead (42)	26.8 ± 6.1 23.2 ± 5.5	≤0.007
Handgrip strength, both hands, right (kg)	Alive (36) Dead (43)	28.5 ± 7.3 24.7 ± 5.9	≤0.013
Handgrip strength, both hands, left (kg)	Alive (36) Dead (43)	26.1 ± 6.2 22.7 ± 5.4	≤0.012
One-leg standing time (s)	Alive (36) Dead (39)	12.2 ± 14.7 11.3 ± 14.3	≤0.792
Stepping rate (steps/10 s)	Alive (36) Dead (38)	35.9 ± 9.2 33.7 ± 7.2	≤0.270
Leg extension strength, single leg (kg)	Alive (33) Dead (36)	29.9 ± 7.9 25.6 ± 8.3	≤0.033
Leg extension strength, both legs (kg)	Alive (33) Dead (36)	54.3 ± 15.5 44.3 ± 15.0	≤0.008
Walking speed (s)	Alive (37) Dead (36)	5.9 ± 1.4 6.3 ± 1.8	≤0.237
Waking step number (steps)/10 m	Alive (37) Dead (36)	13.3 ± 2.6 14.0 ± 2.5	≤0.219
B. Females			
Handgrip strength, single hand, right (kg)	Alive (79) Dead (31)	17.4 ± 3.3 16.1 ± 4.9	≤0.123
Handgrip strength, single hand, left (kg)	Alive (79) Dead (31)	16.5 ± 3.9 15.3 ± 4.4	≤0.167
Handgrip strength, both hands, right (kg)	Alive (79) Dead (32)	17.2 ± 3.2 16.2 ± 4.7	≤0.186
Handgrip strength, both hands, left (kg)	Alive (79) Dead (32)	15.8 ± 3.8 14.4 ± 4.3	≤0.098
One-leg standing time (s)	Alive (65) Dead (23)	5.2 ± 6.1 3.0 ± 1.7	≤0.095
Stepping rate (steps/10 s)	Alive (67) Dead (21)	28.7 ± 5.6 27.6 ± 9.3	≤0.527
Leg extension strength, single leg (kg)	Alive (63) Dead (21)	16.8 ± 5.7 18.8 ± 7.6	≤0.189
Leg extension strength, both legs (kg)	Alive (63) Dead (21)	29.2 ± 9.3 32.2 ± 13.1	≤0.246
Walking speed (s)	Alive (67) Dead (20)	7.5 ± 2.3 7.5 ± 2.3	≤0.949
Waking step number (steps)/10 m	Alive (67) Dead (20)	16.7 ± 5.1 16.0 ± 3.8	≤0.593

died. Of these 81 subjects, 27 deaths were due to cardiovascular disease (9 heart failures, 8 strokes, 5 myocardial infarctions, 2 aortic aneurysms, 1 carotid artery aneurysm, 1 case of hypertensive heart disease, 1 details unknown); 14 were due to cancer (3 gastric cancer, 2 lung cancer, 2 colon cancer, 2 hepatic cancer, 1 uterine cancer, 1 urinary tract cancer, 1 gallbladder cancer, 1 laryngeal cancer, 1 cancer of unknown organ); 11 to respiratory tract disease (10 pneumonia, 1 respiratory failure); 10 to senility; 3 to gastrointestinal disease (1 pancreatitis, 1 liver cirrhosis, 1 details unknown); 3 to exogenous death (2 injury, 1 suffocation); 1 to renal failure, 1 to multiple organ failure, and 11 to unknown causes.

3.3. Association between physical fitness and mortality

Associations between physical fitness measurements and total mortalities were assessed by multivariate Cox regression analyses adjusted for gender difference. These analyses were performed to calculate the risk for mortality associated with a 1 kg, 1 s, 1 step/10 s, 1 step/10 m increase (continuous analysis) in each respective fitness measurement (Table 4A). Another adjustment had both gender and serum level of total cholesterol as confounding factors (Table 4B). Since all subjects were 85 years old at the start of the study, age was not included as a confounding factor in these analyses. The relative hazard ratios (HR) for all-cause mortality adjusted only for gender difference fell 6–7% with each 1 kg increase in handgrip strength, regardless of whether the strength for a single hand or both hands or for the right side or left side was assessed. With a 1-kg increase in the leg extension strength of both legs, HR for all-cause mortality decreased 2% (Table 4A). Similarly, HR for total-cause mortality adjusted both for gender and serum level of total cholesterol fell 5–6% with a 1-kg increase in handgrip strength in a single hand or both hands, whether on the right side or left side. Total mortality also decreased 2% with a 1-kg increase in the leg extension strength of both legs (Table 4B).

3.4. Association between nonparticipation and mortality

Since all subjects were 85 years old, some of them were not able to perform the physical fitness tests. The number of subjects who did not participate in the tests ranged from 9 to 48 as shown in Table 5. The association between total mortality and participation in the fitness tests was also assessed by multivariate Cox analysis, with adjustment only for gender difference or for both gender and serum level of total cholesterol. No association was found between mortality and participation in the handgrip strength test or leg extension strength test, while apparent associations were found between mortality and participation in the walking, stepping rate, and one-leg standing time tests when adjusted for gender difference. Participants in the walking test showed a 62% decrease in mortality compared to nonparticipants. The mortality rates of participants in the stepping rate test and the one-leg standing test also were lower than those in nonparticipants by 53% and 44%, respectively (Table 5A). Similarly, with adjustment both for gender and serum level of total cholesterol, mortality fell by 57% in participants in the walking test and fell by 45% in participants in the stepping rate test compared to mortality in nonparticipants. No association was found in mortality with participation in the handgrip strength test, leg extension strength test, or one-leg standing time test (Table 5B).

Table 3
Baseline characteristics at the age of 85 years in individuals who survived or did not survive the follow-up period.

Basal characteristics	Alive	Dead	*p* value
% men	31.9	60.5	≤0.001
% smokers	4.2	6.5	≤0.485
% alcohol drinkers	53.0	51.3	≤0.810
% complications	76.4	73.1	≤0.608
BMI (kg/m^2)	22.87 ± 3.03	22.26 ± 4.05	≤0.226
SBP (mmHg)	144.1 ± 23.6	144.4 ± 25.2	≤0.926
Total cholesterol (mg/dL)	205.1 ± 37.1	179.1 ± 30.3	≤0.001
HbA1c (%)	5.46 ± 0.57	5.48 ± 0.79	≤0.242
Education period (years)	9.4 ± 2.3	9.6 ± 3.0	≤0.634

BMI, body mass index; SBP, systolic blood pressure.

Table 4
Multivariate Cox analyses of total mortality and physical fitness measurements such as handgrip strength, one-leg standing time, leg extension strength, stepping rate, and walking as assessed at the start of the study. The analyses were adjusted only for gender difference (A) or were adjusted both for gender and serum level of total cholesterol (B).

Physical Fitness Measurements	Hazard ratio	95% CI	*p* value
A. Adjusted for gender difference			
Hand grip strength, single hand, right (kg)	0.925	0.887–0.966	≤0.001
Hand grip strength, single hand, left (kg)	0.925	0.883–0.968	≤0.001
Hand grip strength, both hands, right (kg)	0.936	0.899–0.975	≤0.001
Hand grip strength, both hands, left (kg)	0.927	0.886–0.970	≤0.001
One-leg standing time (s)	0.990	0.964–1.016	≤0.434
Stepping rate (steps/10 s)	0.977	0.948–1.010	≤0.172
Leg extension strength, single leg (kg)	0.962	0.929–1.001	≤0.054
Leg extension strength, both legs (kg)	0.975	0.955–0.995	≤0.015
Walking speed (s)	1.074	0.942–1.223	≤0.286
Walking step number (steps)/10 m	1.015	0.947–1.088	≤0.681
B. Adjusted both for gender and serum level of total cholesterol			
Hand grip strength, single hand, right (kg)	0.940	0.900–0.982	≤0.005
Hand grip strength, single hand, left (kg)	0.938	0.897–0.982	≤0.006
Hand grip strength, both hands, right (kg)	0.949	0.912–0.988	≤0.011
Hand grip strength, both hands, left (kg)	0.942	0.901–0.985	≤0.009
One-leg standing time (s)	0.993	0.968–1.020	≤0.627
Stepping rate (steps/10 s)	0.984	0.953–1.016	≤0.322
Leg extension strength, single leg (kg)	0.967	0.933–1.002	≤0.061
Leg extension strength, both legs (kg)	0.978	0.958–0.997	≤0.027
Walking speed (s)	1.063	0.927–1.218	≤0.385
Walking step number (steps)/10 m	1.011	0.939–1.089	≤0.770

CI, confidence interval.

4. Discussion

In an 85-year-old community-dwelling population, handgrip strength and leg extension strength were greater in males who survived the follow-up period of 6.5 years than in those who died, while no difference was found between survivors and non-survivors among females. With multivariate Cox analysis adjusted for only gender or for the combination of gender and serum level of total cholesterol, mortality was found to have an association with the handgrip strength of a single hand or both hands and the leg extension strength of both legs. The relative risk of mortality fell 5–7% with a 1-kg increase in the handgrip strength of single hand or both hands, and it fell 2% with a 1-kg increase in the leg extension strength of both legs. Participants in the walking test had 57% lower mortality than nonparticipants, and participants in the stepping rate test had 45% lower mortality than nonparticipants,

Table 5
Multivariate Cox analyses of total mortality and participation in fitness tests such as hand-grip strength, one-leg standing time, leg extension strength, stepping rate, and walking as assessed at the start of the study. The analyses were adjusted only for gender difference (A) or were adjusted both for gender and serum level of total cholesterol (B).

Physical Fitness tests	Yes	No	Hazard ratio	95% CI	*p* value
A. Adjusted for gender difference					
Handgrip strength, single hand, right	198	9	0.823	0.331–2.047	≤0.676
Handgrip strength, single hand, left	195	12	0.604	0.288–1.266	≤0.182
Handgrip strength, both hands, right	197	10	0.779	0.335–1.812	≤0.562
Handgrip strength, both hands, left	197	10	0.779	0.335–1.812	≤0.562
One-leg standing time	169	38	0.557	0.330–0.942	≤0.029
Stepping rate	168	39	0.472	0.284–0.784	≤0.004
Leg extension strength, single leg	159	48	0.638	0.391–1.041	≤0.072
Leg extension strength, both legs	159	48	0.638	0.391–1.041	≤0.072
Walking speed	166	41	0.382	0.233–0.626	≤0.001
Walking steps	166	41	0.382	0.233–0.626	≤0.001
B. Adjusted for gender and serum level of total cholesterol					
Handgrip strength, single hand, right	198	9	1.221	0.442–3.372	≤0699
Handgrip strength, single hand, left	195	12	0.780	0.355–1.715	≤0.536
Handgrip strength, both hands, right	197	10	1.065	0.425–2.669	≤0.894
Handgrip strength, both hands, left	197	10	1.065	0.425–2.669	≤0.894
One-leg standing time	169	38	0.633	0.372–1.078	≤0.092
Stepping rate	168	39	0.551	0.329–0.924	≤0.024
Leg extension strength, single leg	159	48	0.754	0.458–1.240	≤0.266
Leg extension strength, both legs	159	48	0.754	0.458–1.240	≤0.266
Walking speed	166	41	0.428	0.260–0.705	≤0.001
Walking steps	166	41	0.428	0.260–0.705	≤0.001

CI, confidence interval; HR, hazard ratio.

when adjusted for gender and serum total level. With an adjustment made only for gender, participants in the one-leg standing test also had a 44% lower mortality rate than non-participants. These findings suggest that not only muscle strength but also other physical abilities may be associated with mortality in a very old population. When assessing physical fitness measurements in a very old population, the influence of participation or nonparticipation on the findings should be considered. No association between fitness measurements and mortality in female may be partly due to much lower score of fitness measurements. Lower mortality in female (28.3%) than in men (56.3%) also may induce a lack of association in mortality with fitness measurement. However, further studies are needed to clarify the gender difference.

Much like our findings for handgrip measurements, associations were previously found between total mortality and handgrip strength in 85-year-old and 89-year-old populations in Holland (Ling et al., 2010). In a Cox regression analysis using handgrip strength as a continuous variable, an increase of 11% in all-cause mortality occurred for each 5-kg reduction at age 85 years and of 24% at age 89 years. In other studies of elderly populations aged 65 and over (Gale et al., 2007) and 70–79 years (Newman et al., 2006a), handgrip strength was a predictor of all-cause mortality. Leg extension strength also predicted mortality in elderly persons (Newman et al., 2006a).

Although walking speed and walking step length were not associated with mortality in our study of 85-year-old persons, participation in the walking test was predictive of long survival, suggesting that poor walking ability may be associated with an increase in mortality. Nonparticipants in the walking test may be more likely than participants to have poor walking ability. This finding was similar to the findings in previous studies of elderly populations. Walking speed was an independent predictor of mortality in older Mexican-Americans aged 65 years and over (Cesari et al., 2009b). In well-functioning older persons with a mean age of 73.6, gait speed was predictive of mortality (Cesari et al., 2009a). Among older women aged 65 years and over, a high level of walking for exercise as assessed by questionnaire was associated with lower all-cause mortality (Gregg et al., 2003). Participants in the stepping rate test had 45% lower mortality than nonparticipants, although no association was found between mortality and stepping rate in the present study. We previously found an association between total mortality and stepping rate in an 80-year-old population (Takata et al., 2007). These findings were suggestive of the presence of similar associations in 85-year-old persons. Not only baseline physical fitness performance but also changes in physical activity have been associated with mortality in elderly populations. Increasing and maintaining physical activity levels were associated with lower mortality among older women aged 65 years or older (Gregg et al., 2003).

In the present study, we found associations between mortality and performance on various physical fitness tests such as the handgrip strength of a single hand and of both hands and the leg extension strength of both legs. We also found associations between mortality and participation in the walking test and the stepping rate test of the legs. These findings suggest that not only handgrip strength, as reported by Ling et al. (2010), but also performance on other fitness tests were independent predictors of total mortality in this population. Thus, we extended the association between physical fitness and mortality to an 85-year-old population.

Although some studies have shown an association between balance and mortality in elderly, we did not find the association. This discrepancy may be partly due to different methods for measuring balance ability. The previous investigators adopted tandem standing (Bandinelli et al., 2009; Blain et al., 2010; Cesari et al., 2009a), while we used one-leg standing with eye open. Age of subjects was also different, subjects of the previous studies were aged between 70 and 79 (Cesari et al., 2009a), 65 and older (Bandinelli et al., 2009), or 75 and older (Blain et al., 2010), whereas our subjects were 85-year-old. Moreover, in the present study, considerably many subjects did not participate in one-leg standing test, inducing no association between mortality and standing time.

There are limitations to our findings. In the present study, the sample size was small ($n = 207$). The potential of the sample is not enough to establish firm conclusions in the analysis of participant and non-participant, especially in some analysis comparing groups with one of the sample size group smaller than 15 subjects. Future

studies with greater sample size are needed to confirm this aspect. There were 7 subjects who were lost to follow-up over the 6 years and 5 months (follow-up rate, 96.6%). Residual confounding factors other than gender and serum level of total cholesterol could have influenced our findings. However, the present findings still clearly indicate that the results of physical fitness tests such as handgrip strength, leg extension strength, and probably also participation in walking and stepping rate tests are predictive of total mortality at the age of 85 in community-dwelling elderly.

5. Conclusion

Poor muscle strength of handgrip or leg extension was found to be an independent predictor of total mortality in an elderly 85-year-old Japanese community-dwelling population. Moreover, nonparticipation in tests of walking or leg stepping rate was independently associated with increased mortality in the very elderly population.

Conflict of interest statement

None

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Mechanisms involved in regulation of osteoclastic differentiation by mechanical stress-loaded osteoblasts

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abstract

Mechanical stress is known to be important for regulation of bone turnover, though the detailed mechanisms are not fully understood. In the present study, we examined the effect of mechanical stress on osteoblasts using a novel compression model. Mouse osteoblastic MC3T3-E1 cells were embedded in three-dimensional (3D) gels and cultured with continuous compressive force (0–10.0 g/cm^2) for 48 h, and the conditioned medium were collected. RAW264.7 cells were then incubated with the conditioned medium for various times in the presence of receptor activator of nuclear factor-j B ligand (RANKL). Conditioned medium was found to inhibit the differentiation of RAW264.7 cells into osteoclasts induced by RANKL via down-regulation of the expression of tumor necrosis factor receptor-associated factor 6 (TRAF6), phosphorylation of Ij Ba, and nuclear translocation of p50 and p65. Interestingly, the conditioned medium also had a high level of binding activity to RANKL and blocked the binding of RANK to RANKL. Furthermore, the binding activity of conditioned medium to RANKL was reduced when the 3D gel was supplemented with KN-93, an inhibitor of non-canonical Wnt/Ca^{2+} pathway. In addition, expression level of osteoprotegerin (OPG) mRNA was increased in time- and force-dependent manners, and remarkably suppressed by KN-93. These results indicate that osteoblastic cells subjected to mechanical stress produce OPG, which binds to RANKL. Furthermore, this binding activity strongly inhibited osteoclastogenesis through suppression of TRAF6 and the nuclear factor-kappa B (NF-j B) signaling pathway, suggesting that enhancement of OPG expression induced by mechanical stress is dependent on non-canonical Wnt/Ca^{2+} pathway.

1. Introduction

Bone mass homeostasis is regulated by an interaction of various factors, including growth factors, hormones and mechanical loading [1,2], and some researchers have reported that mechanical stress applied to several types of cells maintained bone metabolism including bone formation and bone resorption [3–5].

Receptor activator of nuclear factor-j B ligand (RANKL), identified as a membrane-bound protein, is an essential factor for osteoclastogenesis produced by osteoblasts and stimulates osteoclast precursors to differentiate via binding to the receptor, RANK. OPG is a member of tumor necrosis factor (TNF) receptor family that acts as a decoy receptor of the RANKL [6].

RANKL interacts with RANK, resulting in recruitment of intracellular tumor necrosis factor receptor-associated factor 6 (TRAF6) and activation of signaling pathways including nuclear factor of kappa B (NF-j B) and mitogen-activated protein kinase (MAPK) [7–9]. Furthermore, RANKL activates the nuclear translocation and DNA binding of the NF-j B proteins (p50 and p65) via phosphorylation and degradation of Ij Ba [10,11].

Wnt pathway, confirmed to play critical roles in bone development and homeostasis, is classified into 3 pathway groups; the b-catenin-dependent canonical Wnt pathway, non-canonical planar cell polarity pathway, and non-canonical Wnt/Ca^{2+} pathway [12]. It has been demonstrated that canonical Wnt pathway modulates several aspects of osteoblast physiology including proliferation, differentiation, bone matrix formation and apoptosis [13–16]. Findings in a recent genetic study indicated that Wnt/b-catenin pathway is involved in the expression of both RANKL and OPG [17]. On the other hand, mechanical loading was shown to induce differentiation of mesenchymal progenitor cells through the non-

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canonical Wnt/Ca^{2+} pathway [18]. However, the role of non-canonical Wnt/Ca^{2+} pathway in regard to mechanical stress-induce osteoclastogenesis has not been fully elucidated. In the present study, we investigated the mechanisms of the non-canonical Wnt/Ca^{2+} pathway involved in osteoclastogenesis induced by compressive force.

2. Materials and methods

2.1. Reagents

Human recombinant RANKL was purchased from Oriental Yeast Co., Ltd. (Shiga, Japan). Anti-p38 MAPK polyclonal and anti-phosphorylated p38 MAPK polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, CA, USA). Anti-p50 monoclonal, anti-p60 monoclonal, anti-Ij Ba monoclonal, anti-phospho-Ij Ba monoclonal, anti-TRAF6 monoclonal, and anti-RANK monoclonal antibodies were obtained from Santa Cruz Biotechnology. (Santa Cruz, CA, USA).

2.2. Cell cultures

The murine monocyte/macrophage cell line RAW264.7 was maintained in a-minimal essential medium (a-MEM; Gibco, Gland Island, NY, USA) containing 10% fetal bovine serum (FBS), with penicillin G (100 U/ml) and streptomycin (100 l g/ml). The murine osteoblastic cell line MC3T3-E1 was cultured in a-MEM supplemented with 10% FBS and antibiotics. The cells were maintained at 37 °C in an atmosphere containing 5% CO_2.

2.3. Application of compressive force

To examine the effect of static compressive force, MC3T3-E1 cells were cultured in a three-dimensional (3D) cell culture system [19]. Briefly, collagen gel cultures were assembled by mixing 7 volumes of 0.3% type I-A collagen solution (Nitta-gelatin, Osaka, Japan), 1 volume of 20 mM HEPES buffer containing 2.2% sodium bicarbonate and 0.05%sodium hydroxide, and 1 volume of cell suspension to provide a final cell density of 1×10^6 cells/ml. The gel mixtures were allowed to polymerize for 1 h, following transfer to 6-well plates to promote nutrient diffusion from their surroundings. The gel mixtures in each well were cultured with 2 ml of a-MEM containing 1% FBS, and allowed to set for 24 h prior to force loading. Compressive force was applied using a sterile titanium plate (32 mm in diameter) and plastic cylinder placed over the gels, which was adjusted by adding lead granules to the cylinder. In some experiments, KN-93, a selective Ca^{2+}/calmodulin-dependent protein kinase II inhibitor (Sigma–Aldrich, St. Louis, MO, USA), was added to the collagen gels.

2.4. Kinetic analysis using quartz-crystal microbalance (QCM)

A 27-MHz QCM (Affinix Q; Initium Inc., Tokyo, Japan) was employed to analyze the affinity of RANKL and conditioned medium harvested from 3D cultures of MC3T3-E1 cells. RANKL (2 l l; 10^{-11} M) was immobilized directly on the gold electrode surface of the QCM ceramic sensor chip, after which the sensor chip was soaked in a chamber containing 8 ml of distilled water at 25 °C until frequency equilibrium was attained. Conditioned medium (volume 800 l l) was added to the equilibrated solution containing the RANKL-immobilized sensor chip. The binding of conditioned medium to RANKL was determined by monitoring the alterations in frequency resulting from changes in mass on the electrode surface [20].

2.5. Western blot analysis

MC3T3-E1 cells were mixed into the collagen gels and subjected to 7.5 g/cm^2 of compressive force for indicated times, and conditioned medium were collected. Next, RAW 264.7 cells (2.5×10^5 cells/well) were cultured in 6-well plates in a-MEM containing 10% FBS in the presence or absence of RANKL (40 ng/ml) along with the conditioned medium. The cells were then washed twice with phosphate buffer saline (PBS; pH 7.2) and lysed in lysis buffer (75 mM Tris–HCl containing 2% SDS and 10% glycerol, pH 6.8). In some experiments, nuclear factors were isolated using a NucBuster™ Protein Extraction Kit (EMD Biosciences Inc., Darmstadt, Germany) according to the manufacturer's instructions. Protein contents were measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Equivalent sample volumes were subjected to 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Non-specific binding sites were blocked by immersing the membrane in 10% skimmilk in PBS for 1 h at room temperature, after which the membrane was washed 4 times with PBS, followed by incubation with the diluted primary antibody at 4 °C overnight. After washing the membrane, chemiluminescence was produced using enhanced chemiluminescent (ECL) reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) and detected with Hyperfilm-ECL (Amersham Pharmacia Biotech).

2.6. Evaluation of osteoclastic differentiation

RAW264.7 cells were cultured in 24-well plates (7×10^4 cells/well) with RANKL (40 ng/ml) in the presence of conditioned medium from the 3D cultures of MC3T3-E1 cells, for 3 days. Adherent cells were fixed and stained with tartrate-resistant acid phosphatase (TRAP) (Sigma Chemical Co., St. Louis, MO, USA). TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclasts and counted under a microscope.

2.7. Real-time RT-PCR analysis

Total RNA was isolated from compressed 3D-gels using ISOGEN-LS (Nippon Gene, Tokyo, Japan). Briefly, collagen gels containing cells were washed extensively with PBS and minced in ISOGEN-LS, then RNA was isolated according to the manufacturer's instructions. Extracted total RNA was reverse transcribed and subjected to real-time RT-PCR, in which the PCR products were detected using FAST SYBR® Green Master Mix (Applied Biosystems, Foster City, CA). The primer sequences used were as follow; b-actin forward, 5′-CTGAACCCTAAGGCCAACCGTG-3′ and reverse 5′-GGCATACAGGGACAGCACAGCC-3′, and OPG forward, 5′-GCCTGGGACCAAAGTGAATG-3′ and reverse 5′-CTTGTGAGCTGTGTCTCCGTTT-3′. Thermal cycling and fluorescence detection were done using a StepOne™ Real-Time PCR System (Applied Biosystems). Real-time RT-PCR efficiency (E) was calculated according to the equation provided by Rasmussen [21], as follows: E= 10[−1/slope], for b-actin and various target genes. The slope was determined from the graph of ng of the cDNA substrate (x-axis) versus the cycle number at the crossing point (CP) (y-axis). The CP value was the PCR cycle number that represented the CP in SYBR® Green fluorescence intensity above the automatic noise-based threshold. The fold increase in copy numbers of mRNA was calculated as the relative ratio of target gene to b-actin, following the mathematical model presented by Pfaffl [22].

$$\text{Fold increase} = \frac{(E_{\text{TARGET}})^{\text{CP TARGET(MEAN control-MEAN subject)}}}{(E_{b-\text{actin}})^{\text{CP}b-\text{actin(MEAN control-MEAN subject)}}}$$

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2.8. OPG measurement

The amounts of OPG in the conditioned medium were determined using an OPG ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.9. Statistical analysis

Statistical differences were determined using an unpaired Student's t-test with Bonferroni correction for multiple comparisons. All data are expressed as the mean ± standard deviation of three examinations, with similar results obtained in each experiment.

3. Results

3.1. Effect of conditioned medium derived from MC3T3-E1 cells on osteoclastogenesis induced by RANKL

To determine whether mechanical stress in our compression model had effect on osteoclast differentiation, we first evaluated the number of osteoclasts by counting TRAP-positive multinucleated cells (Fig. 1A). Conditioned medium derived from MC3T3-E1 cells inhibited the differentiation of RAW264.7 cells into osteoclast-like cells in a loading force-dependent manner (Fig. 1B). The inhibitory effect of the conditioned medium began to be seen at 7.5 g/cm^2 of loading force (Fig. 1C). In addition, the effect of conditioned medium on proliferation of RAW264.7 cells was examined using a WST-1 assay. However, no effect on cell growth was seen for up to 48 h (data not shown).

3.2. Interaction between RANKL and conditioned medium derived from MC3T3-E1 cells

To examine the interaction of conditioned medium and RANKL, we investigated the affinity between them using a QCM technique. Medium conditioned by 0 g/cm^2 of compressive force decreased the frequency by 745 Hz, while it was decreased by 2200 Hz when we used medium conditioned by 7.5 g/cm^2 of compressive force (Fig. 2A). To investigate whether MC3T3-E1 cells under mechanical stress produce OPG, we examined OPG mRNA expression in MC3T3-E1 cells in the collagen gels. Mechanical stress caused an up-regulation of OPG mRNA expression at 6 h (Fig. 2B). These enhancement was in time- and dose-dependent manners (Fig. 2C).

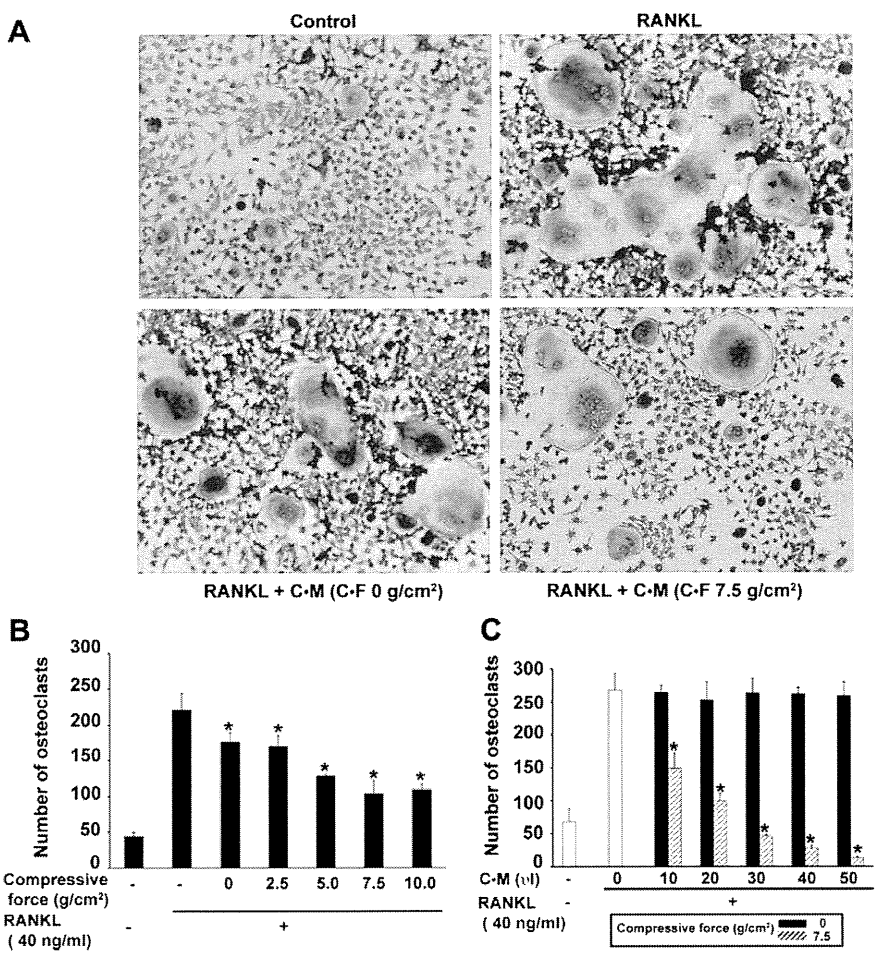

Fig. 1. Effect of conditioned medium derived from MC3T3-E1 cells on osteoclastogenesis induced by RANKL. RAW264.7 cells (7.0×10^4 cells/ml) were cultured with conditioned medium derived from MC3T3-E1 cells subjected to 7.5 g/cm^2 of compressive force and RANKL (40 ng/ml). The number of TRAP-positive multinucleated cells was counted. (A) Images showing osteoclast formation. (B) MC3T3-E1 cells were subjected to 0–10 g/cm^2 of compressive force, then the conditioned medium was collected and used as stimulants. (C) Medium conditioned by 7.5 g/cm^2 of compressive force (0–50 μl) were added to RAW264.7 cells. Data are expressed as the mean ± SD of triplicate cultures. Student's t-test, *$P < 0.05$.

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3.3. Effect of conditioned medium derived from MC3T3-E1 cells on RANKL-induced activation of NF-κB signaling pathway in RAW264.7 cells

We investigated the effect of mechanical stress on signal transduction in the process of osteoclast differentiation in RAW264.7 cells cultured with conditioned medium derived from MC3T3-E1 cells. Addition of medium conditioned by 7.5 g/cm^2 of force suppressed the expression of TRAF6, whereas the expression of RANK was not changed (Fig. 3A). Furthermore, we evaluated the effect of conditioned medium on phosphorylation of p38 MAPK and IκBα and expression of p50/p65, the most common NF-κB dimer, during osteoclast differentiation of RAW264.7 cells. Conditioned medium did not affect the phosphorylation of p38 MAPK (Fig. 3B). In contrast, it inhibited the phosphorylated levels of IκBα and expression of p50/p65 in the nuclear fraction of RAW264.7 cells induced by RANKL (Fig. 3C).

3.4. Involvement of non-canonical Wnt/Ca^{2+} pathway in RAW264.7 cells under the mechanical stress

To investigate the relationship between the non-canonical Wnt/Ca^{2+} pathway and osteoclast differentiation, we examined the effect of KN-93, a selective Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, on the expression of OPG mRNA in RAW264.7 cells. MC3T3-E1 cells in collagen gel were cultured with KN-93 for 24 h and subjected to 7.5 g/cm^2 of compressive force for 12 h. Quantitative real-time RT-PCR analysis revealed that the expression of OPG mRNA induced by mechanical stress was remarkably suppressed by KN-93 (Fig. 4A). Finally, we determined the amount of OPG protein in conditioned medium by ELISA. Mechanical stress increased OPG secretion from MC3T3-E1 cells in a force-dependent manner, which was significantly suppressed by KN-93 (Fig. 4B). To examine the interaction between conditioned medium and RANKL, the affinity between them were determined using a QCM technique. In the presence of KN-93, the reduction of frequency by conditioned medium (7.5 g/cm^2) was recovered by 1618–958 Hz (Fig. 4C).

4. Discussion

It has been reported that mechanical stress functions as a critical regulatory factor in bone metabolism, and is also a postnatal determinant of bone homeostasis and skeletal morphology [23]. Although mechanical stress generates response from mechanosensitive cells, including bone cells, fibroblasts and epithelial cells have also been found to have responsiveness to mechanical stress [23,24]. Furthermore, recent studies have shown that osteoclast differentiation of RAW264.7 cells induced by RANKL was significantly decreased with oscillatory fluid flow [25]. Mechanical stress was also found to inhibit the expression of RANKL by murine stromal cells [26]. We previously reported that compressive mechanical force promoted osteoclast formation through RANKL expression in synovial cells derived from rat knee joints [4], while another study demonstrated that compressive force stimulation increased the levels of soluble RANKL and decreased those of OPG [27]. However, accurate details of the mechanisms by which mechanical

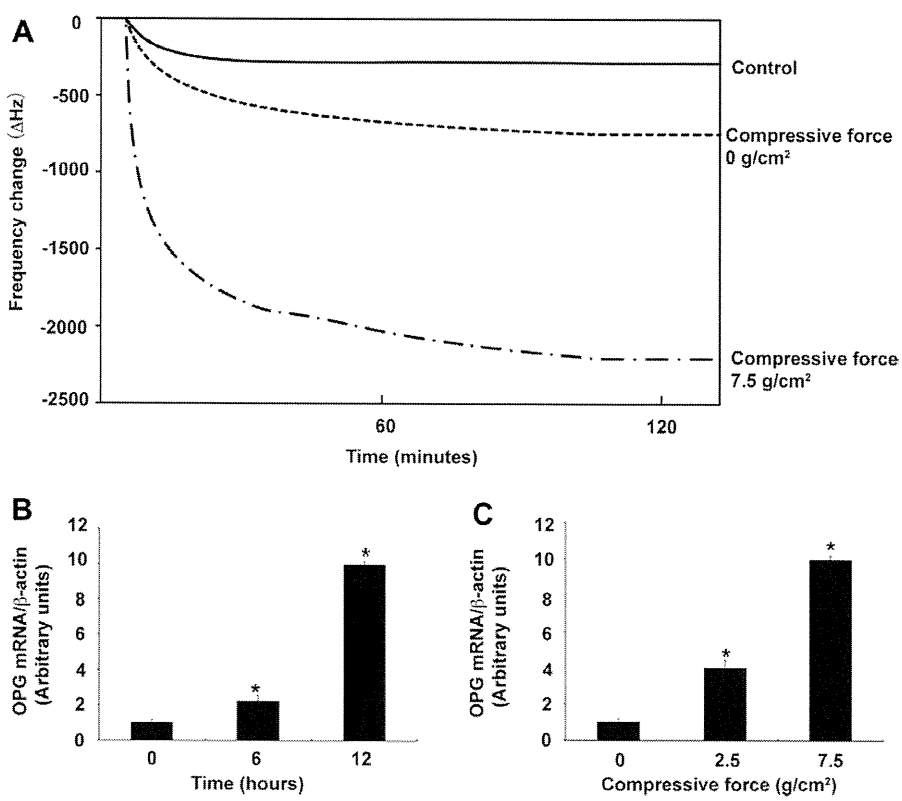

Fig. 2. Conditioned medium derived from MC3T3-E1 cells blocks binding of RANK to RANKL via enhancement of OPG expression MC3T3-E1 cells were cultured in collagen gels and subjected to 7.5 g/cm^2 of compressive force for 48 h, then conditioned medium were collected. (A) The binding ability of RANKL to conditioned medium was assessed using a QCM, as described in Section 2. MC3T3-E1 cells were cultured in collagen gels and subjected to compressive force. The fold change in OPG copy number between control and treated culture was determined by real-time RT-PCR, as described in Section 2. (B) Representative results from a time-dependent experiment with a compressive force of 7.5 g/cm^2. (C) Representative results from a force-dependent experiment at the time point of 12 h. Data are expressed as the mean ± SD of triplicate culture. Student's t-test, *$P < 0.05$.

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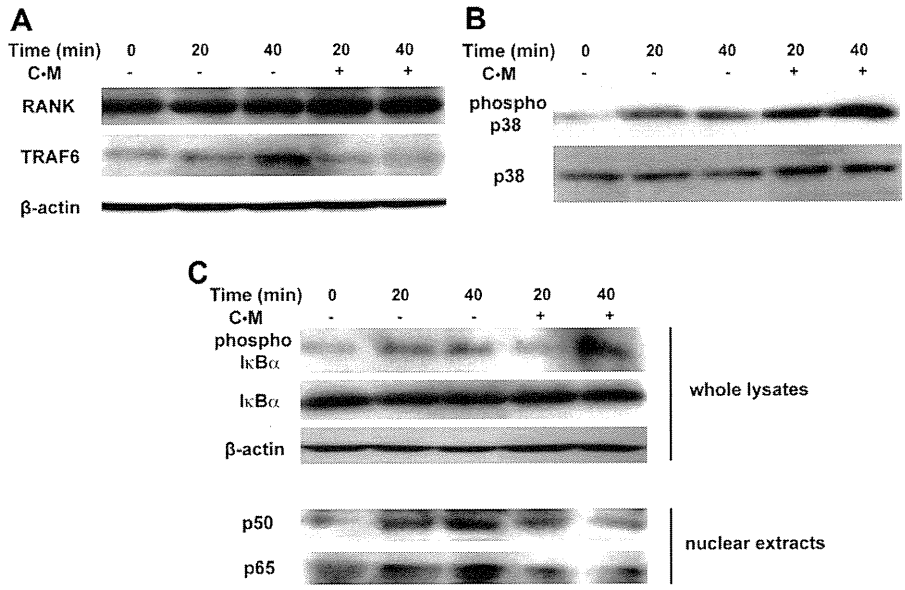

Fig. 3. Conditioned medium derived from MC3T3-E1 cells subjected to compressive force interferes with NF-j B signaling pathway in RAW264.7 cells RAW264.7 cells were stimulated with RANKL (40 ng/ml) in the presence or absence of conditioned medium derived from MC3T3-E1 cells subjected to compressive force for the indicated times. Whole cell lysates or nuclear fractions of RAW264.7 cells were subjected to immunoblotting analysis. (A) Expressions of RANK and TRAF6. (B) Expressions of p38 MAPK and phosphorylated p38 MAPK. (C) Expression of Ij Ba, phosphorylated Ij Ba, and expression of p50 and p65.

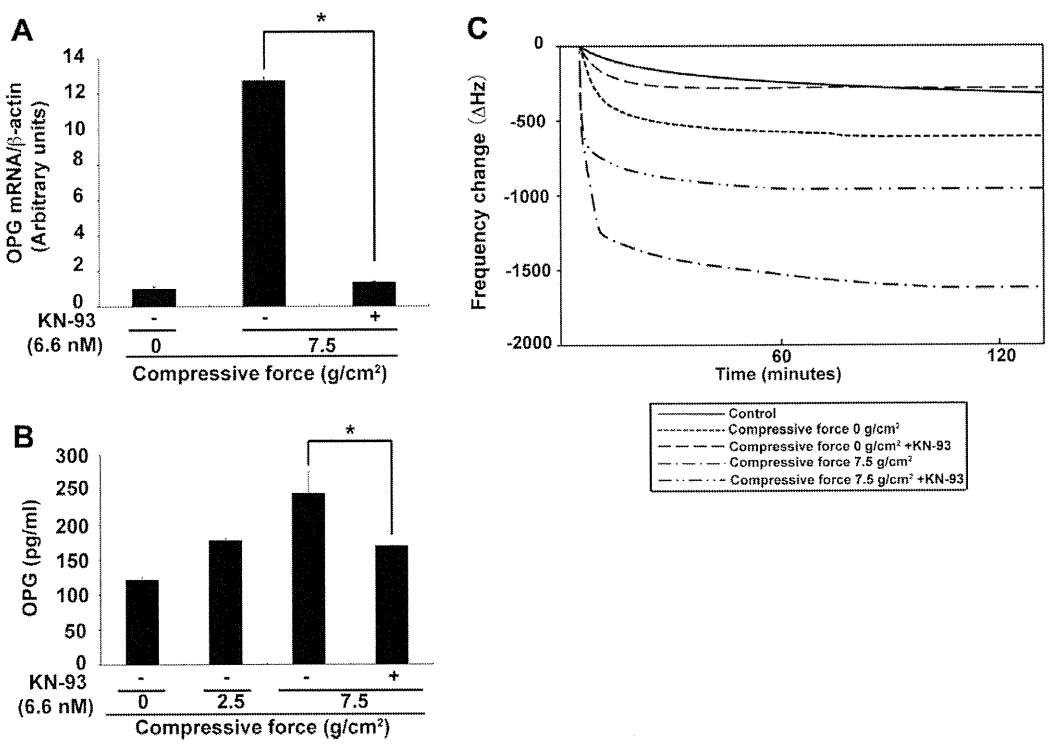

Fig. 4. Involvement of non-canonical Wnt/Ca^{2+} pathway in the induction of OPG in MC3T3-E1 cells by compressive force MC3T3-E1 cells were cultured in collagen gels and subjected to compressive force in the presence or absence of KN-93. (A) The fold change in OPG copy number between control and treated culture was determined by real-time RT-PCR, as described in Section 2. (B) MC3T3-E1 cells were cultured in collagen gels and subjected to 0–7.5 g/cm^2 of compressive force for 24 h in the presence or absence of KN-93, then culture medium were collected. The amount of OPG in the culture medium was determined as described in Section 2. (C) MC3T3-E1 cells were cultured in collagen gels and subjected to 7.5 g/cm^2 for 48 h in the presence or absence of KN-93, then culture medium samples were collected. The binding ability of RANKL to conditioned medium was assessed using a QCM, as described in Section 2. Data are expressed as the mean ± SD of triplicate cultures. The experiment was performed 3 times, with similar results obtained in each. Student's t-test, *$P < 0.05$.

stress affects the productions of RANKL and OPG have yet to be reported.

We previously showed that our 3D culture system made it possible to study the role of loaded osteoblasts in initiation of the bone

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remodeling process, as it partially mimicked the in vivo environment [19]. 3D gel-embedded cultures of various cells were reported to support cell proliferation as well as differentiation into several different types of cells [19,28,29]. In the present study, mouse osteoblast lineage, MC3T3-E1 cells were embedded in 3D gels and cultured with mechanical stimuli, after which the conditioned medium were collected and added to RAW264.7 cells. We found that conditioned medium significantly decreased osteoclast differentiation (Fig. 1). Furthermore, compressive force enhanced the gene expression of OPG in MC3T3-E1 cells in both time- and force-dependent manners (Fig. 2B and C).

OPG is produced by several types of cells including osteoblastic cells, and has been shown to be a soluble decoy receptor for RANKL that blocks osteoclast formation by inhibiting RANK–RANKL interactions. In the present study, we clarified that mechanical stress increases the expression of OPG in MC3T3-E1 cells and then inhibits osteoclastogenesis. Other inhibitory factors for osteoclastogenesis such as interferon-c (IFN-c and Toll-like receptor (TLR) ligands have been shown to function by suppression of RANK signaling in osteoclast precursors [30]. We also evaluated the release of IFN-c protein derived from MC3T3-E1 cells into the conditioned medium, however, no significant stimulation by mechanical stress was observed (data not shown). Together, these results suggest that the inhibitory effect of medium conditioned by mechanical stress on osteoclastogenesis is mainly dependent on the up-regulation of OPG expression.

A number of studies have investigated signaling pathways induced by RANK–RANKL binding. The cytoplasmic domain of RANK was shown to contain a binding site for TRAF6 [31]. In another study, NF-j B, MAPK, c-Jun N-terminal protein kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) were found to be activated downstream of TRAF6 and induced osteoclast differentiation [32]. NF-j B is present in the cytoplasm as an active heterotrimer consisting of p50, p65, and Ij Ba subunits. Upon activation of the complex, phosphorylation and degradation of Ij Ba exposes nuclear localization signals on the p50/p65 complex, leading to nuclear translocation and binding to specific regulated sequences in DNA [33]. We found that mechanical stress suppressed the expression of TRAF6 protein, phosphorylation of Ij Da, and nuclear translocation of p50 and p65 (Fig. 3). These results suggest that down-regulation of TRAF6 and NF-j B-mediated signaling pathway is correlated with inhibition of osteoclastogenesis by conditioned medium of mechanical-loaded osteoblast.

Mechanical stress is also known to stimulate multiple transduction cascades in several types of cells. It has been demonstrated that application of mechanical stress activates MAPKs, JNK, and ERK [34–36]. Furthermore, the canonical Wnt/b-catenin pathway was shown to have an important role in regulating osteoblast and osteoclast functions, as well as involvement in mechanotransduction [12]. Among the three Wnt pathways, non-canonical Wnt/Ca^{2+} pathway is well known to regulate two different downstream signaling pathways, the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and calcineurin, Ca^{2+}/calmodulin-dependent phosphatase (CaMP) pathway [37]. Although calcineurin regulates osteoclast differentiation via activity of the nuclear factor of activated T cells [38], the effect of CaMK in osteoclastogenesis is not clear. Yu et al. [18] reported that mechanical stress-mediated OPG induction was regulated by the non-canonical Wnt/Ca^{2+} pathway and especially the CaMK II-NLK cascade in myoblast lineage cells. Interestingly, we clearly demonstrated that the expression level of OPG mRNA and protein induced by mechanical stress was remarkably suppressed by KN-93, a selective Ca^{2+}/calmodulin-dependent kinase II inhibitor (Fig. 4A and B). Furthermore, the enhanced binding ability of medium conditioned by mechanical stress was diminished by the addition of KN-93 (Fig. 4C). Together, these findings suggest that stimulation of OPG mRNA and protein expression by mechanical stress is dependent on CaMK.

In conclusion, we found that compressive force enhanced the expression of OPG in osteoblasts by activation of the non-canonical Wnt/Ca^{2+} pathway. These results suggest that osteoblasts have the capacity to sense changes in mechanical stress, resulting in regulation of osteoclastogenesis.

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編集後記

本邦の高齢化率は世界に類を見ない速度で上昇しており、すでに 23%を超えた。これに伴い、医療や介護を必要とする要介護高齢者等も増加していると考えられる。とくに、後期高齢者の増加も報告されており、本年年度行った本研究事業における介入研究においても，その平均年齢が 80 歳後半と高かった．後期高齢者の増加は，要介護高齢者の増加ということも言えることから、ケアの充実は重要な課題の一つと考えられる。

要介護高齢者では、障害や疾患などのために単調な日常生活となったり，多くの薬剤を服用していることも少なくない．したがって、口腔に関しても、齲蝕や歯周病という二大歯科疾患だけでなく、口腔乾燥症などの唾液分泌や口腔粘膜状態など口腔環境の問題や、摂食嚥下機能障害など機能的な問題も考慮する必要がある。また、臨床的にはドライマウスによる口腔の違和感や痛みが摂食欲求を減少させたり、唾液の自浄作用の低下により口腔清掃状態が不良になるために口臭などの問題が引き起こされ、良好な介護実施を行うことが難しくなる場合もある。

本研究事業では、高齢者のドライマウスの実態調査と標準的ケア指針の策定を目的に、平成 22 年度から 3 年計画で総合的研究を開始した。今年度は 2 年目でもあり、昨年の実態調査によるリスク要因の解析結果を参考にして標準的口腔ケア方法を確立するために、要介護高齢者に対する全国規模での介入研究を実施し効果を得られた．

今後は、これらの結果をもとに、より効果的な標準的口腔ケア方法の確立と、初年度に実施した実態調査の追跡調査を行い，さらなるリスク要因の検討から、今後の医療および福祉体制に貢献したいと考えている。

最後になりましたが、本研究事業にご協力いただきました関係各位の皆様方、ならびにご助言をいただきました皆様方に深謝申し上げます。

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