(anti-pPer antibody) was raised in accordance with the previous report (29, 30). Briefly, a 10-week-old female WKY/NCrj rat was immunized with a synthetic peptide containing phosphorylated Ser[66] (ARLGpS[66]FRAPRC). Three weeks after immunization, lymph nodes obtained from the rat were dispersed, and lymphocytes were fused with mouse myeloma Sp2/0-Ag14 cells. The phosphorylation-specific antibody was screened by enzyme-linked immunosorbent assay using hybridoma supernatants, and clone 2C2 was selected. Finally, 2C2 hybridoma cells were injected into the abdominal cavity of nude mice, and prepared ascites were used for immunological assays.

Detection of Peripherin Phosphorylation in Cultured Cells—HEK 293T cells seeded on 60-mm culture dishes were grown to ~80% confluence and transfected with pcDNA3-peripherin and pcDNA3-HA-WT-Akt using Lipofectamin 2000 (Invitrogen). After 8 h, the cells were seeded into 12 well culture dishes and cultured for another 24 h. The cells were then serum-starved for 10 h, treated with insulin (Sigma), and subjected to Western blot analysis using the anti-pPer antibody. If necessary, inhibitors were added to the cultured medium 30 min before insulin treatment. As for the phosphorylation of endogenous peripherin, PC12 cells infected with AxCALNLLacZ (MOI 100), AxCALNLHA-WT-Akt (MOI 100), AxCALNLHA-CA-Akt (MOI 100), or AxCALNLHA-DN-Akt (MOI 100) together with AxCANCre (MOI 30) for 48 h were examined.

Immunoprecipitation—HEK 293T cells seeded on 6-well culture dishes were transfected with pcDNA3-HA-WT-Akt together with pcDNA3 empty vector or FLAG-tagged head domain of peripherin (FLAG-Per 1–103) subcloned into pcDNA3 using Lipofectamin 2000. After 32 h, the cells were serum-starved for 10 h and treated with 100 nM insulin for 20 min. The cells were then washed in Tris-buffered saline briefly and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM Na_3VO_4, and 10 mM NaF). After centrifugation at 10,000 × *g* for 10 min at 4 °C, the soluble fractions were collected and reacted with anti-FLAG antibody followed by precipitation using protein G-Sepharose 4B (Sigma). Immunoprecipitates were rinsed four times with lysis buffer and eluted by adding 2× SDS sample buffer.

Immunohistochemistry—Adult male Wistar rats weighing ~150 g were anesthetized with pentobarbital (40 mg/kg) and positioned supine, and their right hypoglossal nerves were crushed with forceps. The rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer 5 days after surgery. The brains were quickly removed, post-fixed overnight at 4 °C in the fixative, and immersed in 0.1 M phosphate buffer containing 25% sucrose for an additional day. Sections were cut on a cryostat (18 μm in thickness), washed once in PBS, and treated with 10 μg/ml proteinase K for 10 min. After two washes in PBS, the sections were blocked with PBS containing 10% normal goat serum for 1 h and subsequently reacted with primary antibodies (anti-peripherin antibody; 1:1000, anti-pPer antibody; 1:1000) in PBS containing 1% normal goat serum overnight at 4 °C. After three washes in PBS, the sections were incubated with secondary antibodies for 1 h and finally washed three times in PBS. The sections were visualized by fluorescent microscopy (AX70; Olympus, Tokyo, Japan).

RESULTS

Identification of Peripherin as an Akt Substrate in Neurons—To identify novel neuronal substrates for Akt, we utilized the anti-phospho-Akt substrate antibody. Akt preferentially phosphorylates Ser or Thr in the R*X*R*XX*(S/T) motif, and the antibody specifically recognizes this motif only when Ser or Thr is phosphorylated. PC12 cells infected with adenovirus expressing LacZ or CA-Akt were subjected to Western blot analysis using this antibody, and proteins exhibiting more intense signal in the CA-Akt-expressing preparation were searched. Our preliminary experiment using ordinary SDS-PAGE demonstrated stacked positive bands where isolation of the individual positive band was impossible (data not shown). We therefore performed two-dimensional PAGE to also separate proteins by their isoelectric points, and the two-dimensional gels were analyzed by Western blotting using the antibody. We initially used a wide pH range gel for the first dimension and found numerous spots were intensely stained in the CA-Akt-expressing preparation; in particular in the region in which the isoelectric point was 5.0–5.5 and molecular mass was ~60 kDa (Fig. 1*A*). We therefore focused on this region and separated proteins more precisely by using narrow pH range gels for the first dimension (Fig. 1*B*). Six spots that exhibited the intense positive immunostaining were identical to the protein spots in the protein-stained gels (*spots 1–4*, *8*, and *9* in Fig. 1*C*). Judging from their sequential spot patterns, we assumed that spots 1–4 were the same proteins, each of which might have different post-translational modifications. Similarly, the spots 8 and 9 were assumed to be the same protein. As representative samples, spots 1 and 9 were punched out from the gel and analyzed by MALDI-TOF mass spectrometry to identify the corresponding proteins. The subsequent data base search revealed that both spots were identical to peripherin. All spots (spots 1–4, 8, and 9) were confirmed as peripherin by Western blot analysis using the anti-peripherin antibody (Fig. 1*D*).

Akt Phosphorylates Ser[66] of Peripherin in Vitro—Peripherin, whose expression is mostly restricted to neurons in the peripheral nervous system, is a member of type III intermediate filament proteins (31). Because peripherin has not been identified as an Akt substrate, we performed further analysis. First, we aimed to determine the phosphorylation site by Akt *in vitro* using recombinant proteins. Although no typical consensus sequence for the Akt substrate, R*X*R*XX*(S/T), was found in peripherin, five potent sequences existed (Fig. 2*A*). Because several previous papers indicated that Akt could possibly recognize some similar sequences as its target (details are described under "Discussion"), we examined the possibility that Akt was able to recognize and phosphorylate some similar sequences. Four types of GST fusion proteins that contained one or two potent sequences were generated and reacted with recombinant CA-Akt protein in the presence of [γ-^{32}P]ATP (Fig. 2*B*). Autoradiography showed that one fragment containing 51–100 amino acids of peripherin (GST-Per 51–100) was exclusively phosphorylated by CA-Akt among four fragments. Because GST-Per 51–100 contained two potent sequences, SARLGS[66]

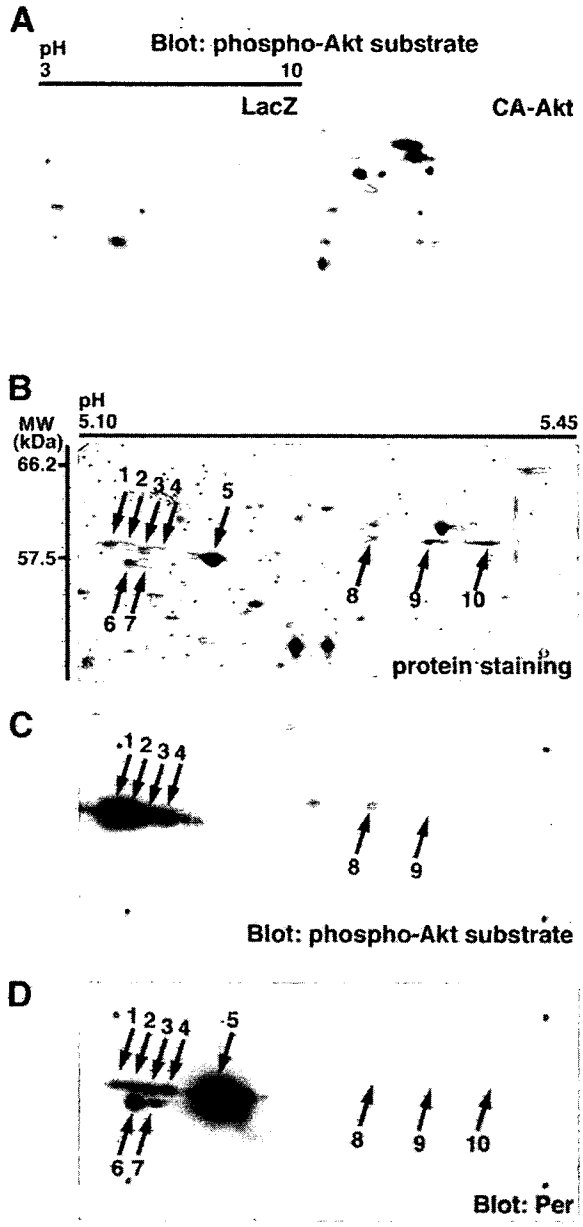

FIGURE 1. **Identification of peripherin as an Akt substrate in PC12 cells.** *A*, immunoblot patterns of PC12 cells expressing LacZ (*left panel*) or CA-Akt (*right panel*). PC12 cells infected with adenovirus expressing LacZ or HA-CA-Akt for 48 h were subjected to two-dimensional PAGE using wide pH range gels (pH 3–10). The gels were then analyzed by Western blotting using anti-phospho-Akt substrate antibody. *B* and *C*, protein staining (*B*) and immunoblotting (*C*) of the gels (pH 5.10–5.45; molecular mass (*MW*), 44–68 kDa) of the CA-Akt-expressing preparation. Extracted proteins from PC12 cells expressing HA-CA-Akt were separated by two-dimensional PAGE using a narrow pH range gel. The gel was stained with SYPRO Ruby, a protein detection reagent (*B*) or transferred to a nitrocellulose membrane followed by immunoblotting using anti-phospho-Akt substrate antibody (*C*). Six spots (*spots 1–4, 8,* and *9*) in *B* were recognized by anti-phospho-Akt substrate antibody in *C*. *D*, peripherin (*Per*) spots in the HA-CA-Akt-expressing preparation. The same membrane used in *C* was reprobed with anti-peripherin antibody. All of the peripherin spots are indicated by *arrows* (*spots 1–10*).

and ALRLPS[79], we then introduced site-directed mutagenesis to GST-Per 51–100 to produce unphosphorylated mutants. These proteins were analyzed by an *in vitro* kinase assay (Fig. 2*C*). The S66A mutation entirely prevented Akt phosphorylation, whereas the S79A mutation did not cause any alterations. These results demonstrate that Akt phosphorylates Ser^{66} of peripherin *in vitro*. The sequence containing Ser^{66} is highly conserved among mammalian species (Fig. 2*D*).

Ser^{66} of Peripherin Is Phosphorylated in Akt-activated Cultured Cells—To evaluate peripherin phosphorylation *in vivo*, a monoclonal antibody (anti-pPer antibody) was raised against the synthetic peptide ARLGpS^{66}FRAPRC. Specificity of this antibody was tested by Western blot analysis using GST-Per 51–100 *in vitro* (Fig. 3*A*). The anti-pPer antibody could detect GST-Per 51–100 only when the fragment was reacted with CA-Akt, and the intense immunoreactivity entirely disappeared when the S66A mutant was used. Using this antibody, peripherin phosphorylation was examined in HEK 293T cells. HEK 293T cells were transfected with WT-Akt and peripherin, because they have no endogenous peripherin, subsequently stimulated with insulin to activate Akt, and peripherin phosphorylation was detected by Western blot analysis. First, HEK 293T cells were treated with increasing doses of insulin, and peripherin phosphorylation was examined (Fig. 3*B*). Both Akt activation, which was evaluated by the phosphorylation state of Akt (32), and peripherin phosphorylation occurred in a dose-dependent manner. Next, we observed changes in peripherin phosphorylation over time after insulin treatment (Fig. 3*C*). Peripherin was phosphorylated in a time-dependent manner, which paralleled Akt activation. To further demonstrate that Akt kinase activity regulated peripherin phosphorylation, we used several inhibitors to modulate Akt activity. Both Akt activation and peripherin phosphorylation were almost prevented by pretreating cells with LY294002 (phosphatidylinositol 3-kinase inhibitor, which inhibited upstream signaling of Akt). In contrast, peripherin phosphorylation was not prevented by Me_2SO (the vehicle for control), U0126 (MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) inhibitor, which prevented mitogen-activated protein kinase signaling), or rapamycin (mTOR inhibitor, which prevented one of the downstream signaling of Akt). We also examined the phosphorylation of endogenous peripherin in PC12 cells by Western blot analysis (Fig. 3*E*). Peripherin phosphorylation was hardly detected in PC12 cells infected with adenovirus expressing LacZ, WT-Akt, or DN-Akt. In contrast, peripherin phosphorylation was clearly observed in cells expressing CA-Akt. Although some minor additional bands were observed at different molecular masses in this blotting using PC12 cells, we assumed those bands would be nonspecific because their intensity was not affected by WT-, CA-, or DN-Akt expression. Together with the results obtained by HEK 293T cells, these results demonstrate that Ser^{66} of peripherin is phosphorylated in an Akt-mediated pathway in cultured cells.

Akt Interacts with the Head Domain of Peripherin in Vivo—It is likely that Akt may directly phosphorylate Ser^{66} of peripherin *in vivo*. To provide further support for this possibility, we examined whether these two proteins could interact *in vivo* using a co-immunoprecipitation experiment. Full-length peripherin, most of which may form intermediate filament in cells, is almost detergent-insoluble (33), and we assumed that peripherin might not be solubilized in a typical lysis buffer for immu-

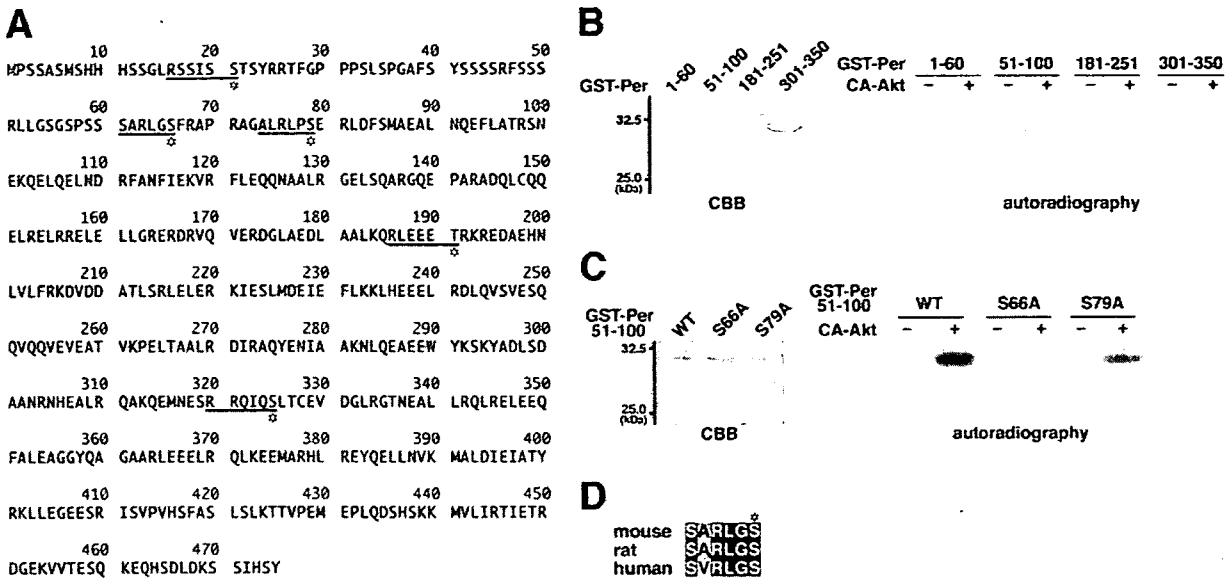

FIGURE 2. **Ser[66] of peripherin is specifically phosphorylated by Akt *in vitro*.** *A*, phosphorylation site candidates in mouse peripherin (*Per*). Peripherin has several potent sequences which are recognized by Akt (*underlined*). Phosphorylation site candidates within respective sequences are indicated by *asterisks* (Ser[21], Ser[66], Ser[79], Thr[191], and Ser[325]). *B* and *C*, *in vitro* kinase assay using recombinant peripherin fragments. GST fusion proteins containing potent sequence(s) were generated. 2 μg of each fragment was subjected to SDS-PAGE followed by Coomassie Brilliant Blue (*CBB*) staining for checking (*left panels*). These fragments were reacted with recombinant His-CA-Akt protein in the presence of Mg^{2+} and [γ-^{32}P]ATP for 30 min, and phosphorylation was detected by autoradiography (*right panels*). Phosphorylation of GST-peripherin 51–100 by CA-Akt was clearly observed (*B*). GST-Per 51–100 carrying the S66A mutation entirely prevented Akt phosphorylation, whereas the S79A mutation did not show any alterations (*C*). *D*, conservation of the motif among different mammalian species. Conserved amino acids between two species and those among three species are shown as *gray* and *black boxes*, respectively. Phosphorylated Ser residue in the motif is indicated by *an asterisk*.

noprecipitation. Therefore, we used a deletion form of peripherin for the immunoprecipitation experiment. Because our preliminary experiment showed that the head domain of peripherin (1–103 amino acids), which contained Ser[66], could be solubilized entirely in radioimmunoprecipitation assay buffer (data not shown), we used the head domain instead of full-length peripherin in this assay. HEK 293T cells transfected with FLAG-Per 1–103 and WT-Akt were treated with or without insulin and subjected to immunoprecipitation using the anti-FLAG antibody (Fig. 4). Akt was co-precipitated with FLAG-Per 1–103, indicating that they could interact *in vivo*. It was of note that this interaction was not dependent on Akt activity because insulin treatment did not enhance the interaction. A similar activity-independent binding has also been reported on several other Akt substrates (34, 35).

Ser[66] of Peripherin Is Phosphorylated in Regenerating Hypoglossal Motor Neurons—Previous reports have revealed that Akt was activated in response to neuronal injury (10, 13, 36). In particular, Akt activation is crucial for nerve-injured motor neurons to regenerate (10). We therefore examined the phosphorylation of endogenous peripherin in nerve-injured hypoglossal motor neurons. We crushed the hypoglossal nerve, and then peripherin expression and phosphorylation were examined by immunohistochemistry 5 days after injury (Fig. 5). Peripherin expression was induced in the cell bodies and also axons of injured neurons (Fig. 5*A*). The higher magnification photographs showed a significant increase of peripherin immunoreactivity in the cell bodies of injured neurons (Fig. 5, *D* and *G*) and a simultaneous induction of peripherin phosphorylation in the cell bodies (Fig. 5, *E* and *H*). However, in injured nerves, immunoreactivity for phosphorylated peripherin was hardly observed, although the peripherin protein was induced and abundantly expressed (Fig. 5, *A–C* and *J–L*). This observation may suggest that enhanced immunoreactivity for phosphorylated peripherin in the cell body of injured neurons is not simply caused by increased peripherin expression. These results indicate that the Ser[66] of peripherin is phosphorylated in regenerating neurons and that phosphorylated peripherin was predominantly localized to their cell bodies but not to axons.

DISCUSSION

We performed a proteomic approach to identify novel Akt substrates in neurons using the anti-phospho-Akt substrate antibody. The present study revealed that Akt phosphorylates Ser[66] of peripherin both *in vitro* and *in vivo*. The antibody we used recognizes the RXRXX(pS/pT) motif, which is preferentially recognized and phosphorylated by Akt. However, the sequence containing Ser[66] (SARLGS[66]) is not typical for Akt substrates where only one Arg residue exists at the −3 position, although the typical one has Arg residues at both −3 and −5 positions. Because a similar variation has been reported for several Akt substrates, the Arg at the −5 position may not always be necessary. For instance, the sequences of PSRTAS in ATP-citrate lyase, LSRRPS in cAMP response element-binding protein, GARRSS in 14-3-3ζ, PMRNTS in p21-activated protein kinase 1, and HVRAHS in Yes-associated protein can be phosphorylated by Akt both *in vitro* and *in vivo* (37–41). As for the +1 position, peripherin has a Phe residue that would be suitable as an Akt substrate because previous studies have shown that a large hydrophobic residue in the +1 position is preferable (42).

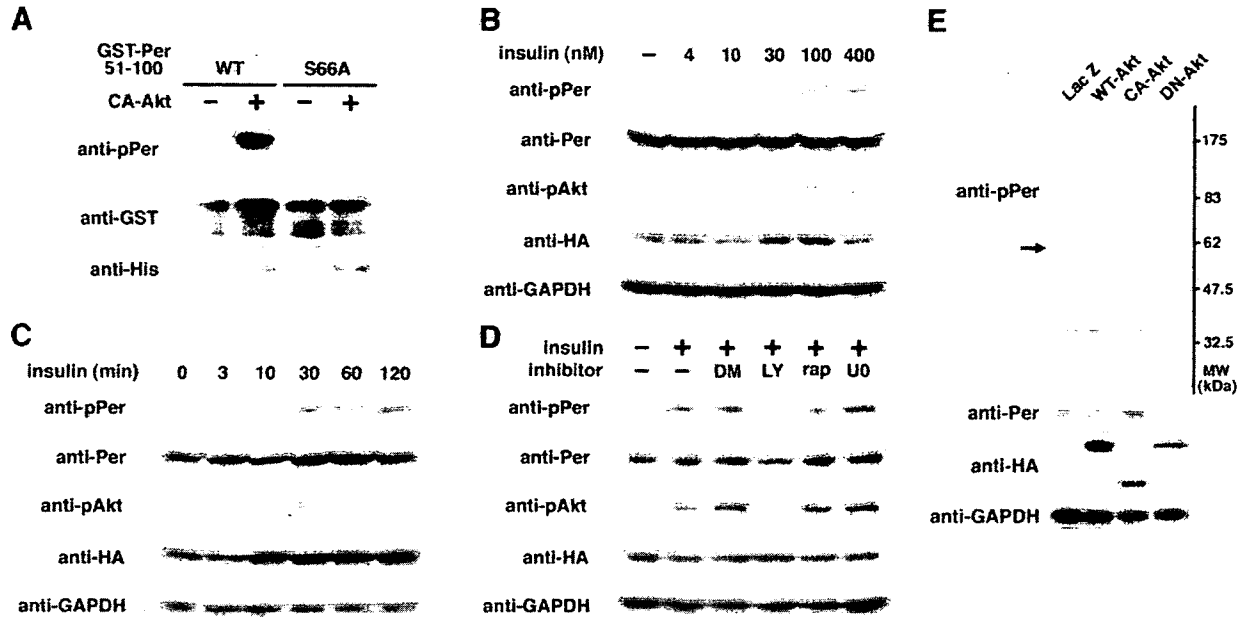

FIGURE 3. **Peripherin phosphorylation in Akt-activated cultured cells.** *A*, Specificity of anti-pPer antibody. GST-peripherin (*Per*) 51–100 WT or S66A was incubated with or without His-CA-Akt for 30 min, and the reaction mixtures were analyzed. Anti-pPer antibody specifically recognized GST-peripherin 51–100 WT reacted with CA-Akt. The antibody did not recognize the S66A mutant even when the mutant was incubated with CA-Akt. *B–D*, HEK 293T cells transfected with peripherin and HA-WT-Akt for 32 h were serum-starved for 10 h and treated with insulin, and peripherin phosphorylation was examined. *B*, dose-dependent effect of insulin on peripherin phosphorylation. HEK 293T cells were treated with different concentrations of insulin for 30 min, and peripherin phosphorylation was detected. The phosphorylation of peripherin, as well as the phosphorylation of Akt, was induced in a dose-dependent manner. *C*, time-dependent effect of insulin on peripherin phosphorylation. HEK 293T cells were treated with 100 nM insulin for indicated times, and peripherin phosphorylation was detected. The phosphorylation of peripherin and Akt was induced in a time-dependent manner. *D*, effect of inhibitors on peripherin phosphorylation. HEK 293T cells were treated with 100 nM insulin for 30 min in the presence or absence of inhibitors. Peripherin phosphorylation was not inhibited by Me_2SO (*DM*), rapamycin (*rap*), or by U0126 (*U0*). In contrast, peripherin phosphorylation was markedly inhibited by LY294002 (*LY*). Note that peripherin phosphorylation was parallel to Akt phosphorylation. *E*, phosphorylation of endogenous peripherin in PC12 cells. PC12 cells infected with adenovirus expressing LacZ, HA-WT-Akt, HA-CA-Akt, or HA-DN-Akt for 48 h were subjected to Western blot analysis, and the phosphorylation of endogenous peripherin was examined. Anti-pPer antibody detected an intense band of ~60 kDa in CA-Akt-expressing preparation (*arrow*), although several nonspecific bands were also detected.

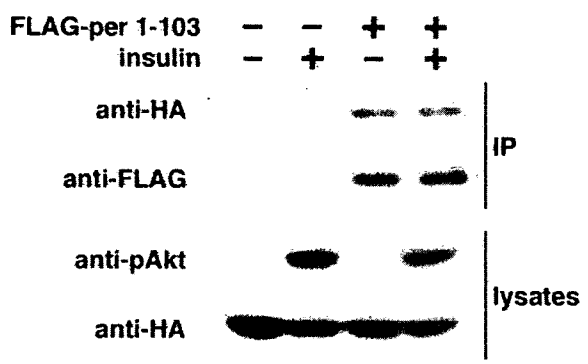

FIGURE 4. **Akt interacts with the head domain of peripherin in HEK 293T cells.** HEK 293T cells transfected with HA-WT-Akt together with empty vector or FLAG-peripherin (*Per*) 1–103 for 32 h were serum-starved for 10 h and treated with or without 100 nM insulin for 20 min. FLAG-Per 1–103 was immunoprecipitated (*IP*) from cell lysates using anti-FLAG antibody, and co-precipitated HA-WT-Akt was detected with anti-HA antibody. As a control, the cell lysates were probed with anti-HA antibody. The same amount of Akt was co-precipitated with FLAG-Per 1–103 with or without insulin.

These studies support the finding that Ser^{66} in peripherin is likely to be phosphorylated by Akt.

The *RXRXX*(S/T) motif is fairly specific as an Akt substrate; however, this motif can also be the phosphorylation target by another evolutionally related kinase, p70 S6 kinase (p70S6K) (43). Because p70S6K is activated downstream of the phosphatidylinositol 3-kinase-Akt pathway (44), the result obtained from our screening raised an alternative possibility in which peripherin might be phosphorylated by p70S6K rather than Akt. This possibility might be disregarded by the results of the *in vitro* kinase assay, where only recombinant proteins were used in these experiments (Figs. 2, *B* and *C*, and 3*A*). However, that result does not entirely rule out a possibility that p70S6K may phosphorylate peripherin *in vivo*, because an *in vitro* kinase is not always an *in vivo* kinase (45, 46). To eliminate this possibility, we examined the effect of p70S6K inhibition on peripherin phosphorylation *in vivo* using cultured cells (Fig. 3*D*). We treated HEK 293T cells with rapamycin, which is known to inhibit p70S6K activation via inhibition of its upstream mTOR kinase (44). The phosphorylation state of peripherin was not changed by rapamycin treatment, whereas it was markedly decreased by phosphatidylinositol 3-kinase inhibitor LY294002 treatment when the cells were stimulated by insulin. Furthermore, we performed a co-immunoprecipitation experiment using the head domain of peripherin and revealed that peripherin was capable of interacting with Akt *in vivo* (Fig. 4). Taken together, these data suggest that Akt directly phosphorylates peripherin *in vivo*.

Although various types of Akt substrates have been identified in a variety of cell types to date, to our knowledge this is the first report that identified an intermediate filament protein as an Akt substrate. Peripherin is a member of type III intermediate filament proteins, which include vimentin, desmin, and glial

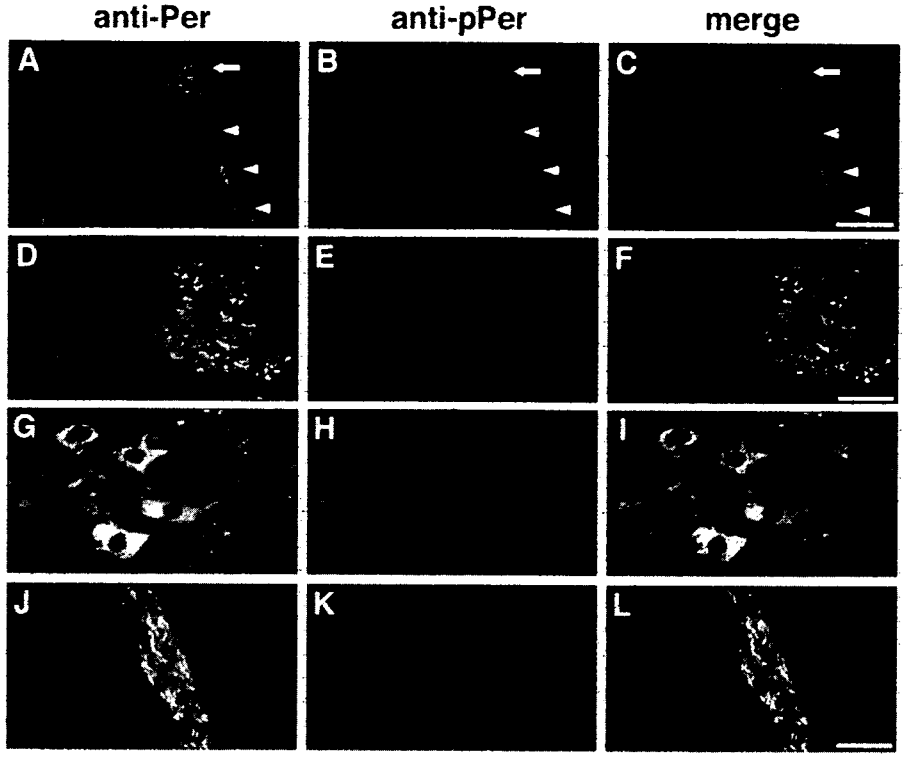

FIGURE 5. **Peripherin phosphorylation in injured hypoglossal motor neurons.** Peripherin (*Per*) phosphorylation was examined in hypoglossal neurons by immunohistochemistry using anti-peripherin (*A*, *D*, *G*, and *J*) and anti-pPer antibody (*B*, *E*, *H*, and *K*) 5 days after unilateral nerve crush injury. *A–C*, low magnification photographs show the entire image of hypoglossal nucleus and nerve in the medulla. Hypoglossal nucleus and nerve on the injured side are indicated by an *arrow* and *arrowheads*, respectively. *D–F*, images of the control nucleus (*left side*) and the injured nucleus (*right side*). Not only peripherin expression, but also peripherin phosphorylation, is significantly induced in the injured nucleus. *G–I*, high magnification photographs of the injured nucleus. Apparent peripherin phosphorylation is observed in cell bodies of injured neurons. *J–L*, high magnification photographs of the injured nerve indicated by *arrowheads* in *A–C*. Phosphorylated peripherin is hardly observed. *Scale bar*, 500 μm (*A–C*), 200 μm (*D–F*), and 50 μm (*G–L*).

fibrillary acidic protein (31). Intermediate filament proteins generally consist of a central coiled-coil α-helical rod domain flanked by a head and a tail domain (47), and Ser^{66} resides in the center of the head domain of peripherin. Generally, head and tail domains of intermediate filament proteins contain several phosphorylation sites, whereas rod domains do not (48). It is well known that a similar neuron-specific intermediate filament, the neurofilament (NF), which belongs to type IV intermediate filament proteins, has multiple phosphorylation sites within its head or tail domains (49). With regard to peripherin, it had been hypothesized that its head domain might contain multiple phosphorylation sites like other intermediate filament proteins (50), and in fact, it has been reported that the N terminus of peripherin, which contains a head domain and a half of rod domain, was phosphorylated in cultured neurons, although the exact phosphorylation sites have not been determined yet (33, 51). Therefore, Ser^{66} could be one of phosphorylation sites previously suggested in those papers.

Of the intermediate filament proteins, peripherin is probably one of the most unknown members in terms of physiological function. However, there are several studies demonstrating its characteristic expression pattern. In contrast to NF proteins, which are widely expressed in various kinds of neurons, peripherin expression is rather restricted to peripheral nervous system neurons (50, 52–54). It is of interest that peripherin expression is transiently induced in injured neurons (55–58). Helfand *et al.* (60) recently revealed that peripherin might be required for both formation and elongation of neurites in PC12 cells, although there remains controversy (59). Therefore, it is likely that peripherin may be involved in neuronal regeneration, probably by contributing to rearrangement of intermediate filaments in neurons during nerve regeneration processes. Although the functional significance of the Akt-mediated peripherin phosphorylation remains unclear, Akt, which is necessary for nerve regeneration, would transfer divergent signals to the substrates, including peripherin, for proper nerve regeneration.

The present immunohistochemical study demonstrated that peripherin phosphorylation occurred endogenously in hypoglossal neurons after nerve injury (Fig. 5). These data suggest that not only peripherin expression but also peripherin phosphorylation may be associated with neuronal regeneration. Although it remains unclear how peripherin phosphorylation plays a role in motor nerve regeneration, immunohistochemical localization of phosphorylated peripherin may provide clues to address this issue. In adult rats, peripherin expression was induced in both cell bodies and axons of injured neurons (Fig. 5, *A*, *D*, *G*, and *J*). However, phosphorylated peripherin was only observed in cell bodies of injured neurons (Fig. 5, *E* and *H*) and could hardly be detected in injured axons (Fig. 5*K*). This cell body-specific localization is reminiscent of phosphorylated NF-L. It has been shown that Ser^{55} within the head domain of NF-L is phosphorylated by protein kinase A (61, 62). This occurred immediately after its synthesis in neuronal cell bodies, and thereafter the phosphorylation of NF-L disappeared, along with its translocation into axons (61). In addition, transgenic mice in which Ser^{55} was replaced with Asp to mimic permanent phosphorylation showed aberrant NF-L inclusions in neuronal perikarya (63). Because NF-L phosphorylation by protein kinase A can lead to disassembly of the filament (64), it is assumed that phosphorylation may block premature assembly of NF-L in cell bodies before transport into axons (49, 61, 63). As mentioned above, Ser^{66} of peripherin is located at the head domain, and some phosphorylation of head domains often causes disassembly of intermediate filaments (48). If Ser^{66} phosphorylation is able to trigger disassembly of peripherin fil-

ament, Akt may control the dynamics of intermediate filament in regenerating axons by phosphorylating newly synthesized peripherin and prevent them from assembly in neuronal cell bodies until the appropriate timing.

It is also possible that peripherin phosphorylation may modulate interactions with other proteins. Previous reports suggest that peripherin has interactive proteins. Peripherin was shown to bind to dystonin (also known as BPAG1-n), which was assumed to be a cross-linker between intermediate filaments and microfilaments (65). Peripherin was also reported to interact with the small heat shock protein αB-crystallin (66). Although it has not been addressed which part of peripherin may be responsible for these interactions, these interactive properties may be regulated by Akt-mediated phosphorylation.

In conclusion, we have identified peripherin as a novel neuronal substrate for Akt both *in vitro* and *in vivo*, and Akt-mediated phosphorylation was induced in regenerating motor neurons. Because Akt is known to play a pivotal role in neuronal regeneration, peripherin would be one of the significant substrates for Akt during nerve regeneration processes. To gain a better understanding of Akt function in regenerating neurons, in particular the functional significance of Akt for the cytoskeletal rearrangement in neurons after nerve injury, further studies, such as how Ser^{66} phosphorylation is capable of changing physiological property of peripherin, are required.

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REVIEW ARTICLE

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Features of limb fractures: a review of epidemiology from a Japanese perspective

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Key words epidemiology · fractures · osteoporosis · incidence · trends

Introduction

Fractures among the elderly which are associated with osteoporosis have become a major health and socioeconomic issue with the rapid increase in the elderly population in both Western and Asian countries. Among the elderly, bone mineral density (BMD) generally decreases with age, and the incidence of fractures increases with age, with the exception of the wrist (Fig. 1) [1]. Among adolescents, BMD increases with age, but the age-specific incidence of fractures does not decrease with age, but rather peaks at the time of the growth spurt (Fig. 1) [2]. These discrepancies between BMD and aging and fracture incidence seem to shed light on the pathogenesis and prevention of fractures.

In this review, the features of geriatric and adolescent limb fractures among the Japanese population will be presented, and an attempt will be made to identify factors for fracture prevention.

Fractures in adolescence

Incidence of fractures in adolescence

Fracture of the distal radius is the most common fracture among children, comprising 20% of total fractures under the age of 17 [3]. The age- and sex-specific incidences under 20 years of age were higher in males than in females, with peaks at 12 or 13 years for males and 11 years for females (Fig. 2) [2]. However, 13-year-old boys and 11-year-old girls are not more active or more prone to falls and trauma than children older or younger than 13 or 11. Therefore, the prominent peak in the incidence of fracture in adolescence has no relation to BMD or physical activity.

Bone mineral density and peak incidence

There is a close relationship between fracture incidence and the relatively low bone density of the radius. The age of peak incidence of distal radius fractures coincides with the age when the metaphyseal/diaphyseal density ratio is lowest (Fig. 2) [2]. The age at which the incidence of fractures peaks almost perfectly matches the age at which the speed of growth in height peaks in both boys and girls. This relative low bone density at the metaphysis is possibly caused by rapid growth during adolescence.

Rauch et al. [4] demonstrated that cortical thickness remains unchanged from 6 to 13 years in girls and from 6 to 15 years in boys, as measured by peripheral quantitative computed tomography (pQCT). The endocortical apposition rate at the distal radial metaphysis should be extremely high in order to maintain cortical thickness during growth. They estimated the endocortical apposition rate at the distal radius metaphysis to be about 9.5–10 μm/day, which is 10–20 times that of the periosteal apposition of the diaphysis. This makes it difficult for the bone to adapt its strength to the increased mechanical needs during growth [5]. After longitudinal growth ceases, the robustness of bone can catch up with the loading needs.

Recent trends

There has been some debate as to whether the incidence of fractures among children in Japan has increased or not. To address this question, all wrist fractures in Tottori Prefecture were surveyed from 1986 to 1995, and the age- and

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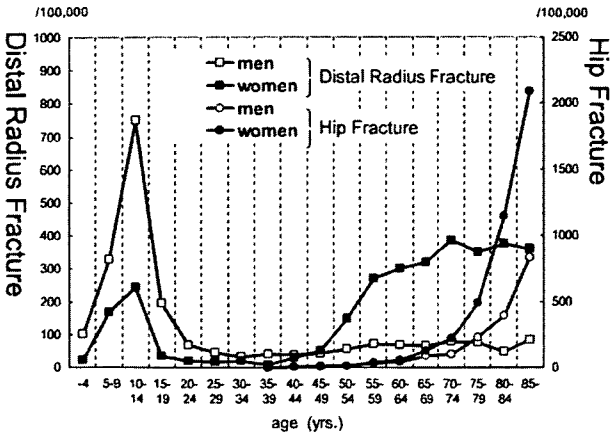

Fig. 1. Age- and sex-specific incidence rates of distal radius and hip fracture. Derived from data in Hagino et al. [1,2,7]

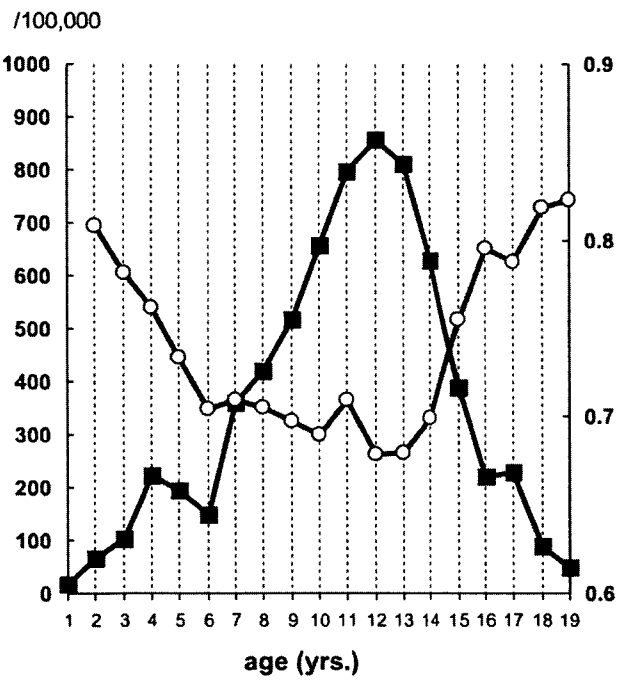

Fig. 2. Fracture incidence and BMD of the distal radial in adolescence for boys. Derived from data in Hagino et al. [2]. —■— Fracture incidence (lt. axis), —○— metaphyseal/diaphyseal density ratio (rt. axis)

sex-specific incidences were calculated, revealing that the incidence increased with time for males between 7 and 12 years of age [6]. Although the changes in incidence among females were not significant, the peak incidence increased during the observation period. Because the mean body height in Japanese adolescents is greater than was previously recognized, this may make the adaptation of bone to loading more difficult than before, and may contribute to weakness at the distal radius with time.

Table 1. Comparison of the incidence of hip fracture in various geographic areas

	Men	Women
Rochester, USA [9]	168.7	382.3
Central Finland [10]	147.8	290.7
Malmo, Sweden [11]	198.1	471.7
Picardy, France [12]	67.4	165.4
Cantabria, Spain [13]	54.2	205.5
Beijing, China [14]	53.3	59.4
Chonnam, Korea [15]	87.2	119.0
Tottori, Japan [7]	58.3	181.3

Data are incidences (per 100000 person-years) adjusted to the population structure of all of Japan (≧35 years, 2000)

Fractures in the elderly

Hip fracture

Lower incidence among the Asian population

There have been many epidemiological surveys of hip fractures all over the world. One of the conclusions derived from these studies is that hip fracture incidence is lower among Asian or African populations than among North American or northern European populations. The age- and sex-specific incidence of hip fractures for both sexes increased exponentially with age after 70 years (Fig. 1) [7,8]. Table 1 shows a comparison of the incidence of hip fracture among different populations based on previous studies. The incidences for both sexes in Asian populations, including Japanese, are substantially lower than those in Caucasian populations living in northern Europe or North America, but not in those in southern Europe.

As bone mass in Asians is known to be lower than or similar to that in Caucasians, the difference in bone mass does not explain the difference in the incidence of hip fractures [16]. Elucidation of the cause of the ethnic differences in the incidence of hip fracture may suggest preventive measures that could protect against osteoporosis-related fractures. Therefore, several different approaches have been tried in order to clarify why the incidence of hip fracture is lower in Asian populations.

Geometry of the proximal femur and fracture type

In the early 1990s, Yoshikawa et al. [17] and Nakamura et al. [18] found that the geometric characteristics of the proximal femoral neck in Japanese women are associated with a lower risk of hip fracture. Faulkner et al. [19] found that a hip axis length (HAL) of 11.0cm corresponds to a doubling of hip fracture risk compared to women with a normal hip length. Duboeuf et al. [20] demonstrated that in the neck fracture group, the HAL was significantly longer than in controls, but this was not true in the trochanteric fracture group.

The incidence of trochanteric fracture is higher than that of neck fracture for individuals over 75 years of age (Fig. 3) [7]. The incidence of neck fracture is substantially lower

than that of trochanteric fractures in Japanese people, but this is not true for Caucasians. The incidence of neck fracture is higher than that of trochanteric fracture in northern European populations, and therefore the neck fracture/trochanteric fracture ratio is different in Japanese and northern Europeans (Fig. 4) [21,22]. The short HAL among Asians affects the lower incidence of neck fractures and might be one possible explanation for a lower incidence of hip fractures.

Risk factors

A case-control study in four Asian countries established the lifestyle factors associated with hip fracture; these are low dietary calcium intake, lack of regular load-bearing activity in the immediate past, no vigorous sports activity between 25 and 49 years of age, cigarette smoking (for men only), daily alcohol consumption, a history of fracture after 50 years of age, a history of falls in the year before a fracture, and a history of stroke [23]. Two studies have examined the risk factors for hip fracture among the Japanese population [24,25]. In one of these studies [23], Suzuki performed a case-control study and found that excessive coffee drinking and sleeping on a bed (as opposed to a futon) were significant risk factors.

In Japan, about 10%–20% of elderly people living in their own homes fall during a year, while 30%–40% fall in northern Europe and North America [26]. The fact that the prevalence of falls among Japanese is half that among Caucasians offers a possible explanation for the difference in the incidence of these fractures [27].

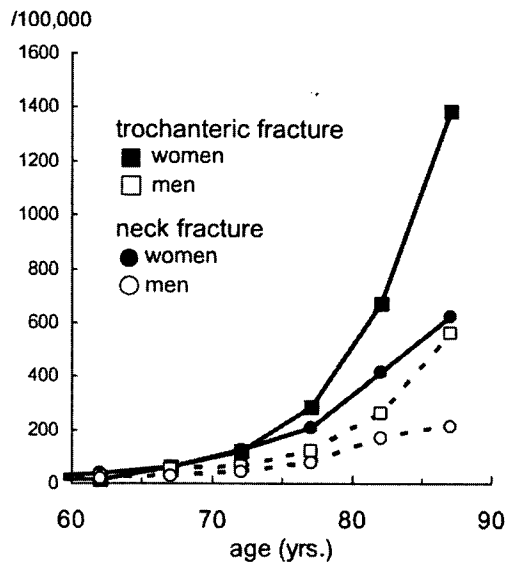

Fig. 3. Age-, sex-, and fracture-type-specific incidence rates of hip fracture in Tottori. Derived from data in Hagino et al. [7]

Changes in incidence over time

Recent trends in the incidence of hip fractures have varied between observation periods or geographic areas. Epidemiological surveys in Europe before 1990 showed that the incidence of hip fractures was increasing [11,28,29]. However, data from the 1990s or later from northern Europe [30,31], North America [32], and Australia [33] indicated that the increase had leveled off [34,35]. On the other hand, most reports from Asia indicate an increase in the incidence of hip fracture with time [7,8,36,37].

The reason for the discrepancies between races has not been clearly elucidated, although changes in lifestyle in Asian countries seem to affect the trend. In Singapore, hip fracture rates from 1991 to 1998 were 5 times higher than rates during the 1960s [38]. In Hong Kong, the fracture rates in 1995 for women 80 years and older were 3 times higher than corresponding rates in the 1960s [39]. However, the incidence rates in these two areas over the last decade have not changed appreciably. The increases were only 1.1 times in Singapore in the 1990s and 1.4 times in Hong Kong from 1985 to 1995. The increase in the incidence rate in Japan of 1.4 times from 1986 to 1998 was very similar. From these points of view, the age-specific incidence of hip fracture among developed and urbanized Asian areas seems to have leveled off over the last decade, even though increases still exist. Urbanization or industrialization, with attendant

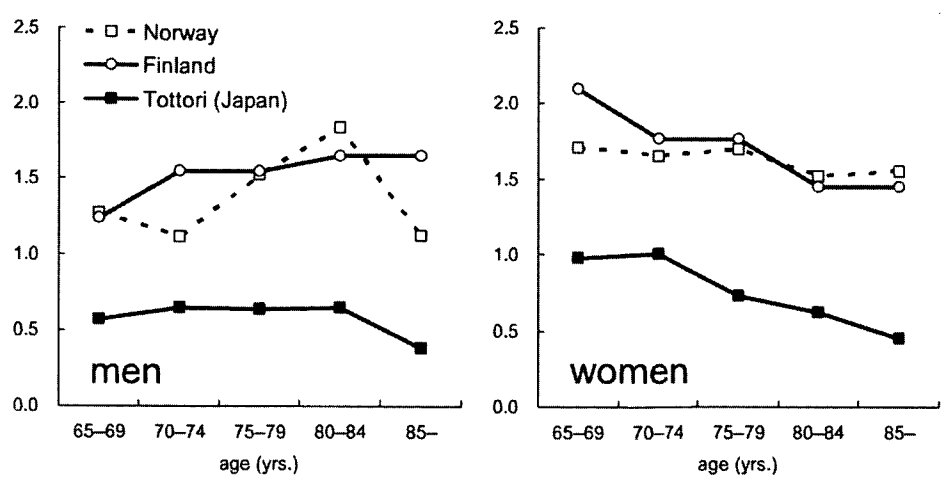

Fig. 4. Neck/trochanteric fracture ratio in different geographic areas. Derived from data in Bjorgul and Reikeras [21], Luthje et al. [22], and Hagino et al. [7]

changes in degree of physical activity, nutrition, alcohol intake, sedative drug use, and lifestyle in Asian countries, may contribute to the rising fracture trend by increasing bone fragility and the risk of falls. On the other hand, adequate diagnosis and treatment for osteoporosis is thought to relate to the decreased incidence of hip fracture in North America [35].

Upper limb fracture

Lower incidence among the Asian population

The incidence of wrist (distal radius) and proximal humerus fractures was surveyed in Tottori Prefecture (Fig. 1) [1]. In women, the incidence of wrist fractures increases after the menopause and plateaus at over 70 years of age; however, the incidence of proximal humerus fractures increases with age over 70 years. This is because elderly people over 75 have difficulty in protecting themselves with their hands when they fall, and instead they injure their hip or shoulder.

Although few epidemiological surveys have been performed to elucidate the incidence among Asian populations, the incidences of wrist and proximal humeral fractures are substantially lower among Japanese than Caucasians. Because 96% of wrist and 95% of humerus fractures are due to falls [40], the lower incidence of upper limb fractures seems to be closely related to the lower incidence of falls.

Risk factors

Epidemiological studies have indicated that risk factors for distal forearm fracture are low bone mass, estrogen deficiency, falls [41], and drinking alcohol [42, 43]. Poor visual acuity, frequent walking [44], and walking at a brisk pace [45] are also risk factors for distal forearm fractures. Among the Japanese population, increased physical activity, in particular increased walking ability, is a risk factor for wrist fractures [46]. This is in agreement with factors identified in previous studies among Caucasians, which concluded that increased physical activity, increased walking ability, and frequent outdoor walking all increase the risk of falls.

On the other hand, a greater frequency of going outdoors significantly decreased the risk of proximal humerus fracture among the Japanese population [46]. This agrees with data from past cohort and case-control studies which identified risk factors for proximal humerus fracture as a low level of physical activity and infrequent walking [47]. The opposite is true for wrist fractures, i.e., wrist fractures are most likely in patients with fragile bones and increased physical activity, while proximal humerus fractures are most likely in patients with fragile bones and decreased physical activity.

One of the significant factors associated with reduced risk of wrist fractures among the Japanese was the use of a futon (as opposed to a bed), which also reduces the risk of hip fracture [24,46]. Futon use might maintain physical activity, resulting in a reduced risk of falls.

Changes in incidence over time

According to our survey performed from 1986 to 1995, the age-adjusted incidence rates of wrist fractures showed a significant increase with time, although no increase was observed among men [1]. Proximal humerus fractures showed a significant increase over time for both sexes. It has been speculated that decreased physical activity associated with a Westernized lifestyle is one possible explanation for the increase in fracture incidence among the Asian population.

Conclusions

In the year 2000, there were an estimated 9.0 million osteoporotic fractures, of which 1.6 million were at the hip, 1.7 million were at the forearm, and 1.4 million were clinical vertebral fractures [48]. It is estimated that the annual number of hip fractures will increase progressively to 2.6 million by the year 2025, and to 4.5 million by 2050 [49]. However, the increase is estimated to be 5 times higher, at 21.3 million, when an annual rate of increase of 3% outside North America and northern Europe is used. This increase will be most marked in Asia.

The percentage of the Japanese population aged 65 years and older will be 23% in 2010, 32% in 2030, and 40% in 2050. Based on the age- and sex-specific incidences observed in the recent study in Tottori, the total number of hip fracture patients in Japan is estimated to be 159000 per year in 2010 and 255000 in 2030. From all these data, we recognize that it is extremely important to implement preventive strategies, which should include the treatment and prevention of osteoporosis, the reduction and prevention of falls, and the maintenance of physical activity among the elderly through lifestyle changes. A reduction in the number of fractures in the elderly is very important in order to reduce the future medical and social burden.

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ORIGINAL

Differences of therapeutic effects on regional bone mineral density and markers of bone mineral metabolism between alendronate and alfacalcidol in Japanese osteoporotic women

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Abstract : We studied the differences of therapeutic effects on regional bone mineral density (BMD) and markers of bone mineral metabolism between alendronate and alfacalcidol in Japanese osteoporotic women. Ninety-two Japanese women suffering from primary osteoporosis without osteoporotic fractures, aged 55 to 81 years, were divided into two groups : women treated orally with alendronate for one-year (5mg/day)(alendronate group, n=35) and women treated orally with alfacalcidol for one year (0.5μg/day) (alfacalcidol group, n=57). The mean BMD of the 2nd to 4th lumbar vertebrae (L2-4 BMD) and regional BMD were measured using dual energy X-ray absorptiometry. In the alendronate group, the percentage changes of L2-4BMD, lumbar spine BMD, thoracic spine BMD, pelvis BMD in the alendronate group were 106.3±4.6%, 104.2±6.6%, 107.1±10.4%, 107.1±10.5%, respectively. The percentage changes of L2-4BMD and regional BMD except for head BMD in the alendronate group were significantly greater than those in the alfacalcidol group. In the alfacalcidol group, L2-4BMD, thoracic spine BMD and lumbar spine BMD were maintained at respective pretreatment levels, whereas other regional BMD were decreased. Both serum bone-specific alkaline phosphatase and urinary type I collagen cross-linked N-telopeptide of the alendronate group were decreased, whereas these markers of bone mineral metabolism of alfacalcidol group were increased compared with the respective pre-treatment levels. The results suggest that one-year treatment with alendronate increased L2-4BMD, lumbar spine BMD, thoracic spine BMD and pelvis BMD, and that markers of both bone formation and bone resorption were decreased following one-year treatment with alendronate. J. Med. Invest. 54 : 35-40, February, 2007

Keywords **:** *alendronate, alfacalcidol, bone mineral density, bone mineral content, markers of bone mineral metabolism*

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INTRODUCTION

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture (1). Osteoporotic fractures are one of significant

complications and an increasing health care burden in osteoporotic patients. As the population of elderly people increases, the incidence of osteoporotic fractures is expected to dramatically rise. At present, large number of postmenopausal women in Japan are at high risk for osteoporosis and its associated fractures, which is an issue of clinical importance. Therefore, in the treatment of osteoporosis, prevention of fractures is undoubtedly a major goal. Smith showed that bone mineral density (BMD) was accounted for approximately 75-80% of the variance in the bone strength (2). It is an undoubted fact that BMD is one of the important determinants of bone strength.

The purpose of this study was to clarify the difference of therapeutic effects of regional BMD and markers of bone mineral metabolism between one-year treatment of alendronate and that of alfacalcidol. Alendronate is a second-generation aminobisphosphonate. Bisphosphonate is an antiresorptive agent of bone and it increases BMD and bone mineral content resulting from inhibition of osteoclast-mediated bone resorption (3, 4), leading to prevention of osteoporotic fractures, hip fractures (5) and vertebral fractures (6). Alfacalcidol, 1 alfa-hydroxyvitamin D_3, is a prodrug of calcitriol, and is one of active vitamin D analog. Alfacalcidol has been widely used in the treatment of osteoporosis in Japan. Alfacalcidol stimulates intestinal calcium absorption, increases urinary Ca excretion and serum Ca levels, and suppresses parathyroid hormone secretion (5). Alfacalcidol is effective to increase BMD and reduces rate of vertebral fracture (7) or hip fracture (8).

MATERIALS AND METHODS

One hundred and three Japanese women suffering from primary osteoporosis without osteoporotic fractures, aged 55 to 81 years, were divided into two groups : women treated orally with alendronate for one year (5mg/day) (alendronate group, n=41) and women treated orally with alfacalcidol for one year (0.5μg/day) (alfacalcidol group, n=62). In the alendronate group, 35 of 41 women completed one-year treatment of alendronate. In contrast, 57 of 62 women in the alfacalcidol group completed one-year treatment of alfacalcidol. All the women had no osteoporotic fractures, no renal injury, no disease of the alimentary tract, no osteomalacia, and no primary or secondary neoplastic bone disease. None of them had received treatments affecting bone metabolism. All the women are allowed to continue their usual diets. Table 1 showed pre-treatment values of body mass index (BMI), age, L2-4BMD, total body BMD. No significant differences were found between the two groups with regard to BMI and age, L2-4BMD and total body BMD.

The mean BMD of the 2nd to 4th lumbar vertebrae (L2-4BMD), regional and total body BMD were measured by DXA using a Hologic QDR 2000 (Waltham, MA, USA). The coefficient of variation (CV) for DXA for total BMD and soft tissue mass was 0.5%-1.0% (9).

The regional BMD (g/cm^2) was measured in the head, arms, ribs, thoracic spine, lumbar spine, pelvis and legs (Figure 1). The regional BMD (g/cm^2) was measured in the head, arms, legs, ribs, thoracic spine, lumbar spine and pelvis. The horizontal line above the shoulders should be just below the chin. The vertical lines at the shoulders should be between the head of the humerus and scapula at the glenoid fossa. The vertical lines on either side of the spine should be moved close to the spine, angled to match the curvature if possible. The small horizontal line should be approximately at the level of L1-T12. The horizontal line above the pelvis should be just above the crest of

Table 1. Comparison of characteristics of alendronate and alfacalcidol groups.

	Alendronat group (n=35)	Alfacalcidol group (n=57)	*P* value
Age	65.2±4.0	66.0±4.5	0.3388
BMI (kg/m^2)	21.5±2.3	22.3±3.1	0.2233
L2-4 BMD (g/cm^2)	0.678±0.082	0.695±0.104	0.427
Total body BMD (g/cm^2)	0.824±0.066	0.836±0.064	0.4061

BMI, body mass index ; L2-4 BMD, the mean bone mineral density of the 2nd to 4th lumbar vertebrae

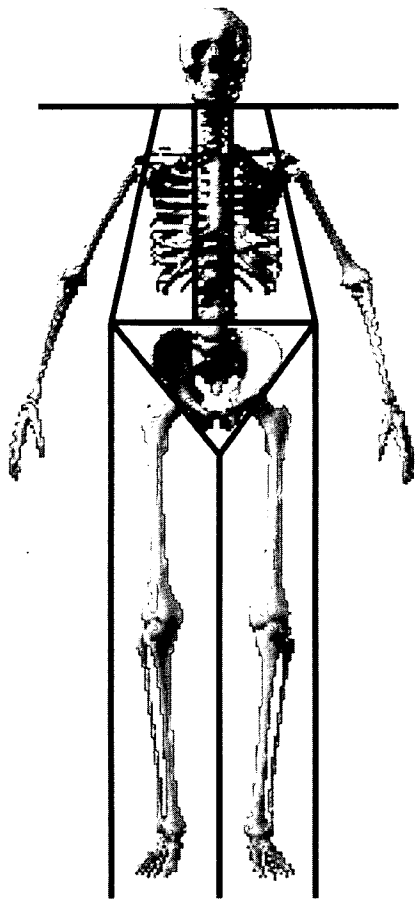

Figure 1. Screen display of total and regional BMD *in vivo*.

the ilium. This line can be extended out at the sides to include soft tissue in the chest and waist. If at all possible, soft tissue from the trunk should not be included with the arms. Due to patient size and placement of the hands, this may not always be possible. The angled lines below the pelvis should bisect both femoral necks. The vertical line between the legs should be adjusted to be between the feet. The vertical lines lateral to (outside of) the legs should be adjusted to include as much of the soft tissue in the thighs as possible.

STATISTICS

Results were expressed as the mean±standard deviation. Unpaired Student's t-test was used to evaluate the differences between the two groups. A P value of less than 0.05 was considered significant.

RESULTS

Fracture

After one-year treatment, 1 of 35 women in the alendronate group and 4 of 57 patients in the alfacalcidol group suffered vertebral fractures.

Regional and total body BMD (Table 2)

Percent change of pre-treatment level of L2-4 BMD and regional BMD of alendronate group were significantly greater than those of alfacalcidol

Table 2. Comparison of percent change of L2-4 BMD, BMD and BMC between alendronate and alfacalcidol groups.

	Alendronate group (n=35)	Alfacalcidol group (n=57)	*P* value
% L2-4 BMD (%)	106.3±4.6	100.7±6.8	< 0.0001
% Lt arm BMD (%)	101.2±4.0	98.7±3.4	0.0019
% Rt arm BMD (%)	100.4±3.4	97.7±3.4	0.0263
% Lt rib BMD (%)	103.2±6.7	98.9±5.5	0.0016
% Rt rib BMD (%)	102.2±4.2	99.6±5.3	0.017
% Thoracic spine BMD (%)	104.2±6.6	98.2±7.0	0.0001
% Lumbar spine BMD (%)	107.1±10.4	100.7±6.9	0.0007
% Pelvis BMD (%)	107.1±10.5	100.6±6.9	0.0013
% Lt leg BMD (%)	100.1±2.6	98.5±3.4	0.015
% Rt leg BMD (%)	100.4±2.2	98.8±3.4	0.0096
% Head BMD (%)	101.3±3.1	101.1±3.5	0.9228
% Total body BMD (%)	101.1±1.8	99.2±2.2	< 0.0001

BMD, bone mineral density ; L2-4 BMD, the mean BMD of the 2nd to 4th lumbar vertebrae.

group except for BMD of head. In the alendronate group, percentage change of L2-4BMD, lumbar spine BMD, thoracic spine BMD and pelvic BMD of alendronate group were greater than those of other regional BMD. There was no significant difference of the percent change of pre-treatment level of head BMD between the two groups.

L2-4BMD, lumbar spine BMD and thoracic spine BMD were maintained at respective pre-treatment levels, whereas other regional BMD were decreased following one-year treatment with alfacalcidol.

Markers of bone mineral metabolism (Table 3, Table 4)

There were no significant differences of pre-treatmet levels of markers of bone mineral metabolism between these two groups (Table 3).

Percent change of urinary type I collagen cross-linked N-telopeptide (NTX) of the alendronate group was decreased to 55.1±30.5% of the pre-treatment level. In contrast, percent change of urinary NTX of alfacalcidol group was increased to 103.3±50.2% of the pre-treatment level. There was significant difference between the two groups (p=0.0002).

Percent change of serum alkaline phosphatase (AP) of alendronate group decreased to 77.4±18.0% of the pre-treatment level, whereas that of alfacalcidol group increased to 106.4±50.9% of the pre-treatment level, which was significantly different (p=0.0073). Percent change of serum bone-specific alkaline phosphatase (BAP) of alendronate group was decreased to 65.6±21.3% of the pre-treatment level, whereas that of alfacalcidol group was increased to 104.8±45.3% of the pre-treatment level. There was significantly difference between the two groups (p=0.0002) (Table 4).

Table 3. Comparison of markers of bone mineral metabolism before treatment.

	Alendronate group (n=35)	Alfacalcidol group (n=57)	*P* value
Urinary NTX (nMBCE/mMCr)	57.0±28.9	45.6±13.5	0.1121
Serum AP (IU/l)	268.3±97.6	281.4±67.8	0.619
Serum BAP (U/l)	36.5±11.9	29.8±14.4	0.0983
Serum Ca (mg/dl)	8.9±1.1	8.6±1.5	0.398
Serum P (mg/dl)	4.0±1.4	4.1±1.5	0.722

AP, alkaline phosphatase ; NTX, type I collagen cross-linked N-telopeptide ; BAP, bone-specific alkaline phosphatase

Table 4. Percent change of markers of bone mineral metabolism.

	Alendronate group (n=35)	Alfacalcidol group (n=57)	*P* value
% Urinary NTX (%)	55.1±30.5	103.3±50.2	0.0002
% Serum AP (%)	77.4±18.0	106.4±50.9	0.0073
% Serum BAP (%)	65.6±21.3	104.8±45.3	0.0002
% Serum Ca (%)	98.0±14.6	97.9±9.4	0.2968
% Serum P (%)	93.5±14.6	94.8±10.4	0.7502

AP, alkaline phosphatase ; NTX, type I collagen cross-linked N-telopeptide ; BAP, bone-specific alkaline phosphatase

DISCUSSION

One-year treatment with alendronate increased L2-4BMD, thoracic spine BMD, lumbar spine BMD and pelvic BMD, and maintained other regional BMD. In contrast, one-year treatment with alfacalcidol maintained L2-4BMD, lumbar spine BMD, pelvis BMD and head BMD, and decreased other regional BMD. Einhorn (10) showed that the relative content of trabecular bone varied among the different parts of the skeleton, and that the content of trabecular bone of vertebra was 66-90%, that of the hip at the intertrochanteric region was 50%, that of the hip at the femoral neck was 25%, that of the distal radius was 25%, that of the mid-radius was 1%, and that of the femoral shaft was 5%. As for bone metabolism, the trabecular bone is approximately eight times as metabolically active as cortical bones, because the surface of trabecular bone is larger than that of cortical bone, and the response to metabolic changes in trabecular bone is faster than that of cortical bone (11). Therefore, the great rate of increase in L2-4BMD, thoracic spine BMD, lumbar spine BMD and pelvis BMD after one-year treatment of alendronate may be due to the high content of trabecular bone compared with other regional bones.

In this study, one-year treatment of alendronate increased lumbar spine BMD, thoracic spine BMD and pelvis BMD. Smith showed that BMD was accounted for approximately 75-80% of the variance in the bone strength (2). In addition to BMD, bone quality is also one of important determinants of bone strength. Alendronate improves trabecular bone microarchitecture of the ileum (greater bone volume, trabecular thickness, decrease in trabecular spacing) (12). Based on these facts, one-year treatment of alendronate increases bone strength and prevents vertebral fracture and hip fracture in osteoporotic patients.

Urinary NTX of the alendronate group were decreased, whereas urinary NTX of the alfacalcidol group was increased. Urinary NTX is one of the markers of bone resorption. This fact showed that alendronate inhibits osteoclast-mediated bone resorption. In addition, one-year treatment with alendronate decreased serum BAP, whereas serum BAP in the alfacalcidol group was increased. Serum BAP is an index of bone turnover. This suggests that one-year treatment with alendronate normalizes bone turnover in patients with osteoporosis.

Previous studies have shown that combination therapy of alendronate and calcitriol are more effective to increase BMD in patients with osteoporosis than monotherapy with either calcitriol or alendronate (13, 14). Recker *et al.* (15) showed that alendronate provides efficacy of bone resorption as measured by serum BAP and urinary NTX, and that alendronate with cholecalciferol increases 25-hydroxyvitamin D (25-OH-D), whereas alendronate without cholecalciferol decreases 25-OH-D. Combination therapy of alfacalcidol and alendronate may be more effective to increase regional BMD and to reduce risk of osteoporotic fractures. Further studies are required to clarify the therapeutic effects of combination therapy of alfacalcidol and alendronate on prophylaxis of osteoporotic fractures.

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Osteoporosis Japan

塩酸ラロキシフェンの新たな骨作用と骨外作用の検索

高田信二郎

はじめに

塩酸ラロキシフェンは，第2世代選択的エストロゲン受容体調整薬（Selected Estrogen Receptor Modulator, SERM）に属する骨粗鬆症治療薬である。その薬理作用は，細胞核内に位置するエストロゲン受容体に結合して初めて発揮される。

塩酸ラロキシフェンは，骨や心臓血管系にはエストロゲン作用を示すが，一方，子宮内膜や乳房組織には，抗エストロゲン作用を発揮するという組織選択性を示すことが[1]，他の骨代謝改善剤とは一線を画するところである。すなわち，塩酸ラロキシフェンは，①破骨細胞機能を抑制して骨量の増加や骨強度を高める，②LDLコレステロールを低下させて抗動脈硬化作用を発揮する，③乳癌細胞の増殖を抑制して乳癌の発生を予防する，④子宮内膜に対する刺激作用がないことから子宮癌の発生リスクを低下させる，という多彩な骨外作用を発揮する。

本研究は，塩酸ラロキシフェンの新たな骨作用と骨外作用をみいだすために企画した。研究成果は，塩酸ラロキシフェンの骨作用と骨外作用について，新たな知見を加えた。

1 方　法

対象は，閉経後骨粗鬆症と診断され，既存骨折がない日本人女性46例であった。年齢は56～72歳，平均年齢は65.5±5.5歳，体格指数body mass index（BMI）は21.6±2.1kg/m^{2}であった。これらを，塩酸ラロキシフェン（60mg）を連日投与したSERM群（n=19），活性型ビタミンD_3製剤としてalfacalcidol（1.0μg）を連日投与したD群（n=27）の2群に分けた。これらの2群では，骨密度に影響を及ぼす因子である年齢と体格指数を一致させた（**表1**）。

表1 SERM群とD群との属性比較

	SERM群	D群	p値
症例数	19	27	
年齢	64.8 ±6.3	65.9 ±4.8	0.4083
体格指数 (kg/m^2)	22.2 ±2.6	21.3 ±1.7	0.3077

身体各部位における骨密度および軟部組織組成の解析，骨代謝マーカーの測定は，治療開始前，塩酸ラロキシフェン投与後6ヵ月の時点で行った。

骨密度および軟部組織組成の解析は，dual energy X-ray absorptiometry（DXA）Hologic社製Delphiを用いた。まず，第2腰椎から第4腰椎正面平均骨密度（L2-4BMD）を測定した。その後，全身骨密度測定プログラムを用いて，身体各部位の骨密度の測定と軟部組織組成の解析を行った。軟部組織組成では，除脂肪量（脂肪を除いた軟部組織重量であり，四肢では，ほぼ筋肉量に相当する）と脂肪量をおのおの測定した。身体各部位の骨密度の測定では，頭部，上肢，肋骨，胸椎，腰椎，骨盤，下肢をその関心領域とし，軟部組織組成の測定領域は，頭部，上肢，体幹，下肢とした（**図1**）。おのおのの測定値は，治療前の値を100%とした百分率で治療開始前後の変化率を求めた。

骨代謝マーカーの測定は，尿中NTX，血清骨型アルカリフォスファターゼ（BAP）を，治療前と治療後6ヵ月の計2回測定した。おのおのの測定値の変動は，治療前の値を100%とした百分率で示した。

治療中における骨折の発生状況は，治療後6ヵ月における胸椎および腰椎の単純X線学的検

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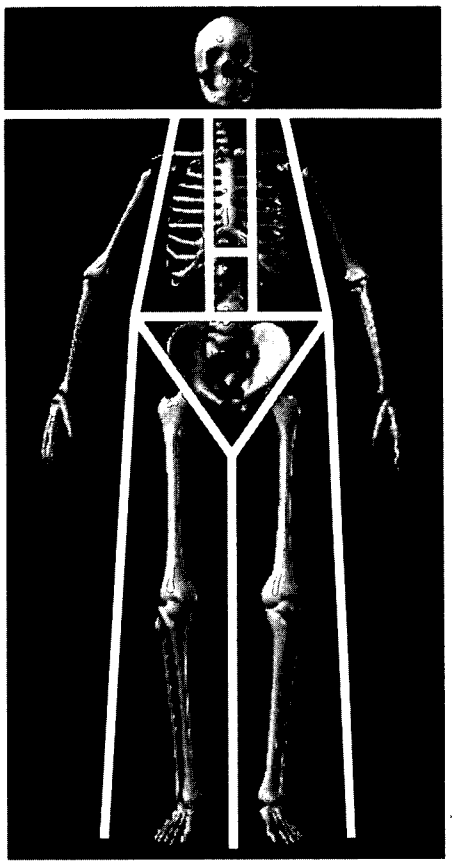

図1　DXAを用いた身体各部位の骨密度および軟部組織組成の関心領域の設定
身体各部位の骨密度，骨塩量の測定における関心領域は，頭部，上肢，肋骨，胸椎，腰椎，骨盤，下肢に設定する。また，軟部組織組成では，頭部，上肢，体幹，下肢を測定領域として設定する。

査を実施して，骨折の有無をみた。

SERM群とD群との間における統計学的比較は，対応のないt検定を用いて行い，その有意水準を5%とした。おのおのの測定値における治療前の値に対する変化率は，対応のあるt検定で統計学的検討を行い，その有意水準は同じく5%とした。

2　結　　果

1）身体各部位の骨密度，除脂肪量，脂肪量の変化

①骨密度の変化

SERM群におけるL2-4BMDは，治療前に比べて増加しており，その変化率は統計学的に有意であった（$p=0.0394$）。一方，D群のL2-4BMDが治療前に比べて減少したが，その変化率には有意差がみられなかった（$p=0.1403$）。

全身骨密度測定プログラムで測定した胸椎骨密度，腰椎骨密度，骨盤骨密度，頭部骨密度，全身骨密度は，SERM群では治療前に比べておのおの増加していた。それらの変化率のうち，骨盤骨密度の変化率のみ，有意な増加がみられた（$p=0.0219$）。

②除脂肪量の変化

SERM群では，左右上肢，左右下肢，全身の除脂肪量は，治療前に比べて増加していた。それらの変化率は，左上肢および右上肢においては有意な変化ではなかった（$p=0.0793$，$p=0.1150$）。しかし，左下肢，右下肢，全身の除脂肪量は，治療前に比べて有意に増加していた（$p=0.0102$，$p=0.0183$，$p=0.0209$）。一方，D群では，左右の上肢および下肢，全身の除脂肪量ともに，治療前に比較して，統計学的に有意な差ではなかった。

③脂肪量の変化

SERM群における左右上肢，左右下肢，全身脂肪量は，除脂肪量の変化とは逆に，治療前に比べて減少していた。それらの変化率のうち，統計学的に有意な減少をみたのは，左右上肢のみであった（$p=0.0378$，$p=0.0466$）。

2）身体各部位の骨密度，除脂肪量，脂肪量のおのおのの変化率の群間比較

①骨密度の変化率の比較（**表2-A**）

L2-4BMD変化率では，SERM群はD群に比べて，有意に高い値を示した（$p=0.0242$）。

②除脂肪量の変化率の比較（**表2-B**）

左上肢，右上肢，左下肢，右下肢，全身の除脂肪量の変化率の群間比較では，SERM群はD群に比べて有意に高かった（$p=0.0299$，$p=0.0351$，$p=0.0128$，$p=0.0412$，$p=0.0114$）。

③脂肪量の変化率の比較（**表2-C**）

脂肪量の変化率では，D群の右上肢の脂肪量変化率はSERM群に比べて有意に高かった（$p=0.0260$）。一方，D群の頭部の脂肪量変化率はSERM群に比べて有意に低い値であった（$p=0.0482$）。

3）骨代謝マーカーのおのおのの変化率の群間比較（表3）

SERM群における尿中NTXおよび血清BAPは，治療前に比べて減少した。一方，D群ではこれらはいずれも治療前の値に比べて増加していた。変化率の比較でも，SERM群の尿中NTXお