Akt, also called protein kinase B, is a serine/threonine protein kinase that plays crucial roles in mediating intracellular signaling of variety of agonists including insulin-like growth factor-I, platelet-derived growth factor and cytokines [12]. Akt regulates biological functions such as gene expression, survival and oncogenesis [12]. Akt is a downstream target of phosphatidylinositol 3-kinase (PI3-kinase) [13,14]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of phosphatidylinositol 3-kinase and activated. As for osteoblasts, insulin-like growth factor-I and platelet-derived growth factor induce translocation of Akt to the nucleus [15]. Recently, Akt is reportedly activated by cyclic stretch or androgen [16,17]. We have recently shown that Akt plays an important role in insulin-like growth factor-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells [18]. However, the correlation between TNF-α and PI3-kinase/Akt in osteoblasts has not yet been clarified.

In the present study, we have investigated whether PI3-kinase/Akt signaling pathway plays a role in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We show here that TNF-α-stimulated IL-6 synthesis through the PI3-kinase/Akt pathway in addition to p44/p42 MAP kinase in these cells.

Materials and Methods

Materials

TNF-α was obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). IL-6 ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Akt inhibitor [1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate], wortmannin, LY294002, PD98059 and rapamycin were obtained from Calbiochem. Co. (La Jolla, CA). Phospho-specific Akt antibodies, Akt antibodies, phosphor-specific p44/p42 MAP kinase and p44/p42 MAP kinase were purchased from New England BioLabs, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Akt inhibitor, wortmannin, LY294002, PD98059 and rapamycin were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1 %, which did not affect assay for IL-6 activity or Western blotting analysis.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [19] were maintained as previously described [20]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35 mm diameter dishes or 90 mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 hours.

Freshly isolated osteoblasts were obtained from the calvaria of new-born (1 or 2-day-old) balb/c mice as previously described [21]. They were seeded into 90 mm diameter dishes (25×104 cells) in α-MEM containing 10% FCS. The medium was changed every 3 days until the cells had reached confluence at about the 5the day. Then, the medium was exchanged for α-MEM containing 0.3 % FCS. The cells were used for experiments after 48 hours.

Assay for IL-6

The cultured cells were stimulated by TNF-α in 1 ml of α-MEM containing 0.3 %. FCS, and then incubated for the indicated periods. The conditioned medium was collected, and IL-6 in the medium was then measured by an IL-6 ELISA kit. Unless otherwise indicated, the cells were pretreated with Akt inhibitor, wortmannin, LY294002, PD98059 or rapamycin, for 60 minutes.

Analysis of Western blotting

The cultured cells were stimulated by TNF-α in α-MEM containing 0.3 % FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2 % sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10 % glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000×g for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [22] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [23] by using phospho-specific Akt antibodies, Akt antibodies, phosphor-specific p44/p42 MAP kinase, or p44/p42 MAP kinase with peroxidase-labeled antibodies raised in goat against rabbit IgG as second antibodies. Peroxidase activity on PVDG membrane was visualized on X-ray film by means of the ECL Western blotting detection system. Unless otherwise indicated, the cells were pretreated with Akt inhibitor, wortmannin, LY294002 or PD98059 for 60 minutes. '

Determinations

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

cDNA Microarray

The cultured cells were washed twice with PBS, and total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Using the obtained total RNAs, cDNA microarray analysis (GeneChip® Expression Analysis; AFFYMETRIX) was performed once by KURABO Industries (Osaka, Japan) with the authorization of AFFYMETRIX Japan K.K. (Tokyo, Japan). The arrays were scanned and the array image was analyzed with GeneChip® operating software (AFFYMETRIX).

Statistical Analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $p < 0.05$. was considered statistically significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effects of TNF-α on the phosphorylation of Akt in MC3T3-E1 cells

To investigate whether TNF-α activates Akt in osteoblast-like MC3T3-E1 cells, we examined the effects of TNF-α on the phosphorylation of Akt. TNF-α significantly induced the phosphorylation of Akt in a time dependent manner (Fig. **1**). The phosphorylation of Akt reached its peak at 15 min, and decreased thereafter.

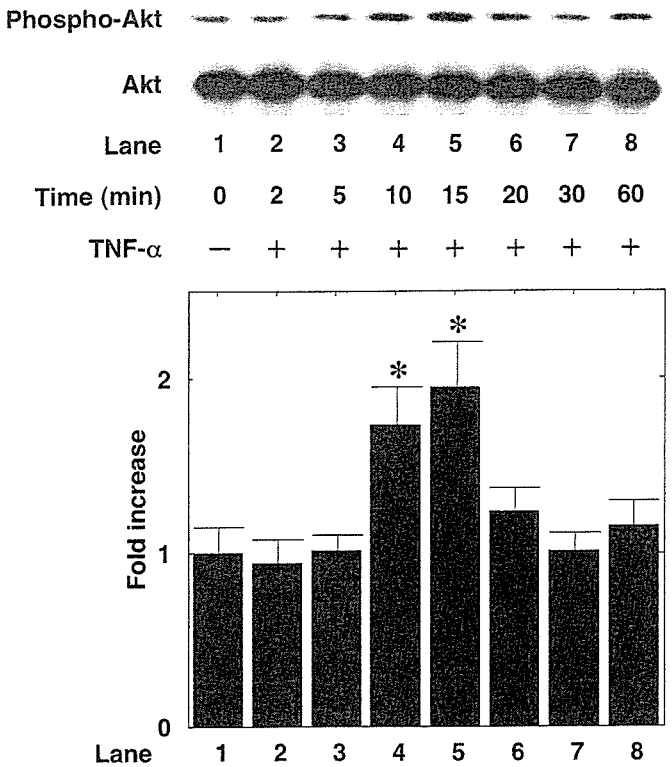

Fig. 1 Effect of TNF-α on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated with 30 ng/ml TNF-α for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of TNF-α-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of control.

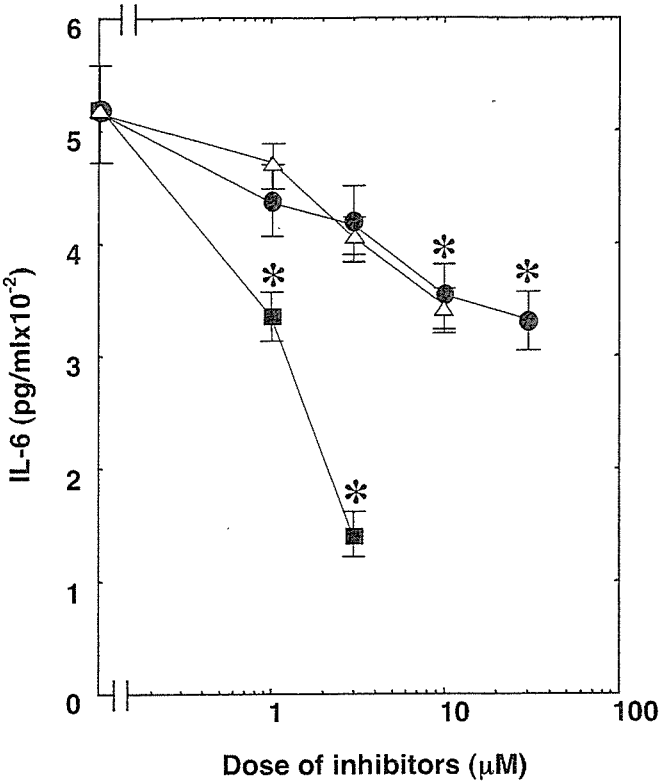

Fig. 2 Effect of LY294002, wortmannin, or Akt inhibitor on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Akt inhibitor (●), LY294002 (■), or wortmannin (△) for 60 min, and then stimulated by 30 ng/ml TNF-α for 48 h. In the absence of TNF-α all IL-6 levels were approximately 25 pg/ml and did not vary with any inhibitor. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of TNF-α alone.

Effect of Akt inhibitor, LY294002, or wortmannin on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells

We next examined the effect of Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate [24], on the TNF-α-stimulated IL-6 synthesis in order to investigate whether Akt is involved in the IL-6 synthesis in MC3T3-E1 cells. Akt inhibitor, which alone did not affect the basal levels of IL-6, significantly suppressed the TNF-α-induced IL-6 synthesis. The inhibitory effect of Akt inhibitor on the IL-6 synthesis was partial and dose dependent in the range between 1 and 30 μM (Fig. **2**). The maximum inhibitory effect of Akt inhibitor at 30 μM caused about 40% reduction in the TNF-α-effect. It is generally known that Akt acts as a downstream effector of PI3-kinase [13,14]. We next examined the effect of LY294002, a specific inhibitor of PI3-kinase [25], on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. LY294002 significantly reduced the synthesis of IL-6 stimulated by TNF-α in a dose-dependent manner between 1 and 3 μM (Fig. **2**). Furthermore, the effect of wortmannin, another inhibitor of PI3-kinase [26], on the IL-6 synthesis was examined. The TNF-α-stimulated synthesis of IL-6 was markedly reduced by wortmannin in a dose-dependent manner between 1 and 10 μM (Fig. **2**).

Effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary culture of osteoblasts

We investigated the effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary culture of osteoblasts. Akt-inhibitor also significantly suppressed the TNF-α-induced IL-6 synthesis (Fig. **3**). The inhibitory effect of Akt inhibitor on the IL-6 synthesis was partial and dose dependent in the range between 1 and 30 μM (Fig. **3**). The maximum inhibitory effect of Akt inhibitor at 30 μM caused about 25% reduction in the TNF-α-effect.

Effects of LY294002 or wortmannin on the phosphorylation of Akt induced by TNF-α in MC3T3-E1 cells

We found that the phosphorylation of Akt induced by TNF-α was markedly attenuated by LY294002 (Fig. **4A**). According to the densitometric analysis, LY294002 (10 μM) caused almost complete reduction of the TNF-α-effect on the Akt phosphorylation. In addition, wortmannin significantly reduced the Akt phosphorylation induced by TNF-α (Fig. **4B**).

Effects of Akt inhibitor, wortmannin or LY294002 on the phosphorylation of p44/p42 MAP kinase induced by TNF-α, and effect of PD98059 on the TNF-α-induced Akt phosphorylation in MC3T3-E1 cells

We have previously shown that p44/p42 MAP kinase plays a part in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-

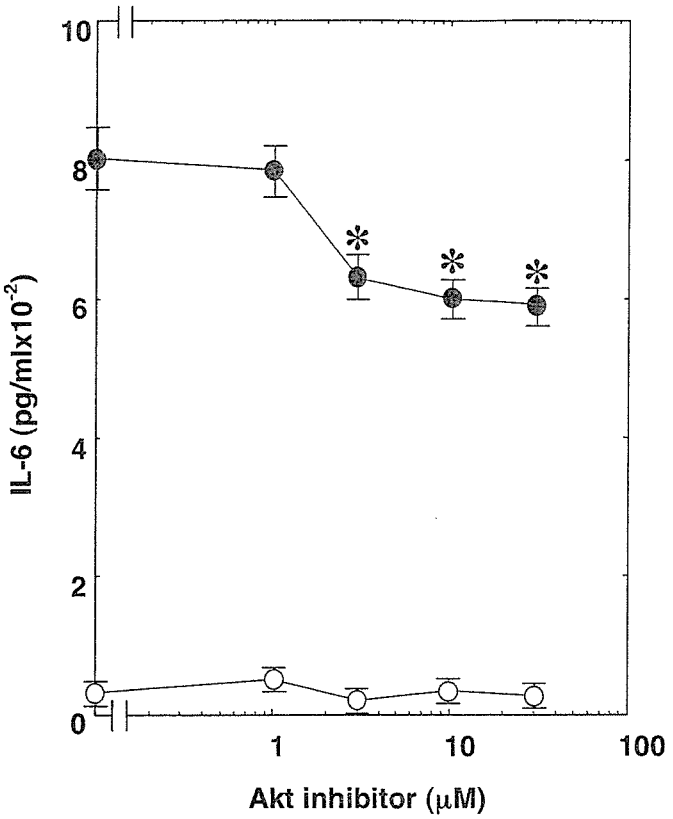

Fig. 3 Effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary osteoblasts. The cultured cells were pretreated with various doses Akt inhibitor for 60 min, and then stimulated by 30 ng/ml TNF-α (●) or vehicle (○) for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of TNF-α alone.

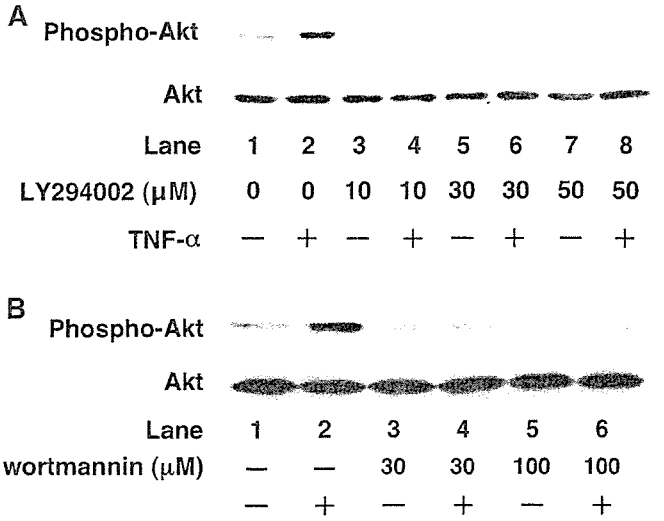

Fig. 4 Effect of LY294002 or wortmannin on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 (**A**) or wortmannin (**B**) for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. Similar results were obtained with two additional and different cell preparations.

E1 cells [10,11], which led us to investigate the relationship between p44/p42 MAP kinase and Akt in these cells. Akt inhibitor had little effect on the TNF-α-induced phosphorylation of p44/p42 MAP kinase (Fig. **5A**). In addition, the TNF-α-induced phosphorylation of p44/p42 MAP kinase was slightly suppressed by wortmannin (Fig. **5B**) or LY294002 (data not shown). These suppressive effects were not statistically significant. Furthermore, PD98059, a specific inhibitor of MEK, upstream kinase that activates p44/p42 MAP kinase [27] failed to affect the TNF-α-induced phosphorylation of Akt (Fig. **5C**).

Effects of rapamycin on TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

It is well known that mammalian target of rapamycin (mTOR) is a downstream target of Akt. We examined the effect of rapamycin on TNF-α-stimulated IL-6 synthesis in these cells. We found that rapamycin markedly increased the TNF-α-induced IL-6 release (25 ± 4 pg/ml for control; 45 ± 9 pg/ml for 10 μM rapamycin; 584 ± 63 pg/ml for 30 ng/ml TNF-α alone; and 1.062 ± 96 pg/ml for 30 ng/ml TNF-α with 10 μM rapamycin, as measured during the stimulation for 48 h).

Effect of TNF-α on mRNA expression of IL-6 receptor and gp130

We next investigated the effect of TNF-α on mRNA expression of IL-6 receptor and gp130 by cDNA microarray. Approximately 45,000 genes were screened. As a result, 1,372 genes showed a greater than 2-fold change in expression level after TNF-α stimulation. The genes of neither IL-6 receptor nor gp130 were included in them. On the other hand, the gene of IL-6 was truly included. It is unlikely that TNF-α affects the IL-6 effect via up-regulation of the receptor or the signal transducer in osteoblasts.

Combined effect of Akt inhibitor and PD98059 on TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

We have shown that the suppressive effect of PD98059 (50 μM) on TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells was partial [10]. We further examined the effect of a combination of Akt inhibitor and PD98059 on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. Combining Akt inhibitor and PD98059 significantly suppressed the TNF-α-stimulated IL-6 synthesis in an additive manner (Table **1**). We have previously confirmed that PD98059 at the concentration used in this experiment is not toxic but specific to MEK1/2 [28].

Discussion

In the present study, we have demonstrated that TNF-α induces the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells depending upon time. Akt mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions such as proliferation and cell survival in a variety of cells [12]. Akt is also activated by phosphorylation of threonine and serine residues [13,14]. Taking these results into account, it is most likely that TNF-α activates Akt in osteoblast-like MC3T3-E1 cells.

We investigated whether Akt is involved in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. Herein, we

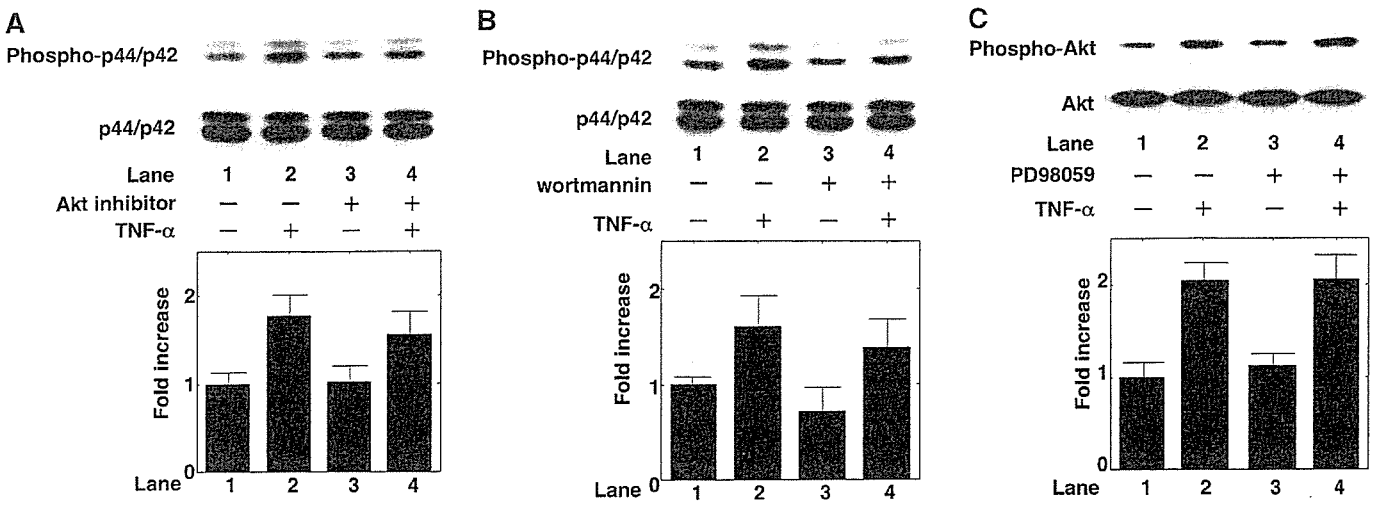

Fig. 5 Effect of Akt inhibitor or wortmannin on the TNF-α-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. Effect of PD98059 on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 50 nM Akt inhibitor (**A**) or wortmannin (**B**) for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. (**C**) The cultured cells were pretreated with 50 μM of PD98059 for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of TNF-α-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean-SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

Table 1 Effect of a combination of Akt inhibitor and PD98059 on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

Akt inhibitor	*PD98059*	*TNF-α*	*IL-6 (pg/ml)*
–	–	–	23 ± 4
–	–	+	506 ± 30*
–	+	–	25 ± 3
–	+	+	388 ± 18**
+	–	–	23 ± 5
+	–	+	359 ± 12**
+	+	–	25 ± 4
+	+	+	257 ± 20***

The cultured cells were pretreated with 30 μM Akt inhibitor, 50 μM PD98059 or vehicle for 60 min, and then stimulated by 30 ng/ml TNF-α for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the control. **$p < 0.05$, compared to the value of TNF-α alone. ***$p < 0.05$, compared to the value of TNF-α with Akt inhibitor or PD98059.

show that a specific inhibitor of Akt [24], significantly suppressed the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. We also confirmed that Akt-inhibitor significantly suppressed the TNF-α-induced IL-6 synthesis also in primary culture of osteoblasts. Thus, it is probable that the activation of Akt is involved in TNF-α-stimulated IL-6 synthesis in osteoblasts. While Akt is a downstream target of PI3-kinase [12–14], we next examined the effects of PI3-kinase inhibitors on the TNF-α-stimulated synthesis of IL-6 in MC3T3-E1 cells. We found that wortmannin [26], significantly reduced the IL-6 synthesis stimulated by TNF-α and markedly attenuated the TNF-α-induced Akt phosphorylation. These findings suggest that PI3-kinase is implicated in TNF-α-stimulated IL-6 synthesis through Akt in MC3T3-E1 cells. We have shown earlier that the IL-6 synthesis induced by TNF-α was significantly suppressed by LY294002 [25]. LY294002 dose dependently reduced the TNF-α-stimulated phosphorylation of Akt. Our results suggest that Akt plays a crucial role as a positive regulator in TNF-α-stimulated IL-6 synthesis at a point downstream from PI3-kinase in osteoblast-like MC3T3-E1 cells.

We have previously reported that the activation of p44/p42 MAP kinase is involved in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells [10,11]. Hence, we investigated to clarify the relationship between p44/p42 MAP kinase and Akt in these cells. We found that Akt inhibitor- and wortmannin-caused minor reductions in TNF-α-induced phosphorylation of p44/p42 MAP kinase did occur in MC3T3-E1 cells, and a specific MEK inhibitor, PD98059 [27] failed to affect the TNF-α-induced phosphorylation of Akt. These results indicate that some cross talk may exist between the PI3-kinase/Akt and the p44/p42 MAP kinase systems, although mostly these are independent. It is not at all unexpected in that Akt will affect many things downstream which may indirectly affect the p44/p42 MAP kinase system. As for the involvement of mTOR, we found that rapamycin markedly increased the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. It is unlikely that TNF-α induces IL-6 synthesis through the PI3-kinase/Akt-dependent activation of mTOR. TNF-α is known to increase the expression of IL-6 genes through activation of nuclear factor-κB (NFκB) in osteoblast-like cells [29]. TNFR2 reportedly facilitates PI3-kinase-dependent NFκB activation [30]. It is possible that the inhibition of PI3-kinase/Akt pathway suppress TNF-α-induced IL-6 synthesis through NFκB inhibition. In addition, from cDNA microarray results, TNF-α truly induced the up-regulation of IL-6 mRNA expression, but influenced the mRNA levels of neither IL-6 receptor nor gp130 in MC3T3-E1 cells. It is unlikely that TNF-α affects the IL-6 effect via up-regulation of the receptor or the signal transducer in osteoblasts.

Additionally, we have shown that the inhibitory effect of PD98059 on the TNF-α-stimulated IL-6 synthesis was partial [10]. In this study, the TNF-α-stimulated IL-6 synthesis was partially reduced by Akt inhibitor (30 μM). We found that a combination of Akt inhibitor and PD98059 additively suppressed TNF-α-stimulated IL-6 synthesis. Based on our findings as a whole, it is most likely that TNF-α stimulates the synthesis of IL-6 via Akt and p44/p42 MAP kinase, and maybe mainly independent of each other in osteoblast-like MC3T3-E1 cells.

The PI3-kinase/Akt pathway plays a pivotal role in several cellular functions, such as proliferation and cell survival in a variety of cells [12]. Our present findings suggest that the PI3-kinase/Akt pathway in osteoblasts has an important role in the regulatory mechanism of the TNF-α-induced production of IL-6 in bone metabolism. IL-6 are potent bone resorptive agent and induces osteoclast formation [1, 4]. Therefore, our present results lead us to speculate that TNF-α-activated PI3-kinase/Akt signaling acts as a positive regulator directing toward bone resorption. It is possible that the PI3-kinase/Akt pathway in osteoblasts might be considered as a molecular target of bone resorption concurrent with various bone diseases. Both TNF-α and IL-6 are well recognized as inflammatory cytokines which play crucial roles in the process of acute and chronic inflammatory diseases. Our present findings might suggest that PI3-kinase/Akt pathway is involved in the process of pathological bone resorption especially in inflammatory bone diseases. In addition, *in vivo* and *in vitro* models of postmenopausal osteoporosis reportedly demonstrate that estrogen deficiency leads to an increase in the adaptive immune function that culminates in an increased production of TNF-α by activated T cells [31]. The signaling molecules contributed to the TNF-α-induced IL-6 synthesis in osteoblasts might be considerable as therapeutic targets of postmenopausal osteoporosis. Further investigation is required to clarify the exact role of PI3-kinase/Akt in osteoblast cell function and bone metabolism.

In conclusion, our results strongly suggest that TNF-α stimulated IL-6 synthesis via PI3-kinase/Akt in addition to p44/p42 MAP kinase in osteoblasts.

Acknowledgements

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Up-regulation by zinc of FGF-2-induced VEGF release through enhancing p44/p42 MAP kinase activation in osteoblasts

Yoshiteru Hanai [a,b], Haruhiko Tokuda [a,b], Eisuke Yasuda [b], Takahiro Noda [b], Toshiki Ohta [c], Shinji Takai [b], Osamu Kozawa [b,*]

[a] *Department of Clinical Laboratory, National Hospital for Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan*
[b] *Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan*
[c] *Department of Internal Medicine, National Hospital for Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan*

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Abstract

We previously reported that basic fibroblast growth factor (FGF-2) activates stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p44/p42 mitogen-activated protein (MAP) kinase resulting in the stimulation of vascular endothelial growth factor (VEGF) release in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether zinc affects the VEGF release by FGF-2 in MC3T3-E1 cells. The FGF-2-induced VEGF release was significantly enhanced by $ZnSO_4$ but not Na_2SO_4. The enhancing effect of $ZnSO_4$ was dose-dependent between 1 and 100 μM. $ZnSO_4$ markedly enhanced the FGF-2-induced phosphorylation of p44/p42 MAP kinase while having little effect on the SAPK/JNK phosphorylation. PD98059 significantly reduced the amplification by $ZnSO_4$ of the FGF-2-stimulated VEGF release. Taken together, our findings strongly suggest that zinc enhances FGF-2-stimulated VEGF release resulting from up-regulating activation of p44/p42 MAP kinase in osteoblasts.

Keywords: Zinc; FGF-2; VEGF; MAP kinase; Osteoblast

Introduction

Zinc is an important regulator of biological functions in humans and many animals (Vallee and Falchuk, 1993). It is generally recognized that growth failure and impaired wound healing occur due to the absence of adequate dietary zinc (Vallee and Falchuk, 1993). In bone metabolism, zinc deficiency results in bone loss. It has been shown that zinc has a stimulatory effect on bone formation and mineralization in vivo and in vitro (Yamaguchi and Yamaguchi, 1986; Hall et al., 1999). Two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption, regulate bone metabolism (Nijweide et al., 1986). As for osteoblasts, it has been reported that zinc increases alkaline phosphatase activity and protein concentration (Hashizume and Yamaguchi, 1994). In addition, the activity of 1,25-dihydroxyvitamin D_3-dependent promoters and the anabolic effect of insulin-like growth factor-I are reportedly enhanced by zinc (Matsui and Yamaguchi, 1995; Lutz et al., 2000). We have recently shown that zinc reduces prostaglandin $F_2\alpha$-stimulated interleukin-6 (IL-6) synthesis via suppression of phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D in osteoblast-like MC3T3-E1 cells (Hatakeyama et al., 2002). However, the exact role of zinc in osteoblasts has not yet been clarified.

Vascular endothelial growth factor (VEGF) is a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells (Ferrara and Davis-Smyth, 1997). VEGF that is produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells (Ferrara and Davis-Smyth, 1997). As for bone metabolism, it has been shown that inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate (Gerber et al., 1999). Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in

* Corresponding author. Tel.: +81 58 230 6214; fax: +81 58 230 6215.
E-mail address: okozawa@cc.gifu-u.ac.jp (O. Kozawa).

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response to various humoral factors (Ferrara and Davis-Smyth, 1997; Goad et al., 1996; Wang et al., 1996; Schlaeppi et al., 1997). During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. Currently, it is well recognized that the activities of osteoblasts, osteoclasts and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism (Erlebacher et al., 1995). Therefore, it is thought that VEGF secreted from osteoblasts may play a crucial role in the regulation of bone metabolism. However, the mechanism behind VEGF synthesis in osteoblasts is not fully known.

Basic fibroblast growth factor (FGF-2) is embedded in bone matrix, and osteoblasts synthesize FGF-2 (Baylink et al., 1993; Hurley et al., 1993). FGF-2 expression in osteoblasts is detected during fracture repair (Bolander, 1992). Therefore, it is thought that FGF-2 may play a pivotal role in fracture healing, bone remodeling and osteogenesis (Marie, 2003). We have previously reported that FGF-2 stimulates VEGF release in MC3T3-E1 cells, and that among the mitogen-activated protein (MAP) kinase superfamily (Tokuda et al., 2003), p44/p42 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) play as positive regulators in the VEGF release (Tokuda et al., 2000, 2003). In the present study, we investigated the effect of zinc on the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. We here show that $ZnSO_4$ up-regulates FGF-2-stimulated VEGF release via enhancing activation of p44/p42 MAP kinase in these cells.

Materials and methods

Materials

FGF-2 and mouse VEGF enzyme immunoassay kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Zinc sulfate ($ZnSO_4$) and Sodium sulfate (Na_2SO_4) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). PD98059 was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from New England BioLabs, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PD98059 was dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or the analysis of MAP kinases.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (Sudo et al., 1983) were maintained as previously described (Kozawa et al., 1992). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes or 90-mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

VEGF assay

The cultured cells were stimulated by FGF-2 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with $ZnSO_4$ or Na_2SO_4 for 20 min. The pretreatment of PD98059 was performed for 60 min before the addition of $ZnSO_4$. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

Analysis of p44/p42 MAP kinase and SAPK/JNK

The cultured cells were stimulated by FGF-2 in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 ×*g* for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli (1970) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996) by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the PVDF sheet was

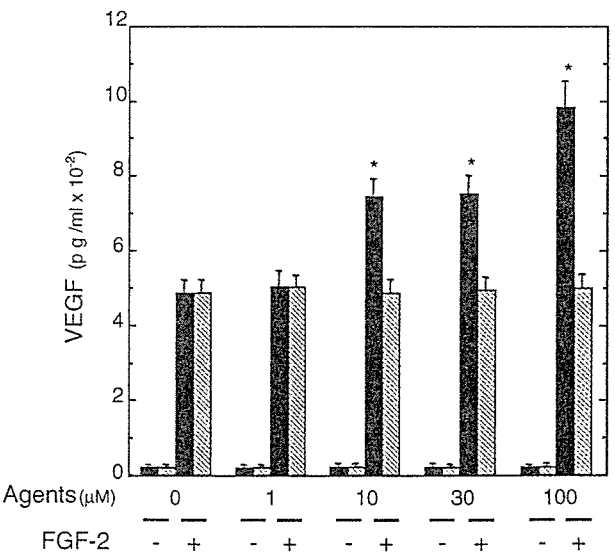

Fig. 1. Effects of $ZnSO_4$ or Na_2SO_4 on FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of $ZnSO_4$ (closed bar) or Na_2SO_4 (hatched bar) for 20 min, and then stimulated by 70 ng/ml FGF-2 or vehicle for 24 h. Each value represents the mean+SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p<0.05$, compared to the value of FGF-2 alone.

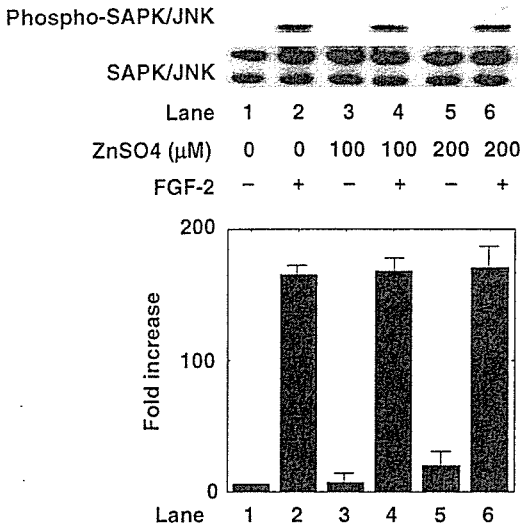

Fig. 2. Effect of $ZnSO_4$ on the phosphorylation of SAPK/JNK induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of $ZnSO_4$ or vehicle for 20 min, and then stimulated by 70 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pretreated with $ZnSO_4$ for 20 min.

Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $p<0.05$ was considered significant. All data are presented as the mean+SEM of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effects of $ZnSO_4$ on the VEGF release by FGF-2 in MC3T3-E1 cells

In our previous study (Tokuda et al., 2000), we have demonstrated that FGF-2 stimulates VEGF release in osteoblast-like MC3T3-E1 cells. To clarify whether zinc affects FGF-2-stimulated VEGF release in MC3T3-E1 cells, we first examined the effect of $ZnSO_4$ on the VEGF release. $ZnSO_4$, which by itself had little effect on the VEGF levels, significantly amplified the FGF-2-stimulated release of VEGF (Fig. 1). The amplifying effect of $ZnSO_4$ was dose-dependent between 1 and 100 μM (Fig. 1). $ZnSO_4$ at 100 μM caused about 110% enhancement in the FGF-2 alone. Na_2SO_4 failed to affect the FGF2-stimulated VEGF release (Fig. 1).

Effects of $ZnSO_4$ on the phosphorylation of p44/p42 MAP kinase and SAPK/JNK induced by FGF-2 in MC3T3-E1 cells

We have previously reported that FGF-2 stimulates VEGF release at least in part via p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (Tokuda et al., 2000, 2003). In order to elucidate if $ZnSO_4$-effect on the FGF-2-stimulated VEGF release is mediated via p44/p42 MAP kinase activation or SAPK/JNK activation in these cells, we next examined the effect of $ZnSO_4$ on the FGF-2-induced phosphorylation of SAPK/JNK kinase. However, $ZnSO_4$ failed to affect the phosphorylation of SAPK/JNK induced by FGF-2 (Fig. 2). On the other hand, the FGF-2-induced phosphorylation of p44/p42 MAP kinase was significantly enhanced by $ZnSO_4$ (Fig. 3). According to the densitometric analysis, $ZnSO_4$ (200 μM) caused about 50% amplification of the FGF-2-effect on the p44/p42 MAP kinase phosphorylation. In addition, $ZnSO_4$ enhanced the p44/p42 MAP kinase phosphorylation in a dose-dependent manner (Fig. 3).

Effect of PD98059 on the enhancement by $ZnSO_4$ of FGF-2-stimulated VEGF release in MC3T3-E1 cells

To furthermore investigate whether the up-regulating effect of $ZnSO_4$ on FGF-2-induced VEGF release is due to

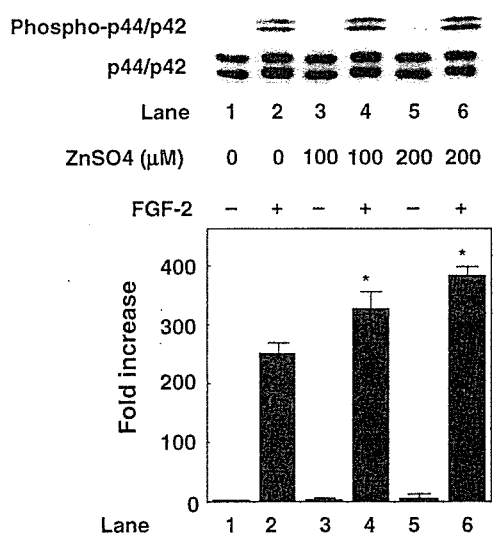

Fig. 3. Effect of $ZnSO_4$ on the phosphorylation of p44/p42 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of $ZnSO_4$ or vehicle for 20 min, and then stimulated by 70 ng/ml FGF-2 or vehicle for 75 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p<0.05$, compared to the value of FGF-2 alone.

Table 1
Effect of PD98059 on the enhancement by $ZnSO_4$ of the FGF-2-stimulated VEGF synthesis in MCT3T3-E1 cells

PD98059	$ZnSO_4$	FGF-2	VEGF (pg/ml)
−	−	−	23±10
−	−	+	502±45*
−	+	−	21±10
−	+	+	955±65**
+	−	−	25±10
+	−	+	263±25**
+	+	−	20±10
+	+	+	272±25***

The cultured cells were pretreated with 10 μM PD98059 or vehicle for 60 min, and then incubated by 100 μM $ZnSO_4$ for 20 min. The cells were stimulated by 70 ng/ml FGF-2 or vehicle for 24 h. Each value represents the mean±SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p<0.05$, compared to the control. **$p<0.05$, compared to the value of FGF-2 alone. ***$p<0.05$, compared to the value of FGF-2 with $ZnSO_4$ pretreatment.

enhancement of p44/p42 MAP kinase activation in MC3T3-E1 cells, we examined the effect of PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase a highly specific inhibitor of MEK (Alessi et al., 1995), on the enhancement by $ZnSO_4$. PD98059, which by itself had no effect on the basal levels of VEGF, significantly reduced the enhancement by $ZnSO_4$ of FGF-2-induced VEGF release to the levels of the FGF-2 with PD98059 (Table 1).

Discussion

In the present study, we showed that $ZnSO_4$, which alone did not affect the levels of VEGF, significantly amplified the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. On the contrary, Na_2SO_4 had little effect on the VEGF release in these cells. Therefore, it is probable that the FGF-2-induced VEGF release is enhanced by zinc in MC3T3-E1 cells.

We next investigated the mechanism of zinc behind the amplification. It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays an important role in cellular functions including proliferation, differentiation, and apoptosis in a variety of cells (Widmann et al., 1999). Three major MAP kinase, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK are known as central elements used by mammalian cells to transduce the diverse messages (Widmann et al., 1999). In our previous studies (Tokuda et al., 2000; Tokuda et al., 2003), we have shown that FGF-2 activates p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells, and these MAP kinases act as positive regulators in FGF-2-induced VEGF release. In the present study, we demonstrated that $ZnSO_4$ did not affect the FGF-2-induced phosphorylation of SAPK/LNK. Therefore, it seems unlikely that $ZnSO_4$ amplified the FGF-2-induced VEGF release through up-regulating the activation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. On the contrary, we showed that the FGF-2-induced phosphorylation of p44/p42 MAP kinase was markedly amplified by $ZnSO_4$ in a dose-dependent manner. It seems that zinc enhances the FGF-2-stimulated VEGF release via up-regulating activation of p44/p42 MAP kinase. We next demonstrated that PD98059 (Alessi et al., 1995) markedly suppressed the enhancement of VEGF release by $ZnSO_4$ almost to the levels of FGF-2 alone. Therefore, it is probable that the enhancement in the FGF-2-induced VEGF release is mediated through the activation of p44/p42 MAP kinase. Based on our findings as a whole, it is most likely that zinc up-regulates FGF-2-stimulated VEGF release through enhancing the activation of p44/p42 MAP kinase but not SAPK/JNK in osteoblast-like MC3T3-E1 cells. Further investigation is necessary to clarify the exact mechanism of zinc in the amplification of VEGF release in osteoblasts.

It is well known that the expansion of capillary network providing microvasculature is an essential process of bone remodeling (Goad et al., 1996). Since VEGF is a specific mitogen of vascular endothelial cells (Ferrara and Davis-Smyth, 1997), it is generally recognized that VEGF secreted by osteoblasts functions as an important intercellular mediator between osteoblasts and vascular endothelial cells in bone metabolism. Moreover, it has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of mouse (Gerber et al., 1999), supporting the significance of VEGF in bone metabolism. On the other hand, in bone metabolism, zinc plays as an important regulator in stimulating bone formation and mineralization (Yamaguchi and Yamaguchi, 1986; Hall et al., 1999). It has been reported that zinc enhances the activity of 1,25-dihydroxyvitamin D_3-dependent promoters and the anabolic effect of insulin-like growth factor-I (Matsui and Yamaguchi, 1995; Lutz et al., 2000). In addition, zinc reportedly induces expression of macrophage colony stimulating factor gene in osteoblasts (Kanekiyo et al., 2002). Based on these findings, it is probable that zinc-enhanced VEGF release from osteoblasts plays a pivotal role in the process of bone remodeling via up-regulating the proliferation of capillary endothelial cells. We have previously reported that zinc reduces prostaglandin $F_2\alpha$-stimulated synthesis of IL-6 in osteoblast-like MC3T3-E1 cells (Hatakeyama et al., 2002). It is well recognized that IL-6 is a potent bone resorptive agent which induces osteoclast formation and stimulates osteoclast activity to resorb bone (Rifas, 1999). Taking our results into account as a whole, therefore, it is most likely that zinc stimulates osteogenesis through increasing VEGF activity in addition to the suppression of interleukin-6 activity in bone metabolism. Further investigations are required to elucidate the precise role of zinc in osteoblasts.

Conclusion

Our present results strongly suggest that zinc enhances FGF-2-stimulated VEGF release resulting from up-regulating activation of p44/p42 MAP kinase in osteoblasts.

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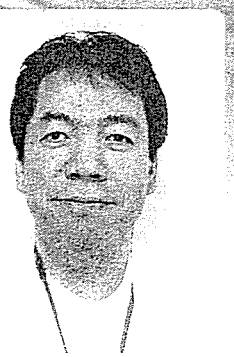
認知症に対する作業療法



ながや まさひろ
長屋政博
国立長寿医療センター病院機能回復診療部骨・関節機能訓練科医長

略歴
1985年：名古屋大学医学部卒業、名古屋記念病院、86年：静岡医療福祉センター、87年：川崎医科大学、92年：国立療養所中部病院、2004年より現職。専門分野：リハビリテーション医学。医学博士

はじめに[1~3]

高齢化社会の進展に伴い、認知症高齢者の数は増加し、その治療やケアが重要な問題となっている。

認知症の中核症状は、「物忘れ」や「時間や今いる場所がわからない」などの記憶・見当識障害であり、副次症状は、うつ状態、夜間せん妄、徘徊、尿・便失禁、易怒などの行動がある。中核症状に対して、薬物療法が症状の進行を遅らせる効果が期待されている。また、周辺症状の問題行動には、介護者をなぐる、ける、かみつくなどの身体的行動、不適切な行動、同じ言葉を繰り返したり、同じような要求をする、大きな声を出したり、悪口雑言を言ったりするなどが含まれる。問題行動は、認知症を有する者のうち80%で起きるとされている。大声は、ナーシングホームで60%の発生率である。問題行動は、認知症ではよく見られ、介護者にとっては非常に大きな負担となっている。過去では、問題行動に対しては、向精神薬、身体拘束、または無視することで対応してきたが、人道上からもQOLの観点からも好ましい対応ではなかった。

認知症を介護していくうえでの目標は、認知症の生活の快適さを向上させること、そして、人間として尊厳ある生活を維持していくことが大切である。つまり、問題行動を向精神薬で穏やかにコントロールされた状態ではなく、むしろ、少々落ち着きがなく騒がしくとも家族や他人と会話したり、ときには逸脱行動があっても表情豊かで元気な状態を求めることにある。このような中で現在、リハビリテーションなどの非薬物療法は、認知症の問題行動の対処法として重要であると考えられている。

認知症に伴うさまざまな問題行動を改善させたり、認知症の精神機能を活性化させ、自発性、集中力や意欲面を向上させたり、認知症の症状を遅らせる目的でさまざまな活動が行われている。

認知症高齢者に対する非薬物療法としては、具体的には、回想法、リアリティ・オリエンテーション、音楽療法、理学療法（筋力強化、バランス訓練、関節可動域訓練）、作業療法（レクリエーション療法、認知トレーニング、家事・家庭内役割作業、手工芸・工作）、園芸療法、演芸療法、社会心理療法、ダンス、散歩、各種体操（ラジオ体操、リズム体操、民謡体操、ストレッチ体操）などがあり、また、環境の整備、介護者への教育・指導なども含まれる。

作業療法とは、身体的・精神的・社会的・職業的な機能の回復を最大限に図り、自立した生活ができる能力を取り戻すことであり、この目的のために作業活動を用いて治療、訓練、指導および援助を行うことである。作業療法で行う作業活動は、日常生活動作の諸動作、仕事・遊び、教育活動など人間の生活全般に関わる諸活動が対象となる。

認知症に対して、現在、当院で行っている具体的な作業療法的アプローチ方法として、レクリエーション療法と認知トレーニングを紹介する。

レクリエーション療法

レクリエーション療法は、音楽、体操、いろいろな種類のゲームなどから構成されている。なかには、回想法、リアリティ・オリエンテーションのプログラムが含まれることもある。当院では、認知症高齢者に対してレクリエーション療法を行ってきた。レクリエーション療法は、リハビリ体操、風船バレーボール、ボウリング、ちぎり絵、ダンスなどからなり、月曜日から金曜日までの週５日、90分間からなり、作業療法士１名と看護師１名で行ってきた。

レクリエーション療法の効果を、脳血管性認知症患者

図　脳血管性認知症のレクリエーション療法前後におけるMMSEの変化

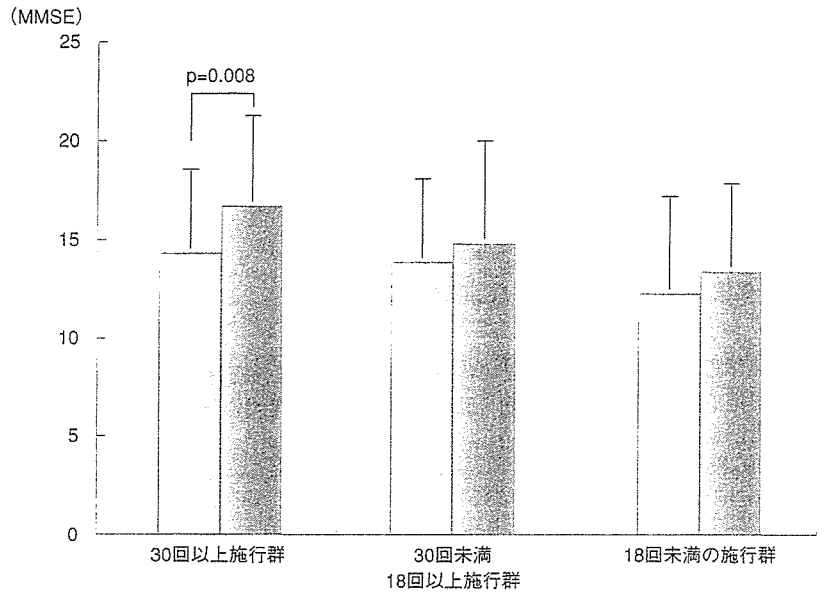


写真1

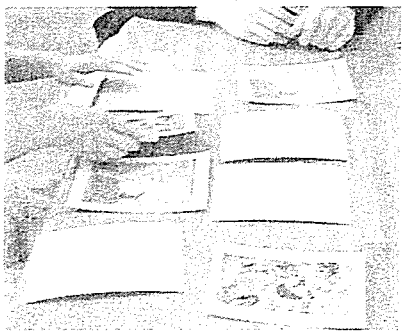
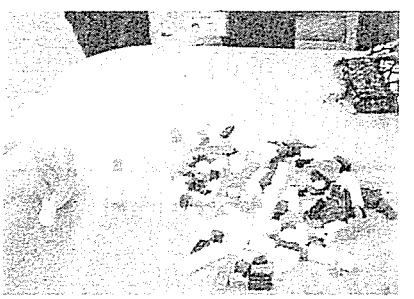


写真2



45名、アルツハイマー型認知症患者37名について、MMSEを用いて比較検討した結果、脳血管性認知症患者では、高頻度に治療した群で(Mini Mental State Examination；MMSE)の有意な改善を認めた[4]。アルツハイマー型認知症患者では、集団訓練の回数ではMMSEに変化がなかった。以上より、脳血管性認知症患者では、レクリエーション療法を施行した場合、MMSEで改善がみられる可能性があり、今後のリハビリ・プログラムとして効果が期待できると考えている(図)。

認知トレーニング

高次脳機能障害に対する作業療法として、生活に必要な時間・物の扱い方・周囲の状況の確認、物事の記憶、計算、動作の順序や方法を決定し遂行していく、などの能力を評価し、治療・訓練する。当院で行っている認知トレーニングの対象者は、MMSEで20から24点前後のもので、問題行動が少なく、作業をこなせる者を目安としている。

具体的な課題としては、絵画記憶課題、計算課題、読み書き課題、迷路課題、ブロック構成課題で、1回20～30分、課題に飽きないように毎日課題内容を変えて施行している。

絵画記憶課題は、神経衰弱カードゲームを簡単にしたものである。4組8枚の絵画のカードを提示して、1分間で位置を記憶してもらい、その後、カードを当てるものである。時間を記録し、正解までの時間が速くなったら枚数を増やすようにしている(写真1)。計算課題としては、ランダムな計算課題を50問から150問前後行ってもらう。1枚50問を2～3枚、時間を記録する。また、サイコロを振ってもらい、出た目を計算する課題を行っている。読み書き課題としては、文章を読む課題として、小学3～4年生が読めるような物語を対象者に読んでもらう。また、読み上げた文章を書き取る課題として、作業療法士が読む物語を対象者に書き取ってもらう。漢字が書けない場合は、ヒントを与えることとしている。迷路課題としては、迷路パズルを1回2～3枚程度行ってもらう。止まった場合、ヒントを与えることとしている。ブロック構成課題としては、動物や自動車などを作り方の手本から作ってもらったり、でき上がったブロックを作ってもらったりしている(写真2)。

課題前後で、MMSE、語想起、数唱(順唱・逆唱)、かなひろい、Trail making test(part A)、記憶力検査(Wechsler Memory scale-Revisedより抜粋)、Barthel indexなどを施行している。対象者はまだ少ないものの、MMSE、語想起、数唱、TMTなど有意な差はみられなかったが、かなひろいテストで有意な改善を認めた。今後、症例を増やして検討していく予定である。

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Comparison of Rat Mandible Bone Characteristics in F344 Substrains, F344/Du and F344/N

Shin TANAKA[1)], Sachi KUWAHARA[1, 2)], Kazutoshi NISHIJIMA[1)], Tamio OHNO[3)], Masahiro NAGAYA[4)], Yasunori NAKAMURA[5)], Yasunori SUMI[5)], Osamu MIYAISHI[6)], Hiroaki AOYAMA[7)], and Nobuo GOTO[8)]

[1)]Animal Facility for Aging Research (AFAR), National Institute for Longevity Sciences (NILS), 474-8522, [4)]Department of Rehabilitation, [5)]Division of Oral and Dental Surgery, Department of Advanced Medicine, National Hospital for Geriatric Medicine, National Center for Geriatrics and Gerontology (NCGG), 36–3 Gengo Morioka-cho, Obu 474-8511, [2)]Department of Anatomy, Hyogo College of Medicine, 1–1 Mukogawa-cho, Nishinomiya, 663-8501, [3)]Division of Experimental Animals, Center for Promotion of Medical Research and Education, Graduate School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, [6)]Chubu Rosai Hospital, 1–10–6 Koraku, Minato-ku, Nagoya 455-8530, [7)]Institute for Environmental Toxicology, 4321 Uchimoriya-cho, Joso 303-0043, and [8)]Japanese Society for Laboratory Animal Resources 2–8–10 Iwamoto-cho, Chiyoda-Ku, Tokyo 101-0032, Japan

Abstract: *The characteristics of the mandible bone were compared through DXA methods between two major substrains of F344 rats, F344/DuCrlCrlj and F344/NSlc at around 60 days of age. Since these two substrains are clearly different in survival and mandible morphology, some genetic differences are supposed to exist. In contrast to a previous microsatellite analysis, clear and significant differences were detected in the body and mandible weights, the mandible bone mineral contents (BMC), bone area (AREA), bone mineral density (BMD) and bone mineral ratio (BMR), between F344/DuCrlCrlj and F344/NSlc, with the mandible molar teeth intact in the bone. Thus, care is needed in the experimental use of these substrains, as results may differ between them. The newly proposed parameter, BMR, may especially contribute to the comparison of bone characteristics among species.*

Key words: *Bone Mineral Density: BMD, Bone Mineral Ratio: BMR, mandible, substrain difference*

Introduction

The longevity sciences, especially the geriatric medicine, need practical and contributive animal models. The most convenient animal models, laboratory rodents, however, sometimes bring unexpected confusion to experimental results due to their species, strain and sex specific biological characteristics, especially their aging properties. Survival, a reliable parameter for the establishment of the aged individuals and the monitor-

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Address corresponding: S. Tanaka, Animal Facility for Aging Research (AFAR), National Institute for Longevity Sciences (NILS), 36–3 Gengo, Morioka-cho, Obu, Aichi 474-8522, Japan

ing of the aging process, was found to be different among F344 substrains and between the USA and Japan [3]. Namely, the survival of F344/N was different from that of F344/Du in Japan and the survival of F344/Du was different between Japan and USA[3, 4]. Microsatellite analysis, however, demonstrated that these two substrains in the two countries were the same [4, unpublished observation]. Morphometric analysis of the mandible shape, known to be effective for the detection of strain differences, demonstrates clear substrain differences among F344 rats [submitted for publication to this journal]. The current research was conducted to compare the mandible bone with special attention to its characteristics including the bone mineral contents (BMC), bone area (AREA) and bone mineral density (BMD) between two F344 substrains available in Japan, F344/DuCrlCrlj and F344/NSlc. These parameters are fundamental to the evaluation of animal models and are related to bone metabolism. Interestingly, these two substrains are identifiable by the shape of their face among F344/NSlc, F344/NHsd and F344/DuCrlCrlj [Tanaka and Miyaishi, unpublished observation].

Materials and Methods

Experimental animals

All animals were male and used at 2 months of age. Two substrains of F344 rats, F344/Du (n=11, Table 1) and F344/N (n=10, Table 1), were purchased from Charles River Laboratories Japan (CRLJ), Yokohama, Japan and SLC Japan (SLC), Hamamatsu, Japan, respectively. Thus, the substrains of "F344" used were F344/DuCrlCrlj and F344/NSlc [1, 3, 4]. The former was fed CRF1 (Crude protein contents: 22.4%, Oriental Yeast, Tokyo) and the latter was fed MRA2 (Current RA2) (Crude protein contents: 22.7%, Nosan, Yokohama), at CRLJ and SLC until the supply, respectively. The substrains are subsequently described only as F344/Du and F344/N, respectively. Animals were housed in groups after weaning at each breeder.

All animal experiments were performed with the permission of the Committee for Animal Ethics NILS according to the Guideline of NILS Animal Experimentation with consideration to animal rights and welfare.

Preparation of the mandible

Animals were sacrificed by an overdose of anesthesia on either 58 or 59 days of age for F344/N, and 61 or 62 days of age for F344/Du on the delivery day after weighing, and the whole tissue around the oral cavity was dissected out. The dissected tissue was autoclaved at 121°C for 5 min. The soft tissue around the mandible was removed carefully. The mandible was incubated overnight in 0.5% papain (Merck, Germany) solution at 37°C and the attaching soft tissue was digested. The incisors were pulled out. After washing and drying, a dried bone specimen of the mandible was prepared.

Measurement of mandible

The mandibles without the incisors were weighed. The whole mandible was measured and surveyed by the DXA method, with DCS-600EX-IIIR, ALOKA, Tokyo.

The bone mineral contents (BMC) in mg, bone area (AREA) in cm^2, and bone mineral density (BMD) in mg/cm^2 were measured. The ratio of the BMC to mandible weight was also calculated. These parameters were measured with the three mandible molar teeth intact in the mandible. The incisors were pulled out in the specimen preparation. The mandible weight and BMC, thus, contained both bone and tooth weight and calcium (hydroxy) apatite.

Statistical procedures

The data obtained were compared by Student's *t*-test after the F test.

Results

Body and mandible weights

As summarized in Table 1, the body weights of F344/Du varied from 181.3 to 196.8 g and the average ± SD was 190.3 ± 4.9 g. In F344/N, the body weights ranged from 167.7 to 181.7 g and the average was 175.7 ± 4.9 g. The body weight was significantly ($P<0.001$) larger in F344/Du than in F344/N.

Similarly, the average mandible weight of F344/Du varied from 173.3 to 184.6 mg and 179.8 ± 2.9 mg, and in F344/N it varied from 162.5 to 175.2 mg and its average ± SD was 170.3 ± 4.8 mg. The mandible weight was significantly ($P<0.001$) larger in F344/Du than in F344/N.

Table 1. Weight, BMC, AREA, BMD and BMR of Mandible on two F344 Substrains, F344/Du and F344/N

Substrain	Days of age	Body weight	Mandible weight	Bone mineral contents	Bone area	Bone mineral density	Bone mineral ratio
		g	mg	BMC: mg	AREA: cm^2	BMD: mg/cm^2	BMR: ratio
F344/DuCrlCrlj							
n=11	61/62						
range		181.3–196.8	173.3–186.4	95.3–103.0	1.509–1.611	62.0–64.3	0.546–0.558
average ± SD		190.3 ± 4.9	179.8 ± 2.9	99.1 ± 2.1	1.558 ± 0.034	63.6 ± 0.7	0.551 ± 0.004
F344/NSlc							
n=10	58/59						
range		167.7–181.7	162.5–175.2	89.4–96.9	1.470–1.550	60.6–63.3	0.542–0.553
average ± SD		175.7 ± 4.9	170.3 ± 4.8	93.2 ± 2.8	1.507 ± 0.030	61.9 ± 1.1	0.547 ± 0.004
t-test		$P<0.001$	$P<0.001$	$P<0.001$	$P<0.01$	$P<0.001$	$P<0.05$

Bone mineral contents (BMC)

The bone mineral contents (BMC) of the F344/Du mandible varied from 95.3 to 103.0 mg and were 99.1 ± 2.1 mg on average. The BMC of the F344/N mandible varied from 89.4 to 96.9 mg and was 93.2 ± 2.8 mg on average. The BMC was significantly ($P<0.001$) larger in F344/Du.

Bone area (AREA)

The area (AREA) of the mandible in F344/Du varied from 1.509 to 1.611 cm^2 and was 1.558 ± 0.034 cm^2 on average. The AREA of the F344/N mandible ranged from 1.470 to 1.550 and was 1.507 ± 0.030 cm^2 on average. The AREA was significantly ($P<0.01$) larger in F344/Du.

Bone mineral density (BMD)

The bone mineral density (BMD), which was calculated by dividing the BMC with AREA of the F344/Du mandible ranged from 62.0 to 64.3 and was 63.6 ± 0.7 mg/cm^2 on average. The BMD of the F344/N mandible ranged from 60.6 to 63.3 and was 61.9 ± 1.1 mg/cm^2 on average. The BMD was significantly ($P<0.001$) larger in F344/Du.

The ratio of BMC to the mandible weight (BMR)

The ratio of the BMC to the mandible weight, which was calculated by dividing the BMC with the mandible weight, was ranged from 0.546 to 0.558 and 0.551 ± 0.004 on average for F344/Du; it ranged from 0.542 to 0.553 and 0.547 ± 0.004 on average for F344/N. This ratio was named BMR, bone mineral ratio, and it was significantly ($P<0.05$) larger in F344/Du.

Discussion

For all of the parameters, body and mandible weights, bone mineral contents (BMC), bone area (AREA), bone mineral density (BMD), and the ratio of BMC to mandible weight (BMR), measured or calculated in the present study, statistically significant differences were detected between the two substrains, F344/Du (F344/DuCrlCrlj) and F344/N (F344/NSlc) of the inbred strain of rats, F344 (Table 1). These two substrains, F344/N and F344/Du, were segregated from the original line at Columbia University by transfer to the NIH (National Institutes of Health) in 1949 and to CRL (Charles River Laboratories) in 1976, respectively [1, 3]. In addition to the present results, the survival and disorders with aging are known to be clearly different [2, 3]. The presence of some genetic differences is strongly suggested to be present between these two substrains. However, microsatellite analysis did not detect differences among the four substrains between Japan and USA [4, unpublished observation]. Thus, care is needed in the experimental use of these two substrains, as results may differ between them.

All parameters measured and calculated, especially the body and mandible weights and BMC, were significantly larger in F344/Du (Table 1). This may, in part, be due to the acute growth in F344/Du. The background of this biological characteristic including the

aging should be investigated with special attention paid to the aging changes. It is generally accepted that the later maturity brings longer survival. However, F344/Du survive longer than F344/N [3]. F344/N is characterized by a high incidence of mono nucleated lymphocyte leukemia [2]. A disorder with aging, that is also inherent, may seriously modify the survival or the aging process. The strain or substrain characteristics should be noted and recognized before the usage of these substrains of rats.

The mandibular bone properties observed in the present study seemed to be dependent on growth, except for BMR (Table 1). Some significant substrain differences seem to depend on growth, especially at the age selected for this experiment. An unexpected finding was that of the BMR. A significant substrain difference was also detected in this index, even though both the mandible weight and BMC were significantly different. This index, BMR, was devised to eliminate the substrain differences of these two parameters. However, the BMR showed a significant substrain difference. This means the ratio or the proportion of calcium apatite per unit of mandible bone weight is significantly different between the two substrains. This significant substrain difference in BMR may reflect a difference in bone metabolism, namely a genetic difference. Microsatellite analysis did not detect differences between the two substrains; however, various physiological differences, including the mandible bone properties strongly suggest the presence of a genetic difference. This also raises questions over the experimental usage of these substrains. Since the BMR was calculated for mandibles containing molar teeth, this index should be reassessed using different bones like the femur.

In the present study, a new index, BMR was devised (Table 1). The BMD was calculated by dividing the BMC by the AREA. Usually, the density is calculated by dividing the mass by the volume. The BMD thus calculated, varies according to the size of the bone. To eliminate this discrepancy, the BMC was divided by the bone weight in the present study. This index is expected to reflect the real and direct bone physiology. The bone mineral ratio, BMR, will contribute to the comparison of bone properties among various animal species. The BMR will contribute to recalculate the bone weight from the group of the BMC, AREA and BMD. As a convenient index, BMR should make a great contribution both aging and osteoporosis researches.

Attention should be paid to the fact that the mandible BMC included the calcium apatite content of three molar teeth. Research is underway now to identify the BMC of the mandible bone proper.

In conclusion, major substrain differences were identified in the mandible bone characteristics of F344 rats. The results strongly suggests that the two substrains, F344/DuCrlCrlj and F344/NSlc, are different to each other not only physiologically but also genetically.

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脳血管障害のリハビリテーション

長屋政博　国立長寿医療センター病院骨・関節機能訓練科

脳血管障害

急性期リハビリテーション
回復期リハビリテーション
維持期のリハビリテーション

脳血管障害は，わが国における死因では第3位を占め，寝たきり者の原因第1位であり，いまだ治療および対応が非常に重要な疾患である。脳血管障害のリハビリテーションは，発症からの時期により急性期リハビリテーションから回復期リハビリテーション，維持期のリハビリテーションへと移行していく（図1）。脳血管障害は，脳梗塞か脳内出血，くも膜下出血などの病型，病巣部位や大きさ，種々の基礎疾患の有無により障害の出現と程度が多様であり，多彩な障害に応じてリハビリテーションプログラムも異なってくる。

障害の評価

リハビリテーションを開始する前に，医学的問題と障害の評価を行う必要がある。リハビリテーション科医の専門職がいれば，コンサルトして，障害の評価，リハビリテーションプログラムの立案を依頼する。障害の評価として，麻痺，筋力，関節可動域，協調運動機能，筋緊張，感覚機能，高次脳機能（失行，失認，失語および知的機能），排泄機能などを評価する（表1）。とくに運動機能評価には，Stroke Impairment Assessment Set（SIAS）やBrunnstrom Stageなどを用いる。また日常生活活動（ADL）や装具や補助具は必要性などを評価する。ADLは，移動能力，食事動作，更衣動作，トイレ動作，入浴動作などからなり，代表的なADLの評価は，Barthel Index，機能的自立度評価法（FIM），Katz Indexなどがある。社会的背景として，家族構成，家族関係，家屋状況，経済状態，職業などに関する情報を収集し，評価する。リハビリテーション医学では，初期評価に基づいて機能予後予測を行い，初期目標を立案し，一定期間のリハビリテーションを施行した後再評価を行い，再度リハビリテーションプログラムを立案していく。

ベッドサイドでのリハビリテーション[1~7)]

脳血管障害患者のリハビリテーションプログラムを示す（表2）[7)]。急性期リハビリテーションを開始する上で最大のリスクは，脳血管障害の再発と神経症状の増悪である。脳浮腫は，4～7日で極期で1～2週間で改善する。脳血流自動調節能の障害は，3～4週間続くといわれている。脳血流が安定せず，血圧が安定しない例，または高血圧が続いたり，坐位で起立性低血圧が生じる場合は，プログラムの進行はゆっくりにせざるをえない。しかし，あまり安静臥床を続けることにより，起立性低血圧が誘発されることも考慮に入れなければならない。リハビリテーションプログラムは神経症候が進展している徴候がない限り，できるだけ早く始めるべきである。12～24時間神経症状が

図1 脳血管障害のリハビリテーションの流れ

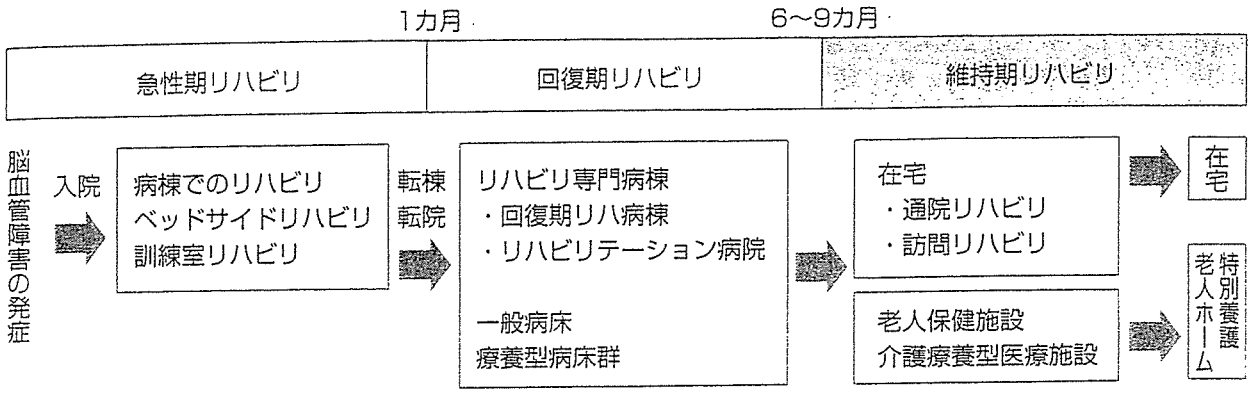

表1 脳血管障害のプログラムリスト

プログラム	評価法
意識障害	Glasgow Coma Scale，Japan Coma Scale
認知障害	MMSE（Mini-mental state examination）， WAIS（Wechsler Adult Intelligence Scale）， HDS（長谷川式簡易知能評価スケール）， 三宅式記銘力検査
失　語	SLTA（Standard Language Test of Aphasia）， WAB（Western Aphasia battery）
失　行	観念運動失行，観念失行，着衣失行，構成失行，習慣的信号動作， 日常物品の使用
注意障害	動作の中で評価する
Motor impresistence （運動維持困難）	閉眼と提舌を30秒以上続けられない
半側無視	線分二等分検査，線分抹消テスト
中枢性麻痺	Brunnstrom stage， SAIS（Stroke Impairment Assessment Set）
運動失調	歩行評価，鼻指鼻試験，踵膝試験
関節可動域	ROM（Range of Motion）
嚥下障害	嚥下ビデオX線検査（Videofluorography；VF），ビデオ内視鏡， 水飲みテスト（原法30mL）10mLでもよい
日常生活活動（ADL）	Barthel Index， FIM（Functional Independence Measure）
参加制約	社会的役割，家族背景，住宅環境，経済状況

安定しておれば，リハビリテーションを始めてよい十分な証である。脳血管障害の症状で，意識レベルや麻痺の悪化は，第14病日までに約25%に認められ，そのうち脳血管障害の再発率は，1.6%でみられる[2]。

脳血管障害の急性期リハビリテーションの目的は，運動機能の回復が第一ではなく，ADLの自立と今後回復期で行われるリハビリテーションの阻害因子となる廃用症候群の予防に主眼が置かれる。最初にベッドサイドで行われるのは，関節拘縮の予防と褥瘡予防に努めるためである。そのためには，ベッド上での他動的な体位変換，良肢位を保つためのポジショニング，適切な

表2 脳血管障害のリハビリテーションプログラム

	ベッドサイドのプログラム		訓練室のリハビリプログラム		退院前プログラム
担当医，リハ医（Dr）	障害の評価 リスクの評価 リハコンサルト リハプログラムの立案・処方 坐位訓練開始時期の判断	訓練室移行の再評価 ソーシャルワーカーへの依頼	下肢装具処方 アームスリング，上肢装具処方 機能予後予測	家族指導 機能予後・訓練期間の説明	外泊指導 家族指導
看護師（Nr）	ポジショニング 良肢位保持 体位変換 関節可動域訓練	嚥下評価・嚥下造影 摂食・嚥下のスクリーニング ADLの指導・介助（排泄訓練，嚥下訓練）	ADL自立支援 心理的支援	病棟内訓練	
理学療法士（PT）	ストレッチング訓練 関節可動域訓練	坐位訓練・坐位耐性訓練 バランス訓練 移乗動作訓練	立ち上がり訓練 基本動作訓練・マット訓練ファミリテーション（筋再教育訓練） 車椅子駆動訓練	平行棒内歩行訓練 杖歩行訓練 応用歩行・階段歩行	ホームプログラムの指導 家屋評価 家族指導
作業療法士（OT）			ADL訓練 機能的作業療法 高次脳機能評価・訓練 ファミリテーション	自助具の作成 利手支援 高度なADL訓練 支持的作業療法	
言語聴覚士（ST）		言語評価 摂食・嚥下評価	言語訓練 嚥下訓練		
ソーシャルワーカー（MSW）			家族状況把握 介護力把握 経済状態・職業の把握	受け入れ体制整備 介護保険の利用 社会資源の利用	

＊リハ医・ソーシャルワーカーなどの専門職がいなければ医師が行う

マットの使用，ときには手または足部にスプリントを使用する。これは，リハビリテーション看護として重要であり，発症当日から可能である。看護師は，口腔ケアを行うとともに摂食・嚥下のスクリーニング評価（水飲みテスト，フードテスト）などを行い，摂食・嚥下訓練も行う。また排泄訓練の前提として，尿意および便意の確認を行い，坐位が可能になれば，介助にてベッドサイドでのポータブルトイレでの排泄を促す。同時に理学療法士および作業療法士による四肢の関節可動域訓練とストレッチングを開始する。拘縮予防として，各関節を5～10回の運動を少なくとも1日2回行う。関節可動域訓練は，意識レベルがJapan coma scale（JCS）分類で2～3桁の状態では他動的に実施する。

次に開始されるのが，坐位訓練および坐位耐性訓練であるが，開始にあたっては，呼吸，血圧，脈拍などのバイタルサインが落ち着き，麻痺の進行が停止したとき，JCSで1桁であることを目安とする。また，病巣の大きさ，病巣の部位，脳浮腫の程度，midline shiftなどのCT所見を参考にする必要がある。初回の坐位訓練としては，ベッドで背もたれ坐位をとらせるか，体幹の安定性があれば，介助でベッドサイドに下肢を下げての端坐位をとらせても