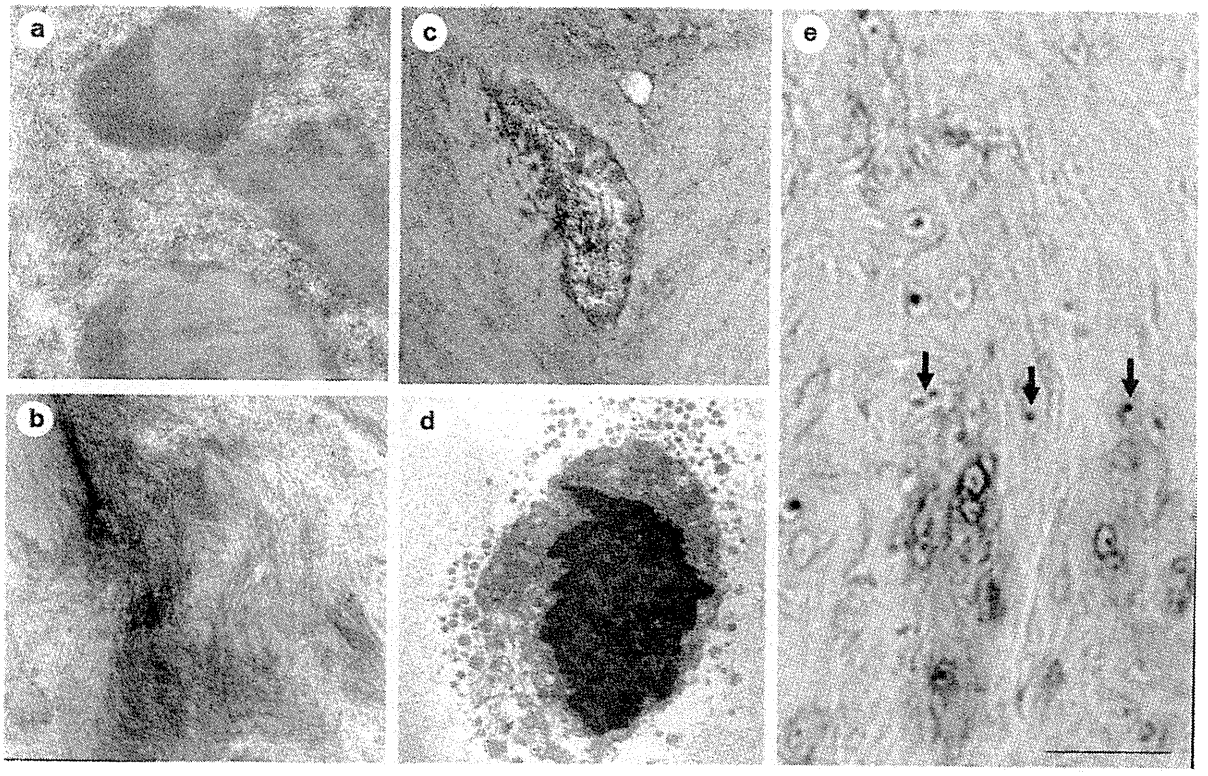

Figure 3 TEM examination and TUNEL staining of the ossification front. The typical structure of the elastic fibers had disappeared with an increase in the number of collagen fibers **(a:** control, **b:** OLF). Note the presence of matrix vesicles containing electron-dense material **(c)** and also the presence of pigmented chondrocytes **(d)** near the ossified layer **(a, b**: magnification ×1,000, **c**; magnification ×4,000, **d**; magnification ×2000). Note the presence of TUNEL-positive chondrocytes near the ossified layer (arrow) **(e**, scale bar = 100 μm). OLF: ossification of the ligamentum flavum; TEM: transmission electron microscopy; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

accounting for 60% to 70% of the dry weight [24], with little blood flow. However, the cross-sectional area and viscoelasticity of the elastic fibers diminish as part of age-related changes or systemic hormonal imbalance, although the elastic fiber bundles retain their normal longitudinal arrangement [25-27]. The pathologic process of degeneration that affects the ligamentum flavum is characterized by disorganization of the elastic fibers with irregular arrangement, a decrease in the number of these fibers, and an increase in collagen fibers [28-30].

In the ossification front of OLF, fiber bundles of the ligament showed disorganization of the elastic fibers and an abundance of collagen fibers. We reported previously that the initial degenerative changes in the ligamentum flavum include reduction in the diameter of elastic fibers together with irregular arrangement and their rupture [25]. The elastic fiber bundles showed gradual fragmentation and extinction, probably because of the action of proteases such as elastase and chymotrypsin [31]. We consider that such matrix changes are important in the development of the ossified plaque, particularly in the fused and tuberous subtypes of OLF, compared with the lateral, extended, and enlarged subtypes [19].

Previous studies examined various aspects of the OLF. Genetic studies identified abnormalities in *COL6A2* and *COL11A1* genes, on chromosome 21, in patients with ossification of the spinal ligaments [14,32]. The expressions of these genes may explain why this pathologic process occurs mainly in East Asian countries. Conversely, the ligamentum flavum is persistently subjected to repetitive variable degrees of tensile stress along its longitudinal axis, and mechanical stress influences the cell biologic properties, such as expression of cytokines and/or transcriptional factors [12,33,34]. Our results showed overexpression of Sox9, Runx2, Msx2, and AP-1 mRNAs in cells cultured from OLF compared with the control samples. Conversely, the mRNA expression levels of Osterix and Dlx5 were not different from the control, although the protein expression of all these factors was positive at the cells gathering in the ossification front *in vivo* environment. Considered together with the previously mentioned studies, we speculate that the

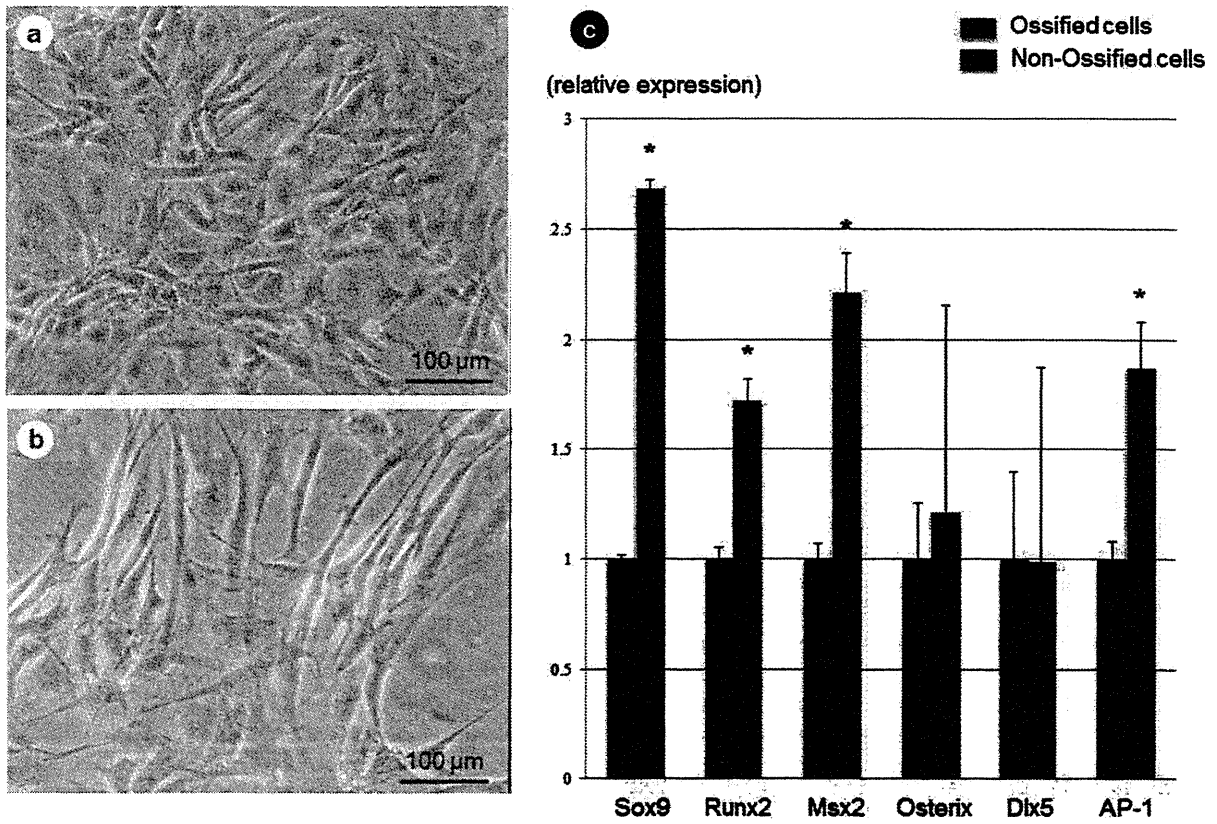

Figure 4 Morphologic findings of cultured cells and relative expression levels of mRNA of transcriptional factors. Cultured cells in OLF **(a)** and control **(b)** showed spindle shape (scale bar = 100 μm). RT-PCR analysis of the ossified and nonossified cultured ligament cells showed that the mRNA expression levels of Sox9, Runx2, Msx2, and AP-1 in OLF were significantly different from those of the control **(c)**. Data are expressed as mean ± SEM values of six samples each. Differences between the two groups were examined with the Student *t* test. *$P < 0.05$, compared with the control. mRNA, messenger RNA; OLF, ossification of the ligamentum flavum; RT-PCR, reverse transcription-polymerase chain reaction.

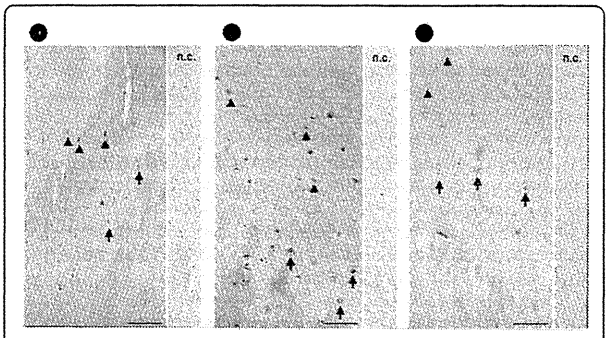

Figure 5 Immunostaining for Sox9, Runx2, and Msx2 of the OLF. Sox9 **(a)** was expressed in proliferating chondrocytes. Runx2 **(b)** was expressed in hypertrophic chondrocytes. Msx2 **(c)** was expressed in proliferating chondrocytes (scale bar = 100 μm; n.c., negative control; black arrow, hypertrophic chondrocytes; black arrowheads, proliferating chondrocytes).

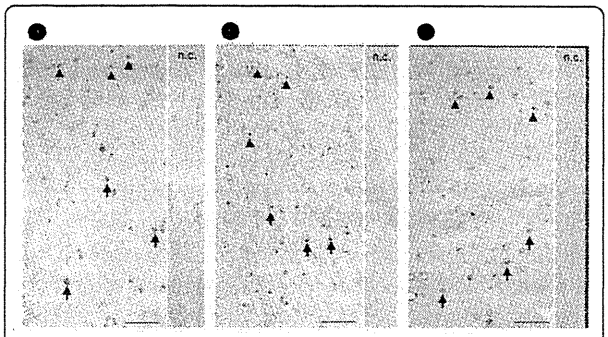

Figure 6 Immunostaining for Osterix, Dlx5, and AP-1 in the OLF. Osterix **(a)**, Dlx5 **(b)**, and AP-1 **(c)** were strongly positive in hypertrophic chondrocytes (scale bar = 100 μm; n.c., negative control; black arrow, hypertrophic chondrocytes; black arrowheads, proliferating chondrocytes). OLF, ossification of the ligamentum flavum.

observed changes are due to the diversity of genetic background as well as spinal mechanical stress, degenerative matrix changes, and/or other yet-unknown factors that influence the expression levels of these transcriptional factors.

Several studies [16,35,36] concluded that the process of ossification of the spinal ligament involves enchondral ossification and clustering of abnormal fibrocartilage or cartilaginous cells, which results in the development of the ossified plaque. Numerous chondrocytes were present, especially around the calcification front, although no such chondrocytes were found in the normal ligamentum flavum [17,25,31]. Yahia *et al.* [37] suggested that the metaplastic chondrocytes in the ligamentum flavum are derived from mesenchymal cells or fibroblast-like cells. In this regard, our study indicates the involvement of certain transcriptional factors in this process. Sox9 is known to promote chondrocyte differentiation, but to prevent hypertrophic changes [38]. Runx2 promotes the differentiation of premature chondrocytes to hypertrophic chondrocytes [39]. In contrast, Msx2 induces differentiation of premature mesenchymal cells and sometimes prevents the maturation of chondrocytes [40]. In the normal ligamentum flavum, these factors might regulate chondrocyte differentiation, preserving homeostasis; however, overexpression of these factors in the OLF violates the regulation of chondrogenesis and differentiation of mesenchymal or fibroblast-like cells to mature chondrocytes in a complex autocrine/paracrine manner.

The maturation of osteoblastic cells and bone formation correlate closely with stromal expansion and neovascularization. We reported previously that hypertrophic chondrocytes around the calcification front regulate angiogenesis by secreting various growth factors, such as vascular endothelial growth factor [19]. Neovascularization promotes infiltration of mesenchymal cells and alters matrix mineralization, particularly in the area of the ossification front. However, many TUNEL-positive hypertrophic chondrocytes were noted in the ossified front, especially around the calcification front. It is not clear whether the chemical change in the matrix induces apoptosis of the hypertrophic chondrocytes or if these cells themselves undergo programmed cell death triggered by biochemical alteration of the chondroid matrix secondary to the degeneration of the ligamentum flavum, or whether apoptosis was induced by the ossified plaque and/or matrix vesicles. We suggest that apoptosis of the hypertrophic chondrocytes induces secondary infiltration of osteoblastic cells through the expression of transcriptional factors such as Runx2, Osterix, Dlx5, and AP-1 [41-43].

Conclusions

Our study showed significant matrix degeneration in the ossification front of OLF and the presence of several ossification fronts between the ossified plaque and non-ossified fiber area. Chondrocyte differentiation was evident in the ossification front and while accumulation of chondrocytes was evident around the calcification front. We suspect that chondrocyte differentiation under the influence of transcriptional factors plays a key role in the ossification process.

Additional material

Additional file 1: Topographic analysis of the expression of transcriptional factors. The table shows the summary of immunohistochemical localization of Sox9, Runx2, Msx2, Osterix, Dlx5, and AP-1 in all 31 cases. The tabulated data represent the distribution of immunopositive areas in the ossification front, based on semiquantitative analysis conducted according to the method described by Kokubo *et al.* [22] and Song *et al.* [35].

Additional file 2: Relation between CT subtype and immunopositivity to each transcriptional factor. The results indicate that the expression of transcriptional factors varied according to the size of the ossified plaque subtype. Immunopositivity for Sox9, Runx2, and Msx2 tended to be more common in the fused and tuberous subtypes (near 100%) than in the lateral and extended subtypes (< 50%). Expression of Osterix, Dlx5, and AP-1 was also high in the fused and tuberous subtypes.

Abbreviations

CCA: calcified cartilage area; CT: computed tomography; Dlx5: distal-less homeobox 5; EDTA: ethylene diaminetetraacetic acid; EVG: Elastica van Gieson; FA: fiber area; FCA: fibrocartilage area; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; H&E: hematoxylin and eosin; MRI: magnetic resonance imaging; Msx2: muscle segment homeobox 2; n.c.: negative control; OA: ossified area; OLF: ossification of the ligamentum flavum; PBS: phosphate-buffered saline; RT-PCR: reverse transcription-polymerase chain reaction; Runx2: runt-related transcription factor 2; Sox9: Sry-type high-mobility group box 9; TEM: transmission electron microscopy; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

Acknowledgements

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Authors' contributions
HB, who did not participate in the histopathologic and immunohistochemical assessments, performed the surgeries. KU and TY (principal authors), together with HC, DS, AG, AY, and KC, were blinded to the surgical findings and were responsible for all histopathologic investigations. HN and SK examined all data. All authors participated in the elaboration of this document, approving the final version of this manuscript for publication.

Competing interests
The authors declare that they have no competing interests.

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Activation of tumor suppressor protein PTEN and induction of apoptosis are involved in cAMP-mediated inhibition of cell number in B92 glial cells

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ABSTRACT

During brain development, cAMP induces morphological changes and inhibits growth effects in several cell types. However, the molecular mechanisms underlying the growth inhibition remain unknown. Tumor suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid phosphatase that inhibits the phosphoinositide 3-kinase (PI3K) pathway. The phosphorylation of Akt, which is one of the key molecules downstream of PI3K, inhibits apoptosis. In this study, we investigated the role of PTEN in cAMP-mediated growth inhibition. B92 rat glial cells were treated with 2 different cAMP stimulatory agents, a phosphodiesterase (PDE) inhibitor and a β-adrenoceptor agonist. Both cAMP stimulatory agents induced marked morphological changes in the cells, decreased cell number, decreased Akt phosphorylation, activated PTEN, cleaved caspase-3, and induced the condensation and fragmentation of nuclei. These results indicate that the cAMP stimulatory agents induced apoptosis. Protein phosphatase inhibitor prevented cAMP-induced dephosphorylation of PTEN and Akt. In addition, cAMP analogs and Epac-selective agonists affected PTEN and Akt activities. These results suggested that cAMP-induced apoptosis may be mediated by PTEN activation and Akt inhibition through protein phosphatase in B92 cells. Our results provide new insight into the role of PTEN in cAMP-induced apoptosis in glial cells.

cAMP stimuli, including the pituitary adenylate cyclase-activating polypeptide, can induce morphological changes and astrocytogenesis of rat cortical precursors [23]. Therefore, intracellular cAMP seems to play an important role in controlling differentiation and proliferation [13,16,22]. However, the signaling cascade involved in the cAMP-regulated proliferation has not yet been fully elucidated. The tumor suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN) has been shown to regulate multiple steps in the development of the central nervous system [8,19]. We hypothesized that PTEN is involved in the regulation of cell proliferation by cAMP. PTEN was initially cloned as a tumor suppressor for gliomas [1], and it is a phosphoinositide-phosphate phosphatase specific for the 3-position of the inositol ring [11]. PTEN and phosphoinositide 3-kinase (PI3K) have opposing effects on cellular PI-(3,4,5)-P3 levels and consequently affect cell proliferation, survival, and differentiation through various signaling molecules, including Akt. Akt activity is negatively regulated by PTEN [3,18]. Here, we found that increased intracellular cAMP levels activated PTEN, which resulted in the inhibition of Akt activity and the induction of apoptosis.

Isobutylmethylxanthine (IBMX), isoproterenol (ISO), and Dulbecco's modified Eagle's medium (DMEM) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 8-Bromoadenosine-3,5-cyclic monophosphate (8-Br-cAMP) and 8-(4-chlorophenylthio)-2-*O*-methyladenosine-3,5-cyclic monophosphate sodium salt (8-CPT-cAMP: Epac inhibitor) were obtained from Biaffin GmbH & Co. KG (Kassel, Germany). H89 and okadaic acid (OA) were obtained from Calbiochem (La Jolla, CA) and LC Laboratories (Woburn, MA), respectively. Hoechst

Abbreviations: 8-Br-cAMP, 8-bromoadenosine-3,5-cyclic monophosphate; 8-CPT-cAMP, 8-(4-chlorophenylthio)-2-*O*-methyladenosine-3,5-cyclic monophosphate sodium salt; IBMX, isobutylmethylxanthine; ISO, isoproterenol; OA, okadaic acid; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PI3K, phosphoinositide 3-kinase; PIP3, phosphoinositide-(3,4,5)-triphosphate; VASP, vasodilator-stimulated phosphoprotein.

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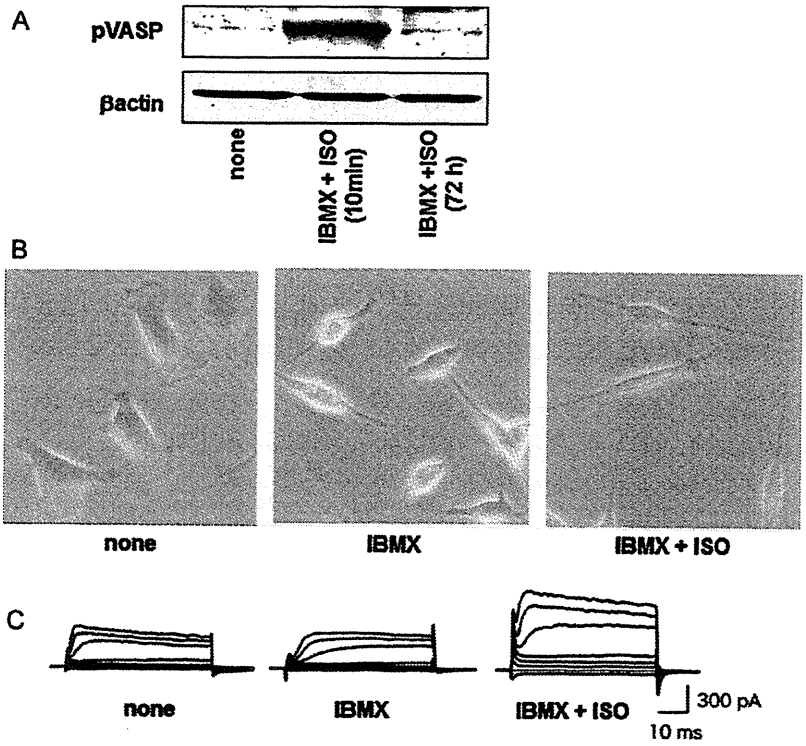

Fig. 1. Changes in the levels of phospho-VASP (A), in the morphology of the cells (B), and in the electrophysiological recordings (C) of B92 cells after treatment with IBMX (1 μM) or a combination of IBMX (1 μM) and ISO (1 μM). Representative current responses to voltage pulses of potentials between −100 mV and +40 mV with a 20 mV step from a holding potential of −80 mV (C).

33342 was obtained from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS) was obtained from Invitrogen Corporation (Carlsbad, CA). Anti-PTEN, anti-phospho-specific PTEN (Ser380), anti-phospho-specific Akt (Ser473), anti-cleaved caspase-3, anti-β-actin, horseradish peroxidase (HRP)-linked anti-rabbit IgG, and anti-mouse IgG were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-phospho-specific VASP (Ser157) was obtained from Calbiochem (La Jolla, CA).

B92 rat glioma cells, which were established by Schubert et al. [17], were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan), and U87MG human glioblastoma cells were provided by Dr. Nakata (Kanazawa University). Both cells were maintained in DMEM containing 10% FBS at 37 °C in a 5% CO_2 incubator.

B92 cells and U87MG cells were seeded on 100 mm dishes at a density of 1×10^5 cells/dish. After 24 h incubation, the cells in DMEM with serum were treated with 1 μM IBMX, and/or 1 μM ISO for 72 h. Cell suspensions were prepared by PBS with trypsin, and the number of cells of the suspension was counted.

To determine the effect of cAMP stimulatory agents on PTEN and Akt activities, we investigated the levels of phospho-PTEN and phospho-Akt by various stimulations. Increases of the levels of phospho-PTEN and phospho-Akt indicate PTEN-inactivation and Akt-activation, respectively. B92 cells and U87MG cells were incubated in DMEM with serum for 24 h, and treated with IBMX (1 μM), ISO (1 μM), 8-Br-cAMP (1 mM) or 8-CPT-cAMP (5 μM) for 10 min with or without H89 (5 μM) or OA (1 μM). Western blotting analyses were performed using the phospho-specific antibodies, phospho-PTEN (Ser380) antibody and phospho-Akt (Ser473) antibody.

Western blotting was performed as described previously [20,21]. Briefly, proteins were extracted from cells, and the protein concentrations were determined using a protein assay. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated with primary antibodies (1:1000), and then incubated with HRP-linked secondary antibodies (1:2000). The blots were developed using the Immobilon Western Chemiluminescence HRP Substrate (Millipore, Billerica, MA).

B92 cells were seeded on 3.5 mm dishes at a density of 1×10^3 cells/dish. After 24 h incubation, the cells in DMEM with serum were treated with 1 μM IBMX, and/or 1 μM ISO for 72 h. Cells were whole-cell voltage clamped using a patch pipette filled with an internal solution containing (in mM) 130 K-gluconate, 15 KCl, 10 HEPES, 0.2 EGTA, 6 $MgCl_2$, 5 Na_2ATP, and 0.2 Na_2GTP (pH 7.3, adjusted with KOH). The external solution contained (in mM) 140 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.3, adjusted with NaOH).

B92 cells were seeded on 3.5 mm dishes at a density of 1×10^3 cells/dish. After 24 h incubation, the cells in DMEM with serum were treated with 1 μM IBMX, and 1 μM ISO for 24 or 72 h. Cells were stained with Hoechst 33342 (10 μg/mL, 15 min). After the incubation, the stained cells were observed by fluorescence microscopy, using UV/355 nm excitation and measuring the fluorescence emission of Hoechst 33342 dye at 460 nm emission. Under these conditions, Hoechst 33342 stains the nuclei of all cells.

Data are presented as means ± SEM from at least 3 independent experiments. Statistical analyses were performed with ANOVA and followed by Dunnett's test.

We first examined the detailed effects of isobutylmethylxanthine (IBMX), which is a phosphodiesterase (PDE) inhibitor, and isoproterenol (ISO), which is a β-adrenoceptor agonist, on the morphology of B92 cells. β2-adrenoceptors, which are positively coupled to adenylate cyclase [10], and IBMX, which inhibits PDE, both increased intracellular cAMP levels. cAMP is therefore a prime candidate for the second messenger that is involved in the mechanisms changing cell morphology and function. Vasodilator-

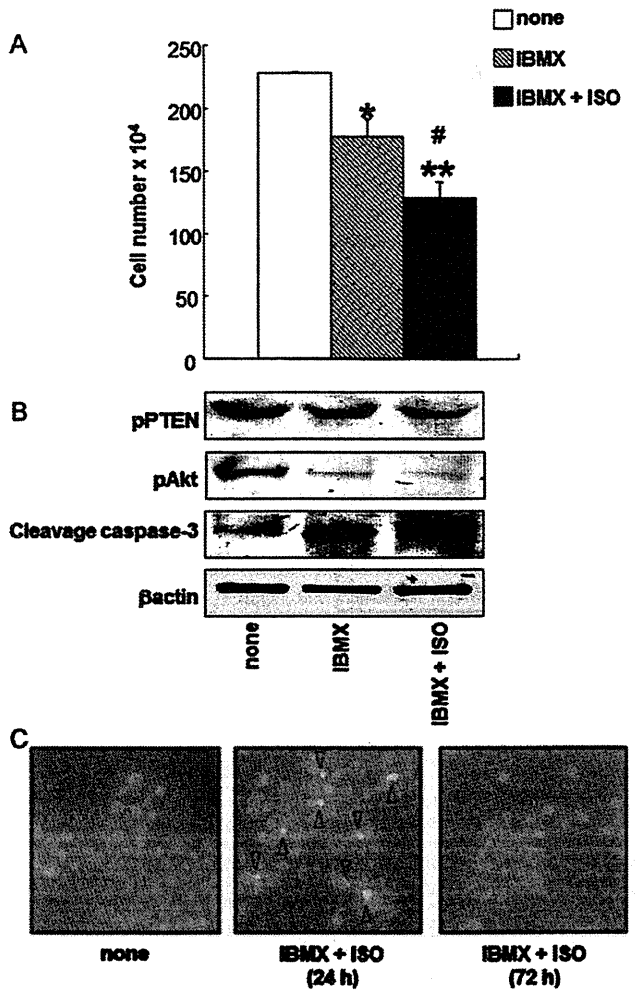

Fig. 2. Changes in cell number (A), in the levels of phospho-PTEN, phospho-Akt, and cleaved caspase-3 (B), and in the nuclear morphology (C) of B92 cells after treatment with IBMX (1 μM) or a combination of IBMX (1 μM) and ISO (1 μM). Each column represents the mean ± SEM. *$P<0.05$, **$P<0.01$ *vs.* untreated controls. #$P<0.05$ *vs.* IBMX treatment group (A). Several condensed or fragmented apoptotic nuclei are indicated by arrows (C).

stimulated phosphoprotein (VASP) is a critical factor in regulating actin dynamics, and an increase in phospho-VASP is related to the increased levels of intracellular cAMP [4,10]. Treatment of B92 cells with IBMX and ISO increased phospho-VASP protein levels significantly in 10 min and slightly in 72 h compared with controls (Fig. 1A).

Microscopic examination indicated that IBMX and ISO affected the cell morphology; cells were extended and spindle-shaped (Fig. 1B). Cell morphology after treatment with a combination of IBMX and ISO were markedly changed compared with treatment with IBMX alone (Fig. 1B). In the electrophysiological experiments (Fig. 1C), an outward current was activated by depolarizing the membrane above −20 mV (300.0 ± 64.9 pA, $n = 10$, at +40 mV) in control cells and (452.9 ± 107.2 pA, $n = 15$, at +40 mV) in IBMX-treated cells. Similar depolarizing pulses induced larger outward currents in the cells treated with a combination of IBMX and ISO (562.2 ± 97.1 pA, $n = 11$, at +40 mV), but there was no sodium inward current, such as that seen in neurons.

To examine whether decreases in cell number were dependent on cAMP, B92 cells were treated with IBMX only or with a combination of IBMX and ISO. IBMX alone and the combination of IBMX with ISO decreased cell number (Fig. 2A). The decrease in cell number after treatment with IBMX and ISO together was greater than that after stimulation with IBMX alone.

Many reports have suggested that PTEN plays a critical role in brain development and cell phenotype changes [7,14,15]. In order to further investigate the role of PTEN in cAMP-regulated cell growth, we performed Western blotting analyses using the phospho-specific antibodies, phospho-PTEN (Ser380) antibody and phospho-Akt (Ser473) antibody. PTEN phosphorylation of various sites, including Ser380, restricted PTEN activity [24]. The dephosphorylation of PTEN also resulted in an increase of PTEN activity [24]. The treatment of B92 cells with IBMX alone or with IBMX in combination with ISO induced the dephosphorylation of PTEN, which indicated an activation of PTEN phosphatase activity (Fig. 2B). This PTEN phosphatase activity resulted in an inhibition of Akt activity (Fig. 2B). Therefore, the apoptotic enzyme caspase-3 was cleaved (Fig. 2B), and cells with condensed or fragmented nuclei were observed by Hoechst 33342 staining 24 h after treatment with the stimuli (Fig. 2C, middle panel), which indicated an induction of apoptosis [9]. However, few cells with condensed or fragmented nuclei were observed 72 h after treatment with the stimuli (Fig. 2C, right panel).

PKA and Epac are molecular players that are downstream of cAMP. Fig. 3A shows the effects of PKA inhibition by H89, which blocked the induction of VASP phosphorylation by cAMP-stimulating agents, but not the ability of cAMP-stimulating agents to dephosphorylate PTEN and Akt. Similarly, H89 failed to block the cAMP analog 8-Br-cAMP-induced inhibition of the dephosphorylation of PTEN and Akt (Fig. 3B, left panel). However, the highly selective Epac agonist 8-CPT-cAMP induced PTEN activation and Akt deactivation (Fig. 3C), confirming that Epac, and not PKA, is involved in cAMP-dependent PTEN activation and Akt deactivation. Although okadaic acid (OA), which is a protein phosphatase inhibitor, increased the level of phosphorylation of PTEN and Akt compared with the control, OA completely abolished the ability of cAMP to dephosphorylate PTEN and Akt in Fig. 3B (right panel), indicating that protein phosphatases might be involved in the cAMP-regulated PTEN activity.

PTEN is not expressed in U87MG human glioblastoma cells (Fig. 3D, left panel). cAMP-stimulating agents increased phospho-VASP protein levels, but they did not inhibit Akt activity (Fig. 3D, middle panel) and cell number (Fig. 3D, right panel) in U87MG cells, indicating that the cAMP-induced inhibition of Akt and cell number was dependent on PTEN activity.

Our results provide a new insight into the activation of PTEN through protein phosphatase during cAMP-induced apoptosis in B92 cells. The effects of PI3K on Akt activation were countered by PTEN, which is a lipid phosphatase of PIP3 [3,18]. The dephosphorylation of PIP3 by PTEN resulted in the inhibition of Akt activity. In response to cAMP stimulation, we observed an increase in the activity of PTEN and a decrease in Akt activity in B92 cells that resulted in the induction of apoptosis. An inhibitory effect of cAMP on the activation of Akt has been described in various cell types [2,5,6,12]. However, the mechanism of cAMP-induced inhibition of Akt is not yet fully understood. Specifically, the role of PI3K in cAMP-mediated Akt inhibition is still controversial; previous studies have indicated either no effect [5] or negative effects [6] of cAMP on PI3K activity. Although PTEN is not a classical target of cAMP action, cAMP-dependent PTEN activation has been reported in alveolar macrophages and human glioma cells [2,12]. PKA and Epac are molecular players downstream of cAMP [2,12]. Epac, and not PKA, is involved in cAMP-dependent PTEN activation and Akt deactivation [2,12]. In this study, we showed that PKA is responsible for cAMP-dependent VASP phosphorylation, but not cAMP-dependent PTEN activation and Akt deactivation (Fig. 3A and B). We also suggest that Epac is responsible for cAMP-dependent PTEN activation and Akt deactivation (Fig. 3C),

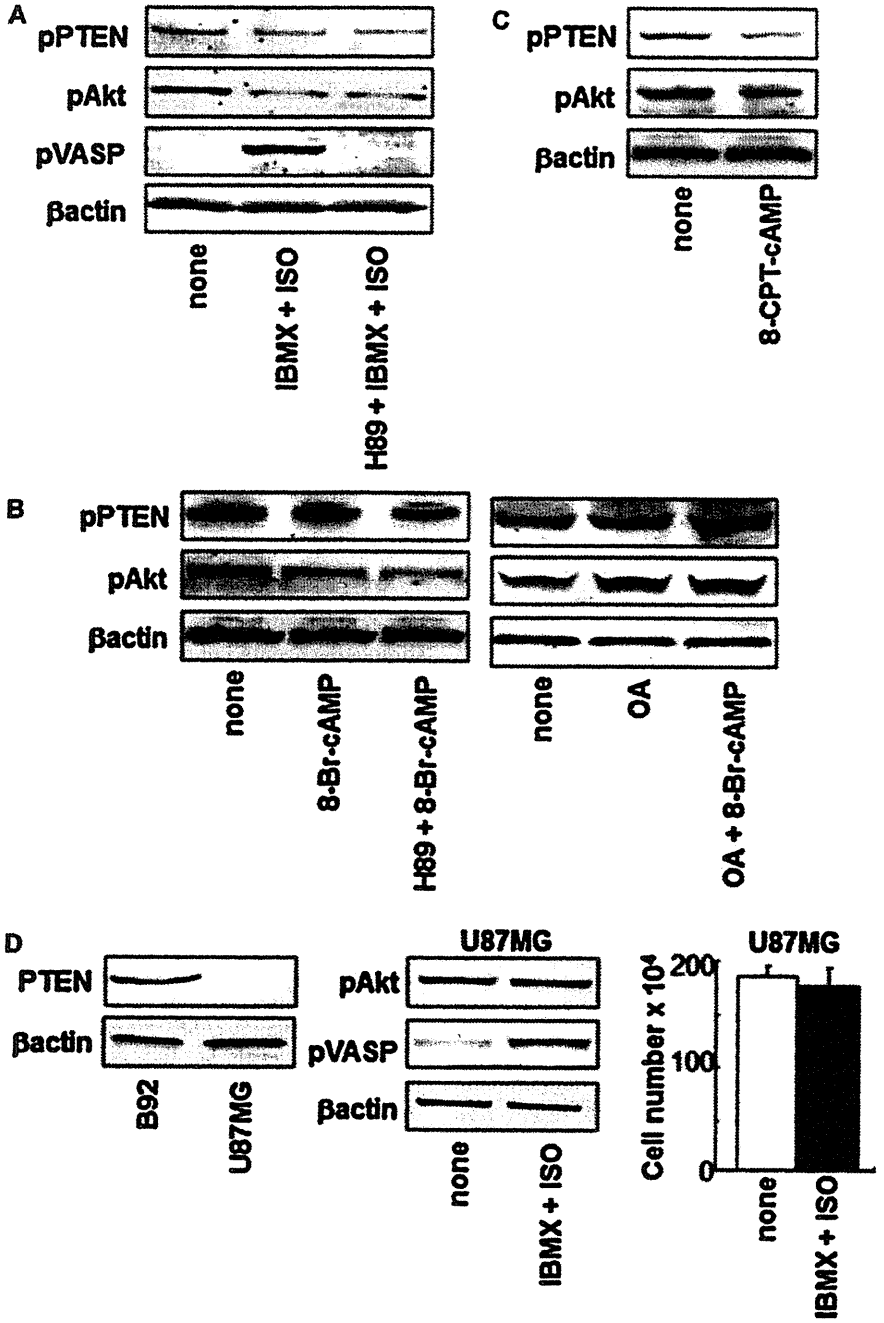

Fig. 3. Changes in the levels of phospho-PTEN, phospho-Akt, and phospho-VASP in B92 cells after treatment with a combination of IBMX (1 μM) and ISO (1 μM) with or without H89 (5 μM) (A), with 8-Br-cAMP (1 mM) with or without H89 (5 μM) or OA (1 μM) (B), and with the selective Epac agonist 8-CPT-cAMP (5 μM) (C). Changes in the levels of phospho-Akt and phospho-VASP, and cell number after treatment of PTEN-depleted U87MG glioblastoma cells with a combination of IBMX (1 μM) and ISO (1 μM) (D).

confirming that cAMP activates PTEN through Epac [2,12]. Moreover, in this study, it was shown that protein phosphatase plays a key role in cAMP-induced PTEN activation (Fig. 3B). Thus, our findings may provide new insight into the mechanistic model by which cAMP activates PTEN in glial cells. However, the details regarding the mechanistic mechanisms underlying cAMP stimulation of PTEN are still unknown. Further studies are required to determine the full mechanism of cAMP-dependent PTEN activation in glial cells.

cAMP has been shown to alter the morphology of primary cortical precursor cells and glioma cells [13,22]. Our results suggested that cAMP acts in B92 cells to induce morphological changes within 72 h of the stimuli (Fig. 1B). B92 cells treated with cAMP-stimulating agents exhibited outward currents when the membrane was depolarized, but not a sodium inward current, like neurons do (Fig. 1C). cAMP activated the apoptotic enzyme caspase-3 and increased the number of apoptotic cells with fragmented and condensed nuclei (Fig. 2B and C). Apoptotic cells were detected 24 h after the stimuli, but not 72 h after the stimuli (Fig. 2C). Probably, all of apoptotic cells which were detected 24 h after the stimuli might be dead and disappear in 72 h after the stimuli, which resulted in the cAMP-induced decrease in cell number (Fig. 2A). From these results, cAMP appears to regulate a number of cell functions, including apoptosis.

In this study, a decrease in cell number appears concomitant with the activation of PTEN by cAMP-stimulating agents in B92 cells (Fig. 2A). It is uncertain whether PTEN activation is necessary or sufficient for the regulation of apoptosis and cell proliferation. However, cAMP failed to inhibit Akt activation in PTEN-depleted U87MG glioblastoma cells (Fig. 3D), and several studies have indicated that PTEN regulates multiple steps in the development of the central nervous system, including apoptosis [8,19]. In this study, it appeared that PTEN might play a role in regulating apoptosis in response to cAMP stimulation. Further studies are needed to fully elucidate the involvement of cAMP in apoptosis and cell proliferation in primary cells and in vivo.

Acknowledgment

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椎間関節の形態が脊椎変性すべり症の発症に及ぼす影響：有限要素法による解析

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The relationship between facet angle orientation and slipping in degenerative spondylolisthesis : A Finite Element Analysis study.

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Toshihiko TAGUCHI, MD.

Abstract

This study investigated how the orientation of the lumbar facet (zygapophyseal) joints influences the etiology of degenerative spondylolisthesis.

Several studies have investigated the etiology of degenerative spondylolisthesis and some investigators have claimed that the disease originates in morphologic abnormalities of the facet joint.

In the current study, a nonlinear three-dimensional finite element program was used to analyze responses of a lumbar motion segment.

Facet joint morphology of the lower lumbar spine in a normal population (34 years, male) was characterized on finite element analysis.

Both CT and MRI scans were digitized, to establish a half finite element model of L4-5.

We changed the facet joint orientation (the angle between the horizontal/frontal/sagittal plane and the plane of the "original" facet joint) to ±15 degree to make 7 variations in the model (original, HP±15°, FP±15°, SP±15°).

As a result, the smaller the angle from the sagittal plane in the frontal plane, the greater the slippage of the anterior L4 vertebra.

These findings support previous reports that patients developing degenerative spondylolisthesis are predisposed by the developmental sagittal orientation of the L4-5 facet joints.

These findings also suggest that the facet angle orientation is a possible causative factor.

Key words : Finite Element Analysis, Degenerative spondylolisthesis, Facet joint morphology, Etiology.

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緒　　言

腰椎変性すべり症は古くから注目されてきた疾患であり，その発生機序については様々な議論がされてきた．発症は40歳以上の女性に好発し，発生部位はL4/5高位が他高位に比べて6倍多く好発すると報告されている．また椎間関節性疼痛や腰部脊柱管狭窄などの頑固な腰痛や下肢症状を来す病態の一因となる．しかしながら発育期腰椎分離症が脊椎関節突起間部の疲労骨折が主な発生機序といわれているのに対して[5]，腰椎変性すべり症は腰椎椎弓に形態学的損傷がないにもかかわらず，上位椎体が椎弓と共に一塊として前方にすべり運動を来すもので，その発生機序はこれまで様々な原因論が議論されているものの未だ確立されていない．発症原因としては大きくは椎間板変性を一次とする前方因子を主とする説と，椎間関節を含めた後方要素を一次とする説に分かれている[1),2)]．

これまで当科では単純X線像から長期自然経過を調査し，後方要素である椎間関節の形態が脊椎変性すべり症の進行に影響を与える因子であることを報告してきた[3)]．本研究では，近年脊椎の運動解析に用いられるようになってきた有限要素解析法（FEM）を用いて，椎間関節の形態，特に椎間関節の方向が脊椎変性すべり，前方すべりを来す影響に関して，静的弾性応力解析を行ったので報告する．

研究方法

臨床所見と画像において正常な成人33歳男性の腰椎をもとに腰椎のFEMモデル化を行った．骨要素は単純CT画像から1㎜スライスで，また軟部組織である椎間板はMRIから各々の要素の輪郭をトレースした．それらを汎用ソフトであるMimics®，3-Matrics®（マテリアライズ社製）を用いてモデル化して，両者を合成して脊椎FEMモデル（図3）を作成した．

まず閾値（Hounsfield Unit値の上限値と下限値）を使用して皮質骨183～1512，海綿骨183～245，髄核0～580，線維輪0～85とし，CT/MRIより関心領域を抽出した．

作成モデルは第4腰椎（L4），椎間板，第5腰椎（L5）からなる運動単位を作成した．さらに解析を簡略化するため矢状面で半切した1/2モデルで解析を行った．

なお椎体と椎間板の接触部は接触係数1として，動きがないよう結合させた．

一方でL4/5の椎間関節部は接触係数を0として解析した．

構成要素は骨要素が皮質骨と海綿骨，椎間板要素は髄核と線維輪とに分けて各々ソリッド要素とし，靱帯（前縦靱帯，後縦靱帯，黄色靱帯，棘間靱帯，棘上靱帯，椎間関節包）はケーブル要素（図4）で構成した．

材料定数（ヤング率（Pa），ポアソン比）はこれまでの文献報告[5)]を参照した．椎体・椎弓は皮質骨（Young's modulus 12000，Poisson's ratio 0.30），海綿骨は（100，0.20），椎間板は線維輪を（4.2，0.45），髄核が（1.0，0.49999）を採用した．軟骨終板は解析では考慮しなかった．

靱帯成分は同じく材料定数を前縦靱帯（15.6-20.0，0.30），後縦靱帯（10.0-20.0，0.30），黄色靱帯（12.0-59.0，0.30），棘間靱帯（9.8-12.0，0.30），棘上靱帯（8.8-15.0，0.30），椎間関節包（8.48-32.9，0.30）とした．

作成したオリジナルのモデルの節点数は65253，要素数は309798であった．

汎用ソフト3-Matrics®はある中心点を設定すると，それを中心に周囲のモデルを球形に切りぬき，さらにはそのくりぬいたモデルを，中心点をもとに自由に360°回転できる機能を持つ（図２）．

今回はこの機能を用いて，作成したオリジナルの相対する上下関節突起面からなる椎間関節の形状をかえることなく，脊椎1/2モデルのL4/5椎間関節部の角度のみ（椎間関節の方向性）をかえた．そしてオリジナルを含めて７つのモデルを作成し，各々FEM解析を行った．

上記ソフトを用いて作成した1/2FEMモデルのL4/5の椎間関節の横断面において椎間関節の長軸の中心をとり，その中心点をもとに椎間関節部を球状にくりぬいた．そしてくりぬいた球体の中心をもとに水平面，前額面，矢状面をとり，各々においてオリジナルの中心点をもとに±15度角度をつけ傾けたモデルを作成した（図１-a～c）．

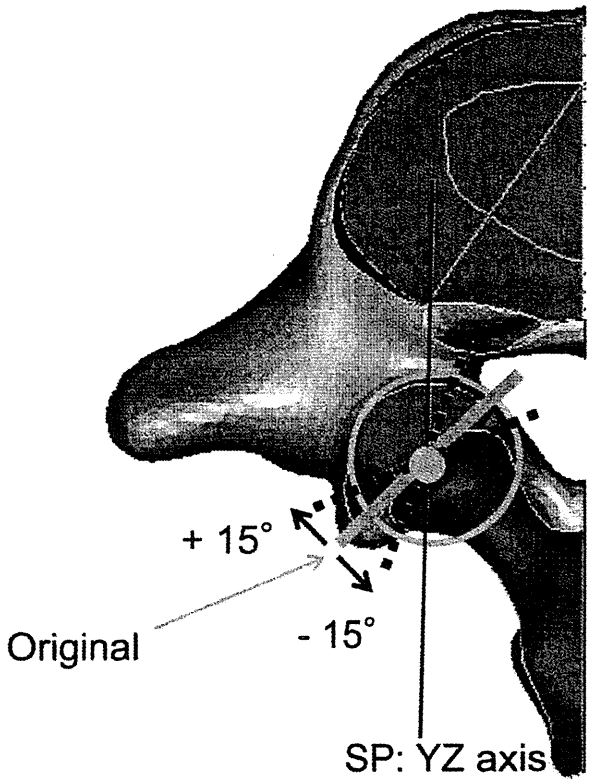

図１－a. HP±15°model.

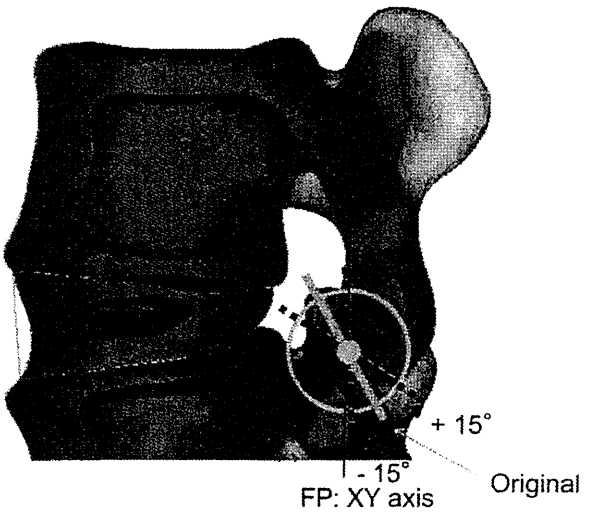

図１－b. SP±15°model.

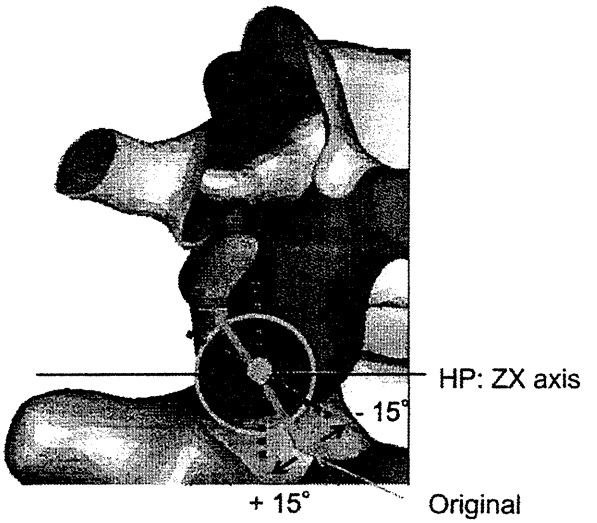

図１－c. FP±15°model.

そして作成したモデルの不連続部分となった部分はスムージングしてモデルを作成した（図２）．

具体的には，まず水平面（HP）では（図１-a），オリジナルの椎間関節を矢状面に近づけるように角度を－15°傾けたモデルをHP－15モデル（矢状化椎間関節）とし，逆に矢状面からはなすように角度を＋15°傾けたモデルをHP+15モデル（前額化椎間関節）とした．

次に矢状面（SP）では（図１-b），オリジ

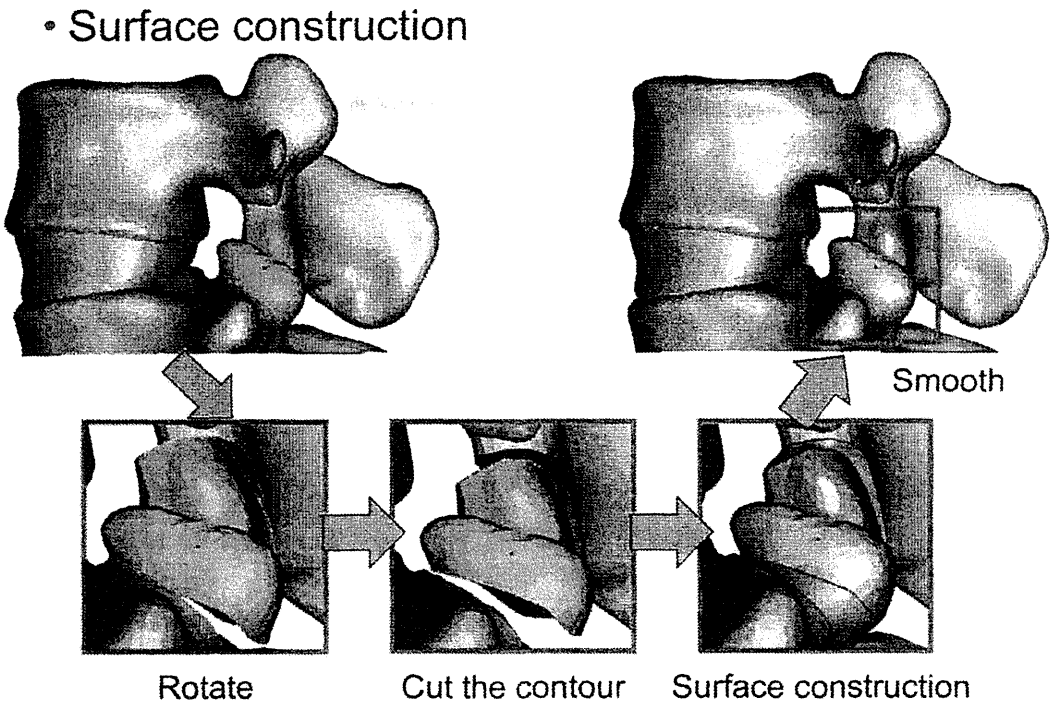

図2．Surface construction.

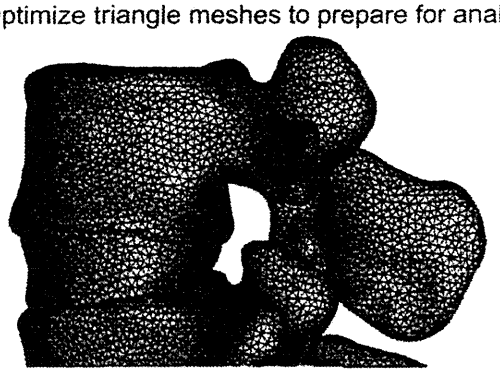

図3．Remesh.

ナルの椎間関節面を前額面に近づけるように角度を－15°傾けたモデルをSP－15モデル（垂直化椎間関節）とし，逆に前額面からはなすように角度を＋15°傾けたモデルをSP+15モデル（水平化椎間関節）とした．

最後に前額面（FP）でも（図1－c），同様に水平面に近づけるように角度を－15°傾けたモデルをFP－15モデル（上向き椎間関節）とし，水平面からはなすように角度を＋15°傾けたモデルをFP+15モデル（内向き椎間関節）とした．

以上のようにして椎間関節の形態・方向性を変化させ，オリジナル（元の椎間関節の方向），HP+15（前額化椎間関節）HP－15（矢状化椎間関節），SP＋15（水平化椎間関節）SP－15（垂直化椎間関節），FP+15（内向き椎間関節）FP－15（上向き椎間関節）を含めて合計7モデルのFEMモデルを作成した．

解析は汎用ソフトウェアのMSC MarcR（MSC Software製）を使用した．拘束条件は，1/2モデルの対称面についてはX方向拘束，L5椎体底面についてはyz方向を拘束した．L4とL5の接触面については椎体と椎間板の間は摩擦係数を1として，L4/5椎間関節の間は摩擦係数を0とした．また各椎体と椎間板の間は共有節点とした．

荷重条件はモデルの体重約60kgの1/6に相当する10kgfを荷重した．L4椎体上面とL4上関節突起椎間関節面に各々軸圧として10kgfの当分布荷重をかけ，さらにはL4椎体上前縁に10kgfの引張り力（点荷重）を加えた（図5）．

そして接触解析を行い，L4椎体の後方上縁をPoint 1，L4椎体の後方下縁をPoint 2として，それぞれのYおよびZ方向の変化量を比較検討した．

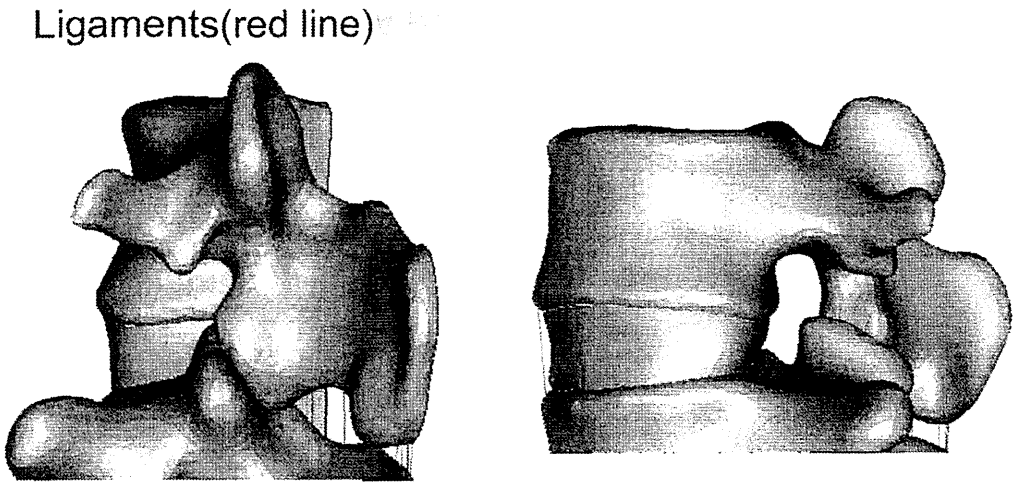

図4．Spine with ligament.

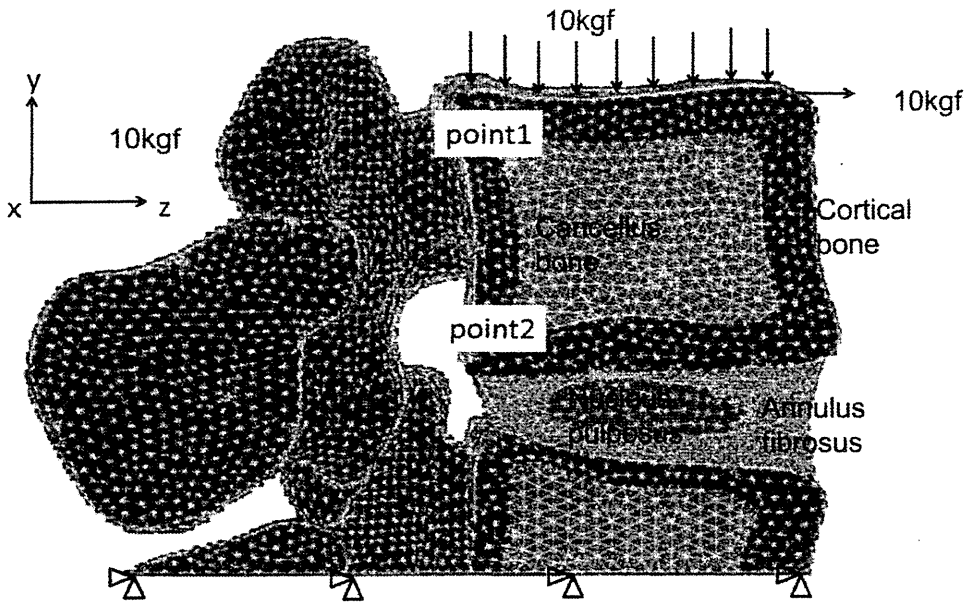

図5．load.

結　　果

各モデルにおけるY，Z方向への移動距離（単位はmm）は以下の通りであった（表1－a～c）．

まずPoint 1（L4椎体後方上縁）では，Y軸方向への移動距離は，オリジナルが2.208，HP－15が2.187，HP+15が2.420，SP－15が2.273，SP+15が1.912，FP－15が1.973，FP+15が2.609であった．

次にZ軸方向への移動距離は，オリジナルが1.460，HP－15が1.653，HP+15が1.297，SP－15が0.677，SP+15が2.472，FP－15が1.674，FP+15が0.412であった．

Point 2（L4椎体後方下縁）では，Y軸方向への移動距離は，オリジナルが1.895，HP－15が1.870，HP+15が2.074，SP－15が2.036，SP+15が1.742，FP－15が1.706，FP+15が2.331であった．

次にZ軸方向への移動距離は，オリジナルが0.555，HP－15が0.663，HP+15が0.584，SP－15が0.434，SP+15が0.509，FP－15が0.530，FP+15が0.359であった．

オリジナルモデルの荷重解析ではPoint 1で，Y軸方向への移動距離が2.208，Z軸方向への移動距離が1.460であり，Point 2では，Y軸方向への移動距離が1.895，Z軸方向への移動距離が

表1－a.

HP	Point1			Point2		
	y	z	distance	y	z	distance
−15	2.187	1.653	2.741	−1.870	0.663	1.984
0(original)	−2.208	1.460	2.647	−1.895	0.555	1.975
15	2.420	1.297	2.745	−2.074	0.584	2.155

表1－b.

SP	Point1			Point2		
	y	z	distance	y	z	distance
−15	2.273	0.677	2.372	−2.036	0.434	2.082
0(original)	−2.208	1.460	2.647	−1.895	0.555	1.975
15	−1.912	1.566	2.472	1.742	0.509	1.814

表1－c.

FP	Point1			Point2		
	y	z	distance	y	z	distance
−15	−1.973	1.674	2.588	−1.706	0.530	1.786
0(original)	−2.208	1.460	2.647	−1.895	0.555	1.975
15	−2.609	0.412	2.641	−2.331	0.359	2.359

0.555であった．この結果からPoint 1の方がPoint 2よりも大きくしかも前方にお辞儀をするような動きをしていると考えられた．正常な腰椎では，上位椎体は矢状前進運動と矢状回旋運動の両方が働き動くとされている．オリジナルモデルの動きはまさにそういった動きであり，今回の荷重解析が正常の動きを模した動きをするモデルであることを示しているといえる．

次に上記の結果をZ方向での移動量でポイントしグラフ化した（図6－a～c）．

HP－15（矢状化），SP+15（上向き），FP－15（水平化）で全てオリジナルよりPoint 1，2ともに値は大きくなった．

しかしHP+15（前額化）がオリジナルの値にくらべて緩やかに小さくなっているのに比べ，一方でSP－15（垂直化）とFP+15（内向き）はPoint 1，2両点の値が極端に小さくなり，また両値が近似していた．

これらの結果はL4椎体が荷重をかけても前方には移動していないことになる．

つまり椎間関節の垂直化と内向きは，Z方向にはほとんど動かずL4椎体の前方すべりを制動している結果を示していると思われる．

考　　察

椎間関節の特徴的な形態は，腰椎運動時にも重要な役割を果たしていると考えられており，そのオリエンテーションは臨床的にも重要であるといわれている．そのため椎間関節の正常な腰椎屈曲運動の解析に加え，脊柱の安定性の破綻を来す結果生じる脊椎変性すべり症への椎間関節の関与に関する研究もこれまで様々報告されてきた．

小田[3]やMatsunagaら[5]の研究は，臨床的に自然経過の研究を行い，その結論すべり発症のリスクファクターを椎間関節の形態が矢状化した椎間関節面を示す角田の分類のM型かW型であり，また椎弓角が110°以上と矢状化関節面の関与を導きだした．

またBodenら[1]は，L4/5の椎間関節の角度が45°よりも矢状方向に傾いた椎間関節を有する症例は，変性脊椎すべり症が発生する可能性が

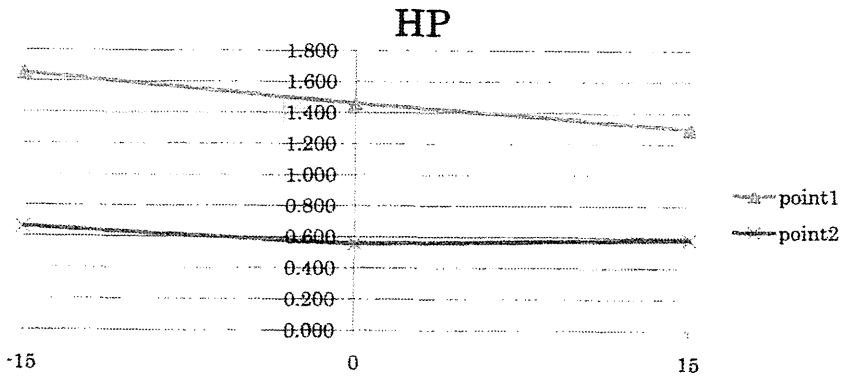

図6－a. Distance to the Z axis in the HP plane.

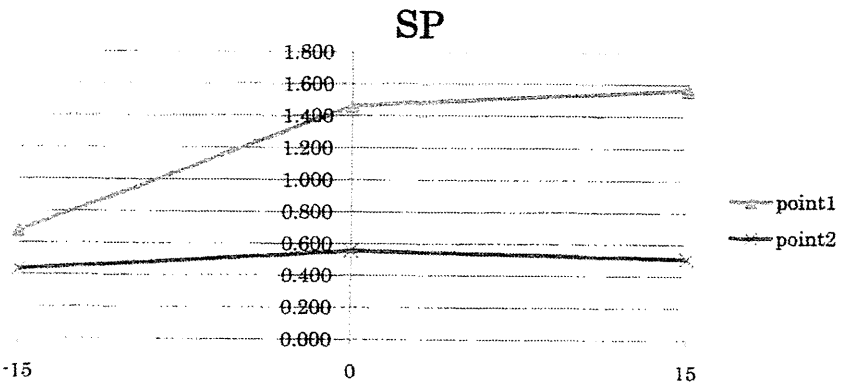

図6－b. Distance to the Z axis in the SP plane.

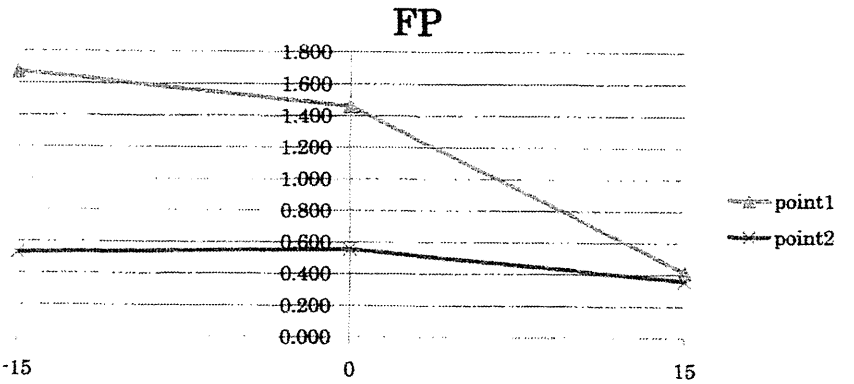

図6－c. Distance to the Z axis in the FP plane.

25倍になると予想されると報告している．

本研究でも矢状化すればするほどL4椎体の前方への動きは制動されなかったことから，椎間関節の矢状化は前方すべりを来す大きな要因であると証明された．また逆に椎間関節が前額化しても前方移動には制動力が働かないことも分かった．

しかしこれまで水平面の方向性のみでしか論じられなかった椎間関節の方向性に加えて，本研究では前額面，矢状面での方向性の違いに関してすべりの発生のしやすさも検討した．

その結果，水平化した椎間関節面は前方すべりを来すが，逆に垂直化すると極端に椎体の動きを止める制動力が働くことが分かった．

また上向きの椎間関節面も前方すべりを来すが，逆に内向きになると極端に椎体の動きを止める制動力が働くことが分かった．

椎間関節を含めた脊椎後方要素の力学的解析は未だ十分には行われているといえない．これには椎間関節が非常に複雑な形状をしており，

実験的な解析が困難なためと思われる．このような実測が困難な系に対する力学的解析手法としては有限要素法を用いたシュミレーションが有効であると思われる．

しかし本研究の問題点は変性すべり症が中年女性に圧倒的に多いにもかかわらず青年男性のデータを元にFEMモデルを作成したことや，異なる椎間関節面の形状であっても同様の結果がでるのか，また各形態間でどの程度お互いの方向性の違いに影響を与えるのかなど不明な点も多い．

しかし，これまで臨床的に矢状化椎間関節が腰椎変性すべり症に多かったということをFEM解析で証明できたことや，一方で水平化，上向きをした椎間関節でもオリジナルに比べてより前方すべりを来しうることをFEMにて証明できた．また逆に垂直化，内向きをした椎間関節ではL4椎体が前方には動かさない制動力が働くことも示唆された．

結　　論

1．本研究は椎間関節面をそのままの形状としながら，椎間関節の角度（方向性）を変化させた異なる形態のモデルを作成，有限要素法を用いて静的弾性応力解析を行った．
2．これまで臨床研究から論じられていた後方要素，特に椎間関節の形態が脊椎変性すべり症の関与する力学的な根拠が得られた．
3．矢状化した椎間関節はL4椎体の矢状回旋運動を増大させ，椎体の前方すべりを来しやすくさせる傾向にあり，変性すべり症の重要な増悪因子と考えられた．
4．垂直化や内向きをした椎間関節は上位椎体の動きを止める傾向があるという結果となった．

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整形外科と災害外科
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ラット頚神経の解剖学的検討

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Anatomical Studies of the Cervival Spine in Rats — Possible Mechanisms of Cervicogenic Headache —

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整形外科診療において，頚部疾患に随伴する後頭部痛をしばしば経験する．これまでに頚性頭痛の要因として，椎間関節性疼痛，頚椎アライメント異常や不安定性など様々な報告があるが，未だ一定の見解を得ていない．今回我々は後頭部痛の一因として，上位頚椎椎間板性疼痛の関連痛に伴う頭痛と考えラットを用いた研究を行ったので報告する．対象はWistar-Rat 220～360 g 6匹を用い，Nembutal麻酔を腹腔内投与（4 mg/kg）した．頚部前方の椎体前面を露出しエバンスブルー溶解液を静脈注射した．さらにカプサイシン1 μgを椎間板前方に投与した．ラットの頚部後方解剖は人間に類似し，C2の後根終末が大後頭神経となっていた．6例中2例においてカプサイシン投与後，大後頭神経領域が選択的に染色され，椎間板性疼痛の関連痛になり得る可能性が示唆された．

Cervicogenic headache is pain perceived in the head but referred from a primary source between the trigeminal afferents and afferents from the upper three cervical spinal nerves.

The possible source lies in the structures innervated by the C1 to C3 spinal nerves. To comfirm this theory, we used capsaicin which causes extravasation in the occipital lesion to intervertebral discs in rats. We administered 10 ug of capsaicin into the anterior portion of the cervical intervertebral discs of rats injected with Evans blue intravenously to cause dye extravasation in the occipital area. The results suggested that the administration of capsaicin may contribute to cervicogenic headaches associated with intervertebral disc lesions.

Key words：rat（ラット），anatomical study of cervical spine（上位頚椎の解剖学的検討），cervicogenic headache（頚原性頭痛），referred pain（関連痛）

はじめに

頚原性頭痛とは1983年にSjaastadらにより提唱された概念[1]で，その後同様の報告が散見されている．Edmeadsらは頭蓋頚椎移行部の異常，上位頚椎の異常に伴う頭痛と述べ，Diezらは頚椎症の約40%に認められる比較的頻度の高い疾患と述べている．現在の国際頭痛分類第2版（ICHD-II）では2次性頭痛に相当し，頚部や顔面・頭蓋の構成組織の障害に起因する頭痛あるいは顔面痛に分類されている．Diezらは頭痛のメカニズムを大後頭神経や小後頭神経の知覚枝の進展により生じる痛み，あるいは三叉神経・後頭神経に伴う頭部への関連痛が要因の1つと述べている．そこで本研究の目的として，ラットの上位頚椎についてヒトとの類似点・相違点を検討すること，また頚原性頭痛の一病態，特に椎間板性頭痛の病態を明らかにすることである．

対象

体重220～360 gのWistar-Rat 6匹を用いた．麻酔方法はnembutalを4 mg/kgで腹腔内投与した．

方法

前方アプローチではC1～C4椎体を露出し，頚長筋

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を左側のみ切離する．C2/3 椎間板腔を露出した後に，エバンスブルー溶解液を大腿静脈より静注する．10分後にカプサイシン 1μg を C2/3 椎間板腔左側の繊維性組織に投与する（図1）．次に後方アプローチでは後頭骨をメルクマールに正中縦切開し大後頭神経を中心に末梢から中枢にかけて展開し，その後 C2 椎弓を椎弓切除し後根神経節を同定する．

結　　果

6例全例でヒトの大後頭神経，小後頭神経に相当する C2, C3 の神経終末が認められた．それぞれの神経は頸部の筋層内を貫通し，C1/2, 2/3 椎間の神経根と連続性が認められた．さらに C2 の椎弓切除を行うと，C2 の後根神経節が確認でき，C2 の神経終末と考えられた（図2）．6例中2例において後頭骨及び左胸鎖乳突筋方向に染色液が拡散した濃い青色の水玉様を呈した（図3）．

図1　前方の展開

考　　察

カプサイシンは 1977 年にカプサイシン受容体遺伝子がクローニングされ発見された[3]．カプサイシン受容体はカプサイシン，熱，酸の3つの痛み刺激を受容する受容体として注目されており，この受容体は脊髄後根神経節，三叉神経節などの知覚神経節に特異的に存在し，疼痛に重要な役割を果たしていると考えられている．チクチクとする鋭い痛みを特徴とする1次痛は細径有髄の Aδ 繊維がジーンとする鈍い痛みを特徴とする2次痛は無髄の C 繊維が伝導し，C 繊維は CGRP，サブスタンス P などの神経ペプチドを有し，カプサイシンはこの C 繊維を特異的に刺激することで，2次痛・関連痛に深く関わっているとされている．さらに交感神経を介し，神経の支配領域の血管透過性を亢進させる性質を利用することが本研究方法である．ヒト頸部の正常の神経解剖では，線維輪外層のみ神経支配をうけ，中間層および内層3分の1，髄核は血管，神経は存在しないはずであるが[5]，椎間板外層までの線維輪にクラックが入り神経終末や後縦靭帯を刺激することで疼痛の要因には成りうると考えられている．Nikolai らは上位頸椎の神経支配の詳細な報告をしているが，椎間板の神経支配については述べていない[1]．Freemont らは椎間板内層への神経細胞の進入[4]，Robert らは椎間板の mechanoreceptor[6]，Burke らは椎間板内の炎症性メディエーターの存在[2]をそれぞれ報告しており，間接的には椎間板も疼痛の要因と成りうると考えられつつある．高橋らはラットを用い，腰椎 L5/6 椎間板にカプサイシンを注入し，腰痛と下肢部痛の関連痛についての研究を報告した[6]．頸椎においても同様に交感神経幹あるいは筋肉内を刺激が上

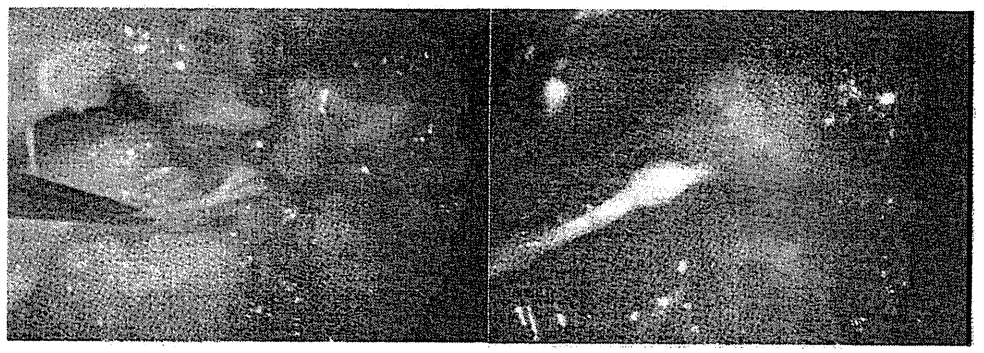

図2　C2後根神経節