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The Role of Microtubule in the Regulation of Endochondral Bone Formation in the Developing Mouse Cranial Base

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Abstract : Endochondral bone formation of the developing cranial base is a complex process. This mechanism requires precise orchestration of many cellular events and cartilage matrix metabolism, such as proliferation, becoming round in shape, termination of proliferation, hypertrophic size-increase, and finally programmed cell death. Active formation and degradation of cartilage matrix take place, in which microtubules are involved for intracellular events; bone apposition follows these events. However, the involvement of microtubules during these changes in the developing cranial base has not been identified yet. Thus, we investigated the involvement of microtubules in the regulation of endochondral bone formation during cranial base development.

Using tubulin-binding drug nocodazole, we examined the effects of altering the structure and function of microtubules during *in vivo* organ culture of the mouse cranial base. Cultured specimens were analyzed with HE staining, immunohistochemistry, and cell counting in order to study the morphological and molecular changes that occurred in the tissues.

Disruption of the microtubular array by nocodazole reduced cells expressing proliferation marker Ki67, osteogenic marker BSP, and BMP4 within the sphenooccipital synchondrosis region; chondrocyte hypertrophy was ceased in the hypertrophic zone; degeneration of cartilage matrix and bone matrix apposition was inhibited in the ossification center of the basooccipital cranial base.

Our data demonstrated that disruption of microtubules by nocodazole have multiple inhibitory effects on the sequential changes that occur during endochondral bone formation, suggesting the importance of normal microtubule-polymerization in cranial base development.

Keywords : Cranial base, Endochondral bone formation, Sphenooccipital synchondrosis, Microtubule, Nocodazole, Bone morphogenetic protein 4, Organ culture

Introduction

The cranial base is formed through endochondral bone formation, and various cellular and molecular events occur in the synchondrosis regions, which serve as growth centers (Nie 2005). These events are regulated by many different genes and signaling molecules (Horton 1990, Kronenberg 2003). At the initiation stage, chondrocytes differentiate from mesenchymal cells (Wright et al. 1995, Ng et al. 1997, Zhao et al. 1997). They proliferate rapidly and synthesize proteoglycans, extracellular matrix, and type II collagen (Col II) in the cartilage matrix. Next, proliferation terminates and chondrocytes begin to hypertrophy and secrete type X collagen (Col X) in the cartilage matrix

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(Gilbert 2006). Finally, these hypertrophic chondrocytes undergo programmed cell death and are degraded (Gerber et al. 2000); cells lining the surface of the cartilage model differentiate into osteoblasts and form bone (Nakashima et al. 2002, Hu et al. 2005). During these sequential changes, the expression level of bone sialoprotein (BSP), a major structural protein of the bone matrix, is low in the resting and proliferating chondrocytes and high in the hypertrophic chondrocytes (Bianco et al. 1991).

Microtubules are one of the major cytoskeleton components. They are involved in many cellular events including mitosis, cytokinesis, and vesicular transport. Particularly in mitosis, it is involved in the action of mitotic spindles. Problems in microtubules lead to diseases such Alzheimer and Shwachman-Diamond syndrome (Iqbal et al. 2005, Austin et al. 2008). One of the symptoms of Shwachman-Diamond syndrome in the skeletal system is metaphyseal dysostosis. However, the involvement of microtubules during skeletogenesis has not been clearly understood, especially in the developing cranial base that undergoes endochondral bone formation. Thus, we investigated how microtubules act by using tubulin-binding drug nocodazole to alter the structure and function of microtubules during *in vivo* organ culture of the mouse cranial base. Morphological and immunohistochemical analyses revealed that sequential events that occur during endochondral bone formation were disrupted by nocodazole, suggesting the importance of normal functions of microtubule in cranial base development.

Materials and Methods

All experiments were performed following the guidelines of the Intramural Animal Use and Care Committee, College of Dentistry, Yonsei University.

1. Preparation of mouse embryos

Adult ICR mice were housed in a temperature-controlled room (22˚C) under artificial illumination (lights on from 05:00 to 17:00 h) and at 55% relative humidity with access to food and water *ad libitum*. The mouse embryos were obtained from time-mated pregnant mice after sacrificing the pregnant mice with cervical dislocation. E0 was designated as the day on which the presence of a vaginal plug was confirmed. Embryos at developmental stage E14 were investigated.

2. *In vitro* organ culture

The cranial bases were isolated from the mouse embryos and cultured in medium at 37˚C and 5% CO_2 for up to 72 h using the culture method reported by Trowell (1959). The culture medium DMEM/F12 (Life Technologies, CA) was supplemented with 20 μg/mL ascorbic acid (Sigma-Aldrich, MO) and 1% penicillin/streptomycin and was renewed every 24 h. 10 specimens were examined in each experiment.

3. Nocodazole treatment

In cultures, nocodazole (Sigma-Aldrich, MO) was dissolved in the culture medium at concentrations ranging from 0 to 10 μg/mL. After 72 h of culture *in vitro*, cranial base specimens were processed for immunohistochemistry and TUNEL assay. 10 specimens were examined in each experiment.

4. Immunohistochemistry and TUNEL assay

The specimens were embedded in paraffin using conventional methods. Sections (4 μm thick) of the specimens were incubated at 4˚C overnight with the primary mouse monoclonal antibody against Ki67 (Thermo Fisher Scientific, MA), Col II (Thermo Fisher Scientific, MA), Col X (Biomeda, CA), BSP (Millipore, MA), and BMP4 (Santa Cruz Biotechnology, CA). After washing with PBS, the specimens were allowed to react with biotinylated goat antimouse immunoglobulins and streptavidin peroxidase at room temperature for two consecutive 10-minute incubations. Finally, the specimens were visualized using the 3,3′-diaminobenzidine (DAB) reagent kit (Life Technologies, CA). TUNEL assay was performed using *in situ* cell apoptosis detection kit (Trevigen, MD) following the manufacturer's instructions. The 4 μm-thick sections were treated with proteinase K [in 10 mM Tris-HCl (pH 8.0)] at a concentration of 20 μg/mL for 15 ~ 20 min at room temperature. The samples were then incubated with the labeling reaction mixture at 37˚C for 1 h and streptavidin-HRP solution for 10 min at room temperature. DAB was used as a substrate solution to detect the sites of *in situ* apoptosis under a light microscope. 10 specimens were examined in each experiment.

Results

1. Nocodazole treatment disrupted chondrogenesis and osteogenesis in the developing cranial base

In order to demonstrate the roles that microtubule plays during endochondral bone formation in the developing cranial base, we employed nocodazole treatment in organ cultures. Cranial bases from E14 mouse embryos were cultured in the media containing 0 or 10 μg/mL nocodazole for 3 days. Since cranial base specimens at E14 exhibited a high rate of proliferation, they were considered to be going under active growth and development. After 3 days of culture with nocodazole, specimens began to show evident dimensional changes. The dorsoventral thickness of the sphenooccipital synchondrosis region in parasagittal sections was lower in the specimens cultured in media containing nocodazole than in the control specimens (n=10/10) (Fig. 1A, A′, brackets). The morphology and arrange-

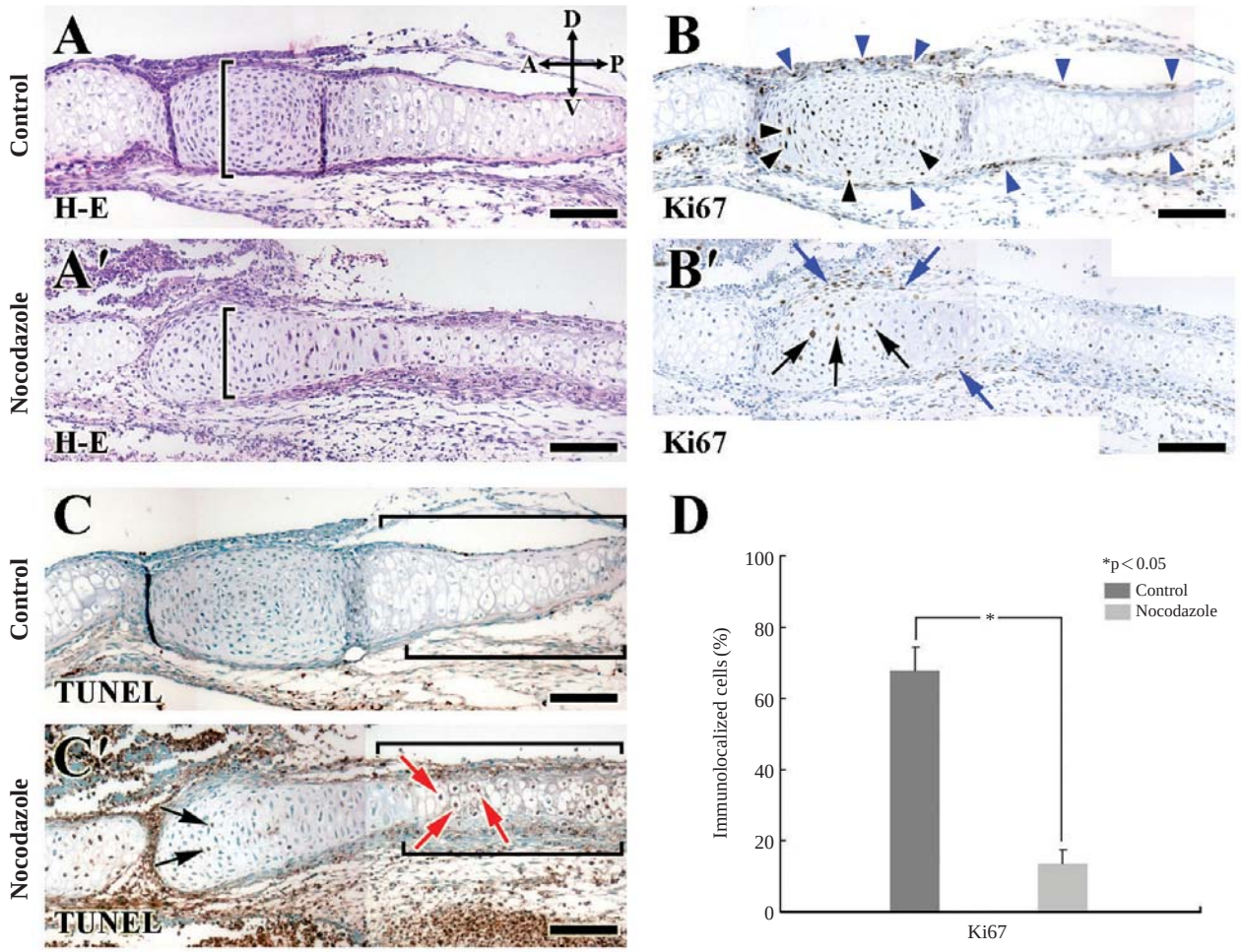

Fig. 1. Nocodazole reduces proliferation in the sphenoocipital synchondrosis and induces programmed cell death in the hypertrophic chondrocytes. Cranial base tissues at E14 were cultured for 3 days in culture medium containing nocodazole (10 μg/mL). (A, A′) H-E staining shows that the dorsoventral thickness of the sphenooccipital synchondrosis region is lower in the nocodazole group than in the control group. In addition, the chondrocytes in the sphenooccipital synchondrosis region lose their concentric arrangement in the nocodazole group. The point where the thickness of the sphenooccipital synchondrosis region was measured is indicated with a bracket in each group. (B, B′, D) Ki67 staining demonstrates the inhibitory effect of nocodazole on cell proliferation in the perichondral cells (blue arrowheads, blue arrows) and chondrocytes (black arrowheads, black arrows). In addition, Ki67-positive chondrocytes that exhibit larger nuclei within the sphenooccipital synchondrosis region are located in the periphery of the sphenooccipital synchondrosis region in the control group (black arrowheads) and are randomly located in the nocodazole group (black arrows). (C, C′) TUNEL staining shows that nocodazole treatment induces apoptosis of chondrocytes in the sphenooccipital synchondrosis region (black arrows) and hypertrophic chondrocytes (red arrows). The zones corresponding to bone-forming regions are marked with brackets. (D) Chondrocytes expressing Ki67 are counted within a 2,500-μm^2 region. *$P < 0.05$ as determined by ANOVA. Scale bars: A-C, A′-C′, 100 μm.

ment of the chondrocyte nuclei also showed marked differences. The shape of chondrocyte nuclei in the control group varied with the zone, and the nuclei were concentrically arranged within the sphenooccipital synchondrosis (Fig. 1A), whereas most of the nocodazole-group nuclei were similar in size and shape and did not show concentric arrangement within the sphenooccipital synchondrosis region (Fig. 1A′). In the control group, Ki67-positive nuclei that were larger or beginning to show hypertrophic morphology were found in the periphery of the sphenooccipital synchondrosis region (Fig. 1B, black arrowheads), whereas nocodazole-group nuclei stained with Ki67 were not only in the periphery but also in the center of the sphenooccipital synchondrosis region (Fig. 1B′, black arrows). The density of Ki67-positive chondrocytes in the sphenooccipital synchondrosis region was lower in the nocodazole group than in the control group (Fig. 1B, B′, D). A similar reduction of Ki67 localization was also evident in the perichondral cells (Fig. 1B, blue arrowheads; B′, blue arrows).

Cells undergoing programmed cell death were confirmed by TUNEL staining. None of the chondrocytes in the sphenooccipital synchondrosis region of the control group were

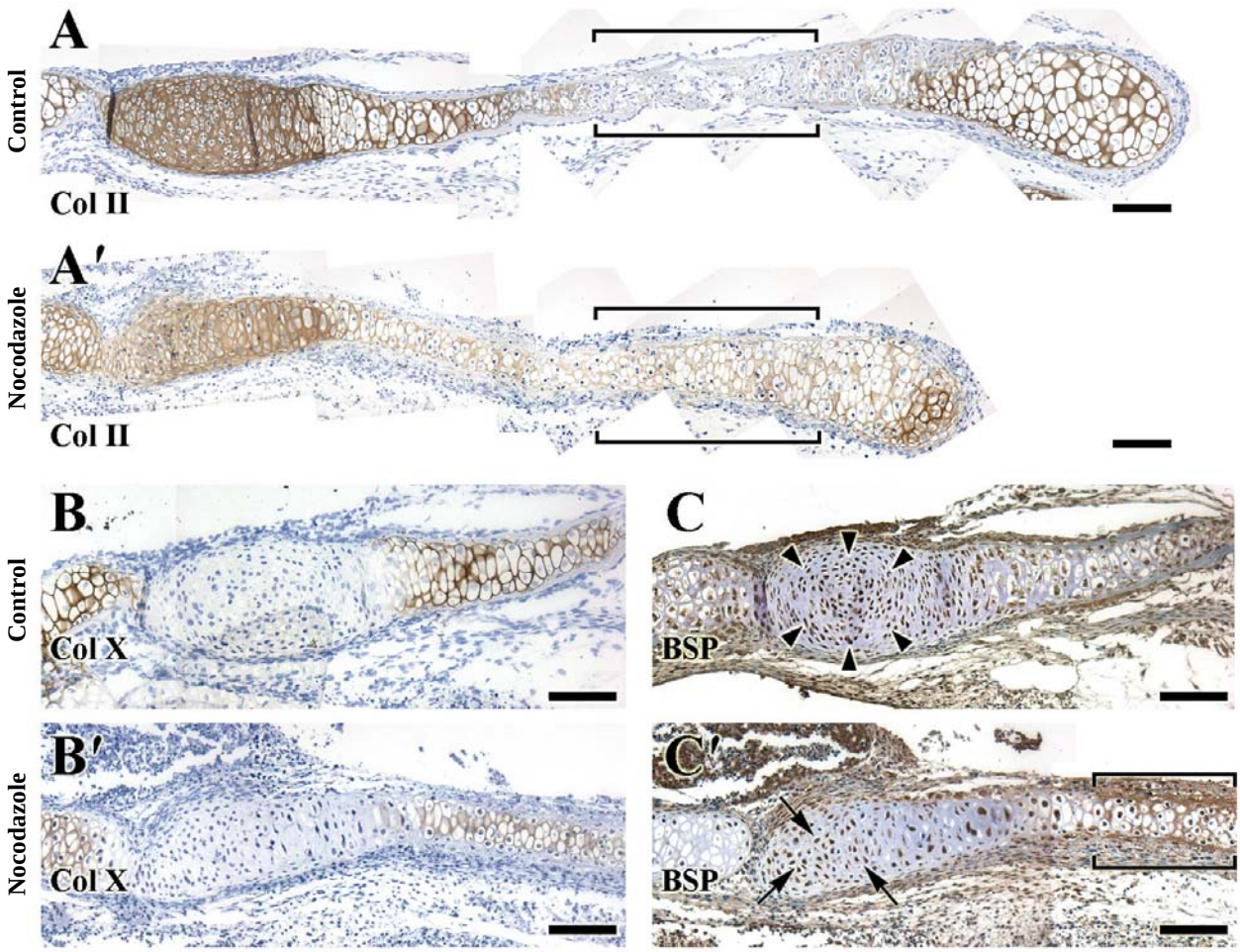

Fig. 2. Nocodazole reduces chondrogenic markers (Col II and Col X) and osteogenic marker (BSP) in the sphenoocipital synchondrosis. (A, A′) Col II stains the cartilage matrix strongly in the control group and weakly in the nocodazole group. The zone in the center of the basioccipital bone overlapping the ossification center and lacking Col II-stained cartilage matrix is only found in the control group (A, brackets). In addition, the anteroposterior length of the cranial base is shorter in the nocodazole group than in the control group. (B, B′) Col X is localized around the hypertrophic chondrocytes in both the control and nocodazole groups. However, the localization is stronger in the control group. (C, C′) Most of the chondrocytes are BSP-positive in the control group (arrowheads), whereas the number BSP-positive chondrocytes (arrows) is lower in the nocodazole group. In addition, cartilage matrix stained by BSP is found only in the nocodazole group (C, brackets). Scale bars: A-C, A′-C′, 100 μm.

labeled by TUNEL staining (Fig. 1C), whereas TUNEL-stained chondrocytes were found within the sphenooccipital synchondrosis region in the nocodazole group (Fig. 1C′, black arrows). Another group of chondrocytes undergoing apoptosis was located in the posterior region within the basioccipital bone (Fig. 1C′, red arrows). This region corresponded to the zone where the bone matrix began to form on the dorsal and ventral surfaces of the cranial base cartilage plate in the control group (Fig. 1C, bracket).

In the control group, Col II was densely localized in the cartilage matrix, clearly showing the outline of lacunae surrounding the chondrocytes (Fig. 2A), but was absent in the ossification center of the basioccipital region where bone matrix was formed (Fig. 2A, brackets). However, in the nocodazole-group cartilage matrix, Col II localization was weak but remained present throughout the cranial base cartilage plate and was even evident in the region corresponding to the ossification center, and bone matrix was not fomed in this region (Fig. 2A′, brackets). In addition, the anteroposterior length of the basioccipital bone was shorter in the nocodazole group than in the control group (Fig. 2A, A′). The cartilage matrix surrounding hypertrophic chondrocytes were stained with Col X in similar regions of the cranial base in both groups, although the staining was weaker and the lacunae containing hypertrophic chondrocytes were smaller in the nocodazole group (Fig. 2B, B′). Immunohistochemistry of BSP revealed that nocodazole reduced the number of chondrocytes stained with BSP within the synchondrosis region (Fig. 2C, C′). Most of the chondrocytes in the control-group synchondrosis region were BSP-positive (Fig. 2C, arrowheads), and fewer chondrocytes were BSP-positive in the nocodazole group (Fig. 2C′, arrows). Another difference in BSP staining was evident in a region in the nocodazole-group cranial base where cartilage was diffusely stained (Fig. 2C′, brackets). This region was not stained in the control group, with newly formed bone being found instead (Fig. 2C).

All chondrocytes strongly expressed BMP4 in the control group, and markedly fewer BMP4-positive chondrocytes were evident in the nocodazole group (Fig. 3A, A′, B). The localization of BMP4 in the cytoplasm was strong enough to mask the counterstaining in the nuclei in control-group chondrocytes (Fig. 3A, arrowheads, box), whereas nocodazole-group chondrocytes exhibited weak localization of BMP4 in the cytoplasm, exposing hematoxylin-stained nuclei (Fig. 3A′, arrows, box).

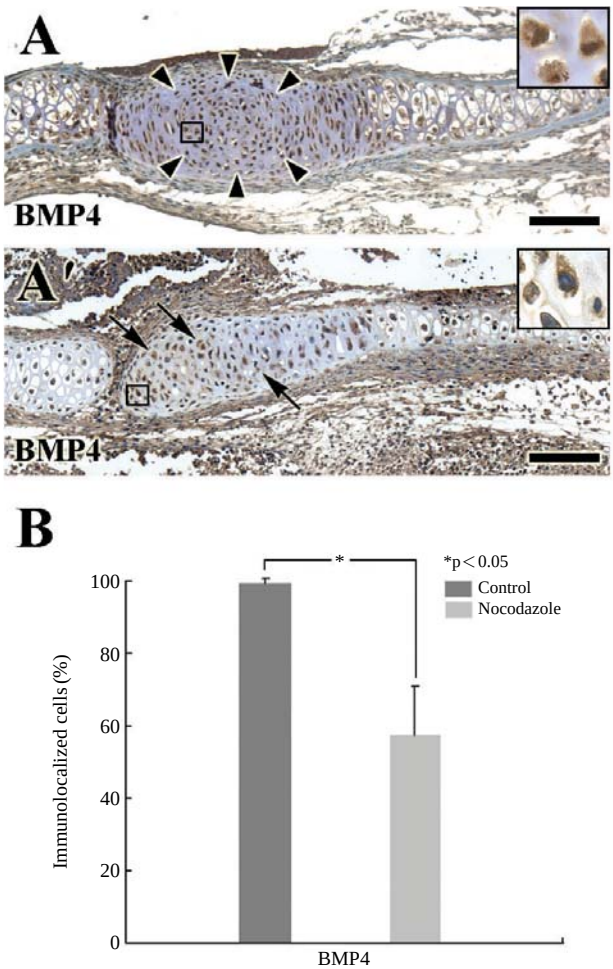

Fig. 3. Nocodazole reduces BMP4-expressing cells in the sphenooccipital synchondrosis. (A, A′, B) Chondrocytes exhibit strong localization of BMP in the control group (arrowheads), with staining in both the nucleus and cytoplasm in most chondrocytes (A, box), whereas the number of BMP-positive chondrocytes (arrows) is markedly lower in the nocodazole group, with BMP staining only in the cytoplasm, leaving the nuclei counterstained by hematoxylin (A′, box). (B) Chondrocytes expressing BMP4 are counted within a 2,500-μm^2 region. *$P < 0.05$ as determined by ANOVA. Scale bars: A, A′, 100 μm.

Discussion

Our study demonstrated generalized cessation of endochondral bone formation in the developing cranial base at E14 after disrupting the structure and function of microtubules with nocodazole in cultured cranial base specimens. Expression of proliferation marker Ki67, osteogenic marker BSP, and BMP4 were reduced within the sphenooccipital synchondrosis region. Chondrocyte hypertrophy was ceased in the hypertrophic zone of the sphenooccipital

synchondrosis, and bone matrix apposition was inhibited on the dorsal and ventral surfaces of the cranial base. Programmed cell death was found in the chondrocytes, particularly in the hypertrophic zone.

Lower number of Ki67-positive cells in the nocodazole compared to the control group is a direct effect of nocodazole treatment since one of the uses of nocodazole is to arrest the cell cycle in prometaphase (Fig. 1B, B′; De Brabander et al. 1976). This finding may be related to lower dorsoventral thickness of the sphenooccipital synchondrosis region and lower anteroposterior length of the cartilage plate that would later become the basioccipital bone in the nocodazole group compared to those in the control group (Figs. 1A, A′ and 2A, A′). Lower dorsoventral and anteroposterior dimensions of the cranial base may also occur due to inhibition of cartilage matrix synthesis. Col II staining and Col X staining were in the nocodazole group (Fig. 2A′, B′). This may be another effect of inhibiting cartilage matrix synthesis since cartilage synthesis depends on normal function of the Golgi apparatus, in which microtubules play an important role (Mellman and Simons 1992, Farquhar and Palade 1998). In addition, hypertrophy appeared to be retarded in the nocodazole group. The size of hypertrophic chondrocytes in the nocodazole group was not as large as that in the control group (Fig. 2A, A′, B, B′). Nor did it become any larger compared to the size of the hypertrophic-zone chondrocytes in the beginning of the organ culture. This finding supported the previous cell study on microtubule as a regulator of chondrocyte hypertrophy (Farquharson et al. 1999) and was in line with the previous study on microtubules in the joint cartilage (Benjamin et al. 1994). Thus, microtubules may be important in dimensional growth of the cranial base by modulating chondrocyte proliferation and hypertrophy, and cartilage matrix synthesis. However, the relation of programmed cell death and dimensional growth of the cranial base needs to be further clarified.

Interestingly, localization of Col II was weak but remained throughout the cartilage plate in the nocodazole group (Fig. 2A′), whereas there was a zone in the central region of the basioccipital-bone cartilage in the control group where Col II localization was absent (Fig. 2A, brackets). This indicated that Col II degradation was inhibited after nocodazole treatment, which was in line with a previous study discussing the inhibitory effect of cytoskeleton disruption on cartilage matrix degradation (Blain et al. 2006). Based on this finding and the finding that nocodazole-group specimens lacked bone-layer apposition on the dorsal and ventral surfaces of the cartilage plate, we suggest that nocodazole exerts a negative effect on the transitional process where replacement of cartilage matrix with bone (the main event of endochondral bone formation) occurs. The numbers of chondrocytes positive to BSP and BMP4 also decreased in the nocodazole group (Fig. 2C, C′). This finding indicated a reduction of osteogenic potential in both chondrocytes and perichondral cells, which might explain why bone formation was absent in the ossification center in the specimens in the nocodazole group (Fig. 2C′, brackets).

In addition, there was a region where the cartilage matrix was diffusely stained by BSP in the nocodazole group but not in the control group (Figs. 2C, C′ and 3). This finding was unexpected since we have already confirmed that the levels of the osteogenic markers BSP and BMP4 were lower in the nocodazole group. The absence of localization in the control group may be related to the mineralization level of the cartilage matrix being higher in that group. In their immunolocalization study of bone matrix proteins, Hosoya et al. (2005) found that BSP was present in the bone matrix only after decalcification, suggesting that the negative immunolocalization observed in our study was due to calcification occurring only in the control group. Further studies on decalcified specimens may be necessary to confirm this. In summary, altering the structure of microtubule may retard endochondral bone formation by inhibiting cartilage removal and suppressing osteogenic marker proteins, such as BSP, resulting in the inhibition of bone formation.

Taken together, we suggest that microtubules play important roles in many different parts of the developing cranial base. The results of this investigation of the unique developmental processes underlying cranial base development help to elucidate the cellular mechanisms underlying craniofacial anomalies in the cranial base. Future studies should examine the factors that play fundamental roles in chondrogenesis during cranial base development by using genetic manipulation to explore the precise molecular mechanisms underlying cranial base development.

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발생 중인 쥐 머리뼈바닥의 연골속뼈발생 중 미세관의 역할

권혁제, 이종민, 조경원, 신정오, 조성원, 이민정, 정한성

연세대학교 치과대학 구강생물학교실 해부 및 발생생물학 연구실

간추림 : 머리뼈바닥의 발생 중에 일어나는 연골속뼈발생은 다양한 세포현상과 연골바탕질 대사가 관여하는 매우 복잡한 과정이다. 연골세포에서는 증식, 형태변화, 증식 종료, 비대, 세포자멸사가 일어나고, 연골세포 주변으로는 연골바탕질이 활발하게 생성되었다가 제거되면서 뼈조직에 의해서 대체됨이 밝혀졌다. 그러나 이러한 복잡한 변화가 일어나는 동안 미세관이 어떠한 역할을 하는지에 대해서는 뚜렷하게 밝혀진 바가 없다. 그러므로 본 연구는 머리뼈바닥 발생 중 연골속뼈발생 동안의 미세관의 역할을 밝히고자 하였다.

미세관의 구성 단백질인 튜불린에 결합하는 약물 노코다졸을 사용하여 머리뼈바닥 조직을 기관배양 함으로써 미세관과 머리뼈바닥 발생의 관계를 조사하였다. 배양된 조직에서 일어나는 형태학적, 분자적 변화를 확인하기 위하여 헤마톡실린-에오신염색, 면역조직화학염색, 세포 개수 계산을 통한 분석이 이루어졌다.

나비뒤통수결합 부위 내의 미세관에 가해진 변화에 의해서 Ki67, 뼈발생표지자 BSP, BMP4의 발현이 감소하였다. 비대층 구역에서는 연골세포의 비대과정이 억제되었고, 뼈발생중심에서는 연골조직의 제거 및 뼈조직의 생성이 억제되었다.

본 연구는 미세관이 연골속뼈발생의 다양한 과정에 관여함을 밝혔다. 이러한 결과는 머리뼈 성장 및 머리뼈바닥 기형과 관련된 질병을 이해하는 자료로 사용될 수 있을 것으로 사료된다.

찾아보기 낱말 : 머리뼈바닥, 연골속뼈발생, 나비뒤통수결합, 미세관, 노코다졸, BMP4, 조직배양

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