Original article

Effects of electrical stimulation of the lateral pterygoid muscle on the localization of glycosaminoglycans in mandibular condyles in rats

Ichiro TAKAHASHI, Itaru MIZOGUCHI, Yasuyuki SASANO, Shuji SAITOH, Manabu KAGAYAMA and Hideo MITANI

Department of Orthodontics, School of Dentistry, Tohoku University

Key words: Lateral pterygoid muscle, electrical stimulation, glycosaminoglycan, immunohistochemistry, mandibular condylar cartilage

Abstract: The lateral pterygoid muscles were electrically stimulated to evaluate the effects of biomechanical stress on the distribution of glycosaminoglycans (GAG) in mandibular condyle. The lateral pterygoid muscles of 3-week-old male Sprague-Dawley rats were electrically stimulated by repeated application (5-sec on/5-sec off) of 5-Hz current, 10 msec in duration, at an intensity of −1 to −3V for 1, 2, 4, and 7 days. The mandibular condyles were processed for immunohistochemistry using the monoclonal antibodies 2B6, 3B3 and 5D4 that recognize chondroitin-4-sulfate, chondroitin-6-sulfate and keratan sulfate, respectively.

Three epitopes were found in the central and peripheral areas of the mandibular condyles at the transitional, maturative and hypertrophic cell layers. The residual cartilage matrix embedded in the primary spongiosa of bone also showed immunoreactivity for 2B6.

In the central area, immunoreactivity for 3B3 increased, whereas that for the 2B6 and 5D4 epitopes decreased during days 1 and 2 of the experiment, when the thickness of the cartilage increased remarkably. On day 7, the content of GAGs returned to the control level in association with a reduced cartilage thickness.

In contrast to the central area, the posterior area showed a gradual decrease of the three GAG epitopes during the investigation, and these were almost nonexistent on day 7. This change was associated with the transdifferentiation of progenitor cells into osteoblasts rather than chondroblasts.

The present results strongly suggest that the expression of GAGs is controlled by the biomechanical stress on the tissue and may be related to the differentiation of progenitor cells in mandibular condyles.

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ラット外側翼突筋の電気刺激が下顎頭軟骨におけるグリコサミノグリカンの局在き及ぼす影響

抄録：下顎頭軟骨の形態形成と外側翼突筋の機能との関連性を解明することを目的として以下の実験を行った．3週齢雄性 Sprague-Dawley 系ラットの外側翼突筋に単極針電極を挿入し5秒毎に5秒間，刺激頻度 5Hz，刺激電圧 −1～−3V の電気刺激を負荷し，実験期間を1，2，4，および7日間とした．下顎頭軟骨を含む顎関節部の矢状断連続切片を作製して3種類のグリコサミノグリカン (GAG)，すなわちコンドロイチン4硫酸，コンドロイチン6硫酸，ケラタン硫酸について免疫組織化学的手法を用いてその局在を検討した．

組織学的観察から，関節円板狭窄部に対向する下顎頭軟骨部では軟骨層の一過性の肥厚と骨梁構造の改変が認められた．また，外側翼突筋の間接的あるいは直接的付着部にあたる下顎頭軟骨前方部および後方部では軟骨組織の消失とそれに続く膜性骨基質の形成が認められた．これらの変化に伴って後方部ではすべての GAG の分布範囲が顕著に減少し，中央部では分化段階を反映した局在の変化が認められた．これらのことはバイオメカニカルストレスとしての外側翼突筋の電気刺激が下顎頭軟骨における GAG の局在や軟骨細胞の分化過程に大きな影響を与えることを示唆しているものと考えられる．

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Introduction

Mandibular condyles are growth site for mandibles and play a significant role in temporomandibular joint (TMJ) function. The mandibular condylar cartilage has been characterized as a secondary cartilage[1], the growth and development of which are affected by both genetic and environmental factors[2,3], and collagen types I and II[4] are both present in the extracellular matrix (ECM). The progenitor cells in the proliferative cell layer can proliferate and differentiate into either chondrocytes or osteoblasts[5,6], and it has been suggested that their differentiation largely depends on their biomechanical environment[7~12].

The mechanical stress loaded on the mandibular condyle is mainly due to mastication. Among the masticatory muscles, the lateral pterygoid muscle (LPM) has been suggested to be one of the most important factors in regulating the growth of the mandible[7,8,13~15], since changes in the activity of the LPM have been shown to induce several changes in the condyles, such as the mitotic activity of the progenitor cells, the thickness of the condylar cartilage, and the rate and amount of bone formation[7,8,13,15]. Further, resection of the LPM has been shown to cause osteogenic differentiation of progenitor cells[9]. We previously examined the effect of electrical stimulation of the LPM on the rat mandibular condyles, and observed several morphological changes ; i. e., the disappearance of cartilaginous cell layers and new bone formation in both the anterior and posterior areas, a transient increase in the thickness of the cartilage in the central area, and changes in the phenotypic expression of collagen types I and II[7,8].

Cartilage contains a large amount of proteoglycans (PG) which are composed of one or more glycosaminoglycan (GAG) chains and a core protein[16]. Due to the highly anionic nature of GAGs, GAGs of PGs are believed to be related to tissue viscoelasticity and biomechanical stress[17]. GAGs in the mandibular condyles have been analyzed biochemically[18], and the localization of GAGs has been described histochemically[19]. However, there is little information available concerning the effect of the altered activity of the LPM on the distribution of GAGs in the mandibular condyles.

The purpose of the present study was to determine the distribution of the three predominant GAGs of PG in hyaline cartilage immunohistochemically in rat mandibular condyles that were subjected to electrical stimulation of the LPM.

Materials and Methods

Experimental animals

The experimental animals used were male 3-week-old Sprague-Dawley rats, which were in the active growing stage soon after weaning. Seven and 12 animals were used in the control and experimental groups, respectively.

Electrical stimulation

The electrical stimulation in this experiment has been described previously[7,8]. Briefly, electrical stimulation consisted of the repeated application (5-sec on/5-sec off) of 5-Hz current, 10 msec in duration, and at an intensity of −1 to −3V. The two monopolar teflon-coated stainless electrodes were inserted into the bilateral LPMs under pentobarbital anesthesia (50 mg/50 g of body weight).

Electrical stimulation was applied for 1, 2, 4 and 7days, and 3 animals were used in each group. The control experiments were 1) 3-week-old and 4-week-old rats, 2) electrodes inserted into the LPM without electrical stimulation, and 3) electrodes inserted into fibrous tissue running out of the LPM with application of the electrical stimulations for 7days. Each group in the control experiments was composed of 3 to 5 animals.

Tissue preparations

After the respective experimental periods, all of the animals were perfused from the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at a rate of 3 m*l*/min under pentobarbital anesthesia. The temporomandibular joints were dissected and the specimens were further fixed in the same fixative. After thoroughly rinsing with 0.01 M PBS, the specimens were decalcified in 10% ethylenediaminetetraacetate in 0.01 M PBS (pH 7.4) for 2 weeks at 4°C. After decalcification, the specimens were dehydrated in a graded series of ethanol and embedded in Spurr's resin. One μm-thick sections were cut by using of Reihert-jung Ultracut with grass knife. The sections were placed on glass slides, treated with saturated sodium ethylate in absolute ethanol for 15min, rehydrated and dried at room temperature.

Immunohistochemistry for GAGs

After 3 rinses in 0.01 M PBS for 5 min, the sections were incubated with chondroitinase ABC and chondroitinase ACII (Seikagakukogyo, Tokyo, Japan) in a moisture chamber for 60 min at 37°C. After thorough rinses with 0.01 M PBS, sections were incubated with 10% normal goat serum (Cappel, Westchester, U. S. A.), 5% bovine serum albumin (Sigma, St. Louis, U. S. A.), 0.1% NaN_3 and 0.025% Triton-X 100 in 0.01 M PBS (solution A) for 1 h in a moisture chamber at room temperature to reduce nonspecific reaction. The sections were incubated with primary antibodies for GAGs and the control sections were incubated with solution A for 2h at room temperature. The antibodies used in this study were mouse monoclonal antibody, 2B6 : anti chondroitin-4-sulfate, 3B3 : anti chondroitin-6-sulfate, and 5D4 : anti keratan sulfate (Seikagakukogyo, Tokyo, Japan). The enzymatic digestions corresponded to chondroitin-6-sulfate and keratan sulfate (chondroitinase ABC) and chondroitin-4-sulfate (chondroitinase ACII). The antibodies were diluted to 1 : 200 for 2B6, 1 : 100 for 3B3 and 1 : 200 for 5D4[20~22] in solution A. Thorough rinsing with 0.01 M PBS was followed by incubation with FITC-conjugated anti-mouse IgG and IgM antibody (TAGO, Burlingame, CA, U. S. A.) for 1. 5h at room temperature. The secondary antibody was diluted 1 : 50 with solution A. The sections were rinsed in 0.01M PBS, mounted in modified PVA[23,24] and observed under a fluorescence microscope (Olympus, BH2-RFK). The immunostaining methods were standardized as described above and all of the sections for each antibody were put on the same glass slides for simultaneous staining. Photomicrographs were taken under identical conditions.

Histological observations

Toluidine blue staining was performed to observe the histological features of condylar cartilage.

Results

The condyles were divided into 2 parts for observation purposes (Fig. 1) : 1) the central area of the condyles, which corresponded to the thinnest part of the articular disc, and 2) the posterior area of the condyles, which was attached to the retrodiscal pads. The condylar cartilage was divided into 5

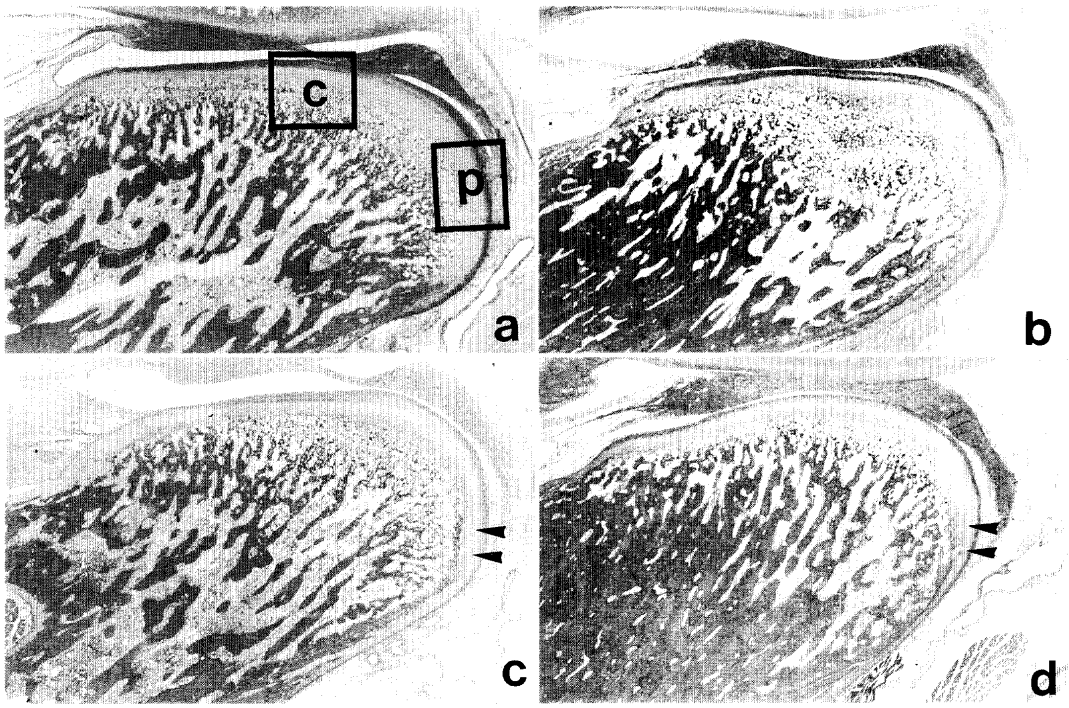

Fig. 1 Microphotographs of the condyles from a : 4-week-old Sprague-Dawley rats, b : 1-day experimental animals, c : 2-day experimental animals, and d : 7-day of experimental animals. Square "c" indicates the central area and "p" indicates the posterior area of the condylar cartilage. Arrowheads indicate the area that was replaced by bone. (Original magnification : ×8)

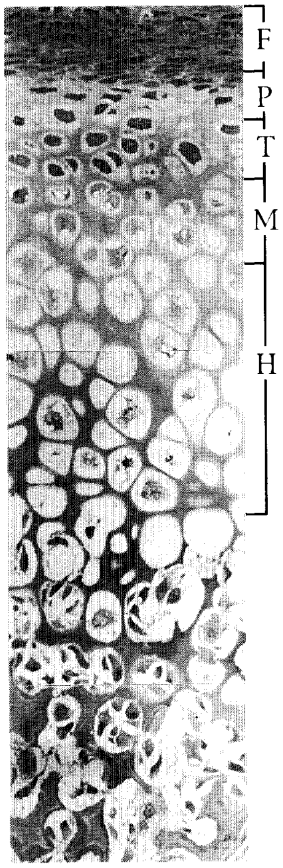

Fig. 2 The cell layers of the condylar cartilage. F : fibrous layer, P : proliferative cell layer, T : transitional cell layer, M : maturative cell layer, and H : hypertrophic cell layer. (Original magnification : ×160)

Table 1 Immunoreactivity and its changes in the cetral areas

	Cell layers	C4S	C6S	KS
Control	Fibrous	−	++	−
	Proliferative	±	+	−
	Transitional	++	++	−
	Maturative	+++	+++	++
	Hypertrophic	+++	+++	+++
Day 1	Fibrous	−	++	−
	Proliferative	±	++↑	−
	Transitional	+↓	++	−
	Maturative	+↓	+++	+↓
	Hypertrophic	+↓	+++	+↓
Day 7	Fibrous	−	++	−
	Proliferative	±	+↓	−
	Transitional	++↑	++	−
	Maturative	++↑	+++	++↑
	Hypertrophic	++↑	+++	++↑

C4S : Chondroith-4-sulfate, C6S : Chondroitin-6-sulfate, KS : Keratan sulfate

layers from the articular surface : the fibrous layer, the proliferative cell layer, the transitional cell layer, the maturative cell layer and the hypertrophic cell layer. The fibrous layer is the superficial layer of the condyle that includes spindle-shaped fibroblasts. The proliferative cell layer has the highest activity of cell division and polygonal-shaped, osteo-chondro progenitor cells. The transitional cell layer is the intermediate layer between the maturative and proliferative cell layers, without type II collagen. The maturative cell layer includes

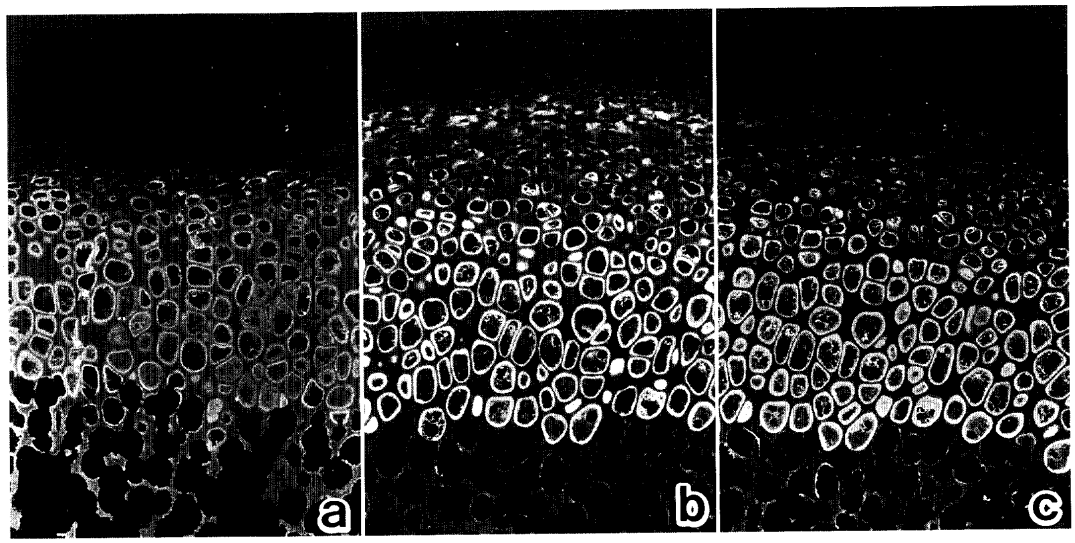

Fig. 3 Immunohistochemical localization of a : chondroitin-4-sulfate (2B6), b : chondroitin-6-sulfate (3B3) and c : keratan sulfate (5D4) in the central areas of the control sections. (Original magnification : ×62.5)

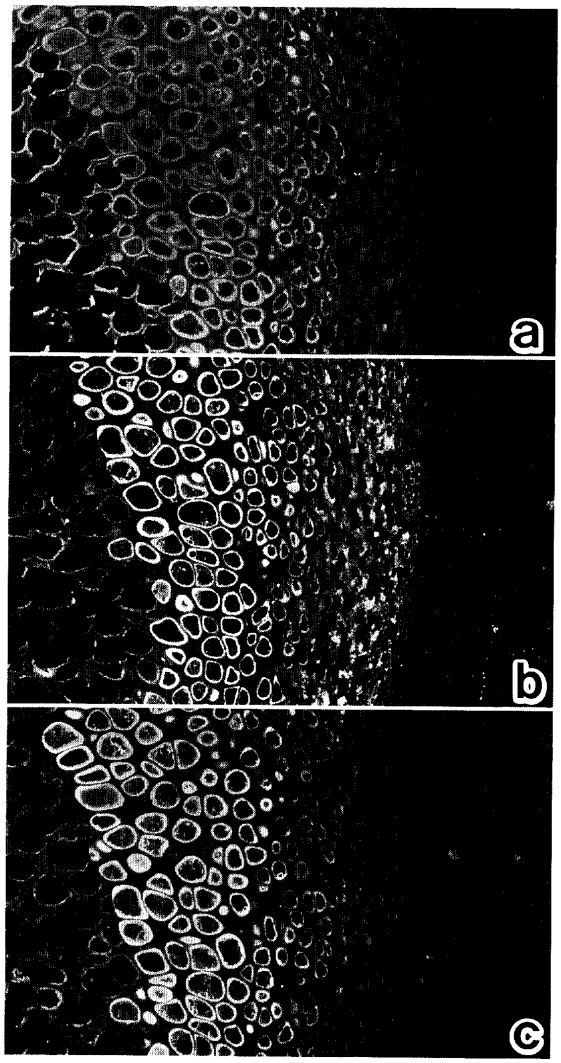

Fig. 4 Immunohistochemical localization of a : chondroitin-4-sulfate (2B6), b : chondroitin-6-sulfate (3B3) and c : keratan sulfate (5D4) in the posterior areas of the control sections. (Original magnification : ×62.5)

mature chondrocytes where type II collagen was deposited. The hypertrophic cell layer is the most differentiated layer of the cartilage, and contains hypertrophied chondrocytes and partially calcified matrix (Fig. 2).

Control groups (Figs. 3~4, Tables 1, 2)

No significant histological differences were observed among the three control experiments. The cartilage in the central and posterior areas of condyles consisted of the five layers described above. Immunoreaction for 2B6 was mainly localized in the transitional, maturative, and hypertrophic cell layers. Calcified cartilage matrix showed a weak response to 2B6, however, residual cartilage matrix in the primary spongiosa showed stronger reaction than that in the calcified cartilage matrix just below the hypertrophic cell layer. 3B3 reacted with all of the layers, but weaker in the transitional cell layer. The epitope of 5D4 was found at the maturative cell layer and the below. Generally, GAGs were much more abandant in the pericellular and territorial matrix of chondrocytes than in the interterritorial matrix.

In the posterior area (Fig. 4), the staining patterns and distribution of 2B6, 3B3 and 5D4 were similar to those in the central area, while the interterritorial matrix showed less reaction than that in the central area. The staining intensity for 3B3 in the proliferative cell layer was weaker in the posterior area than in the central area.

Experimental groups (Figs. 5, 6)

1) Central area (Fig. 5, Table 1)

The cartilaginous layers of the central area were increased in thickness on days 1 and 2, and returned to the control level on day 7. The transitional, maturative and hypertrophic cell layers were generally thicker during the early experimental periods. On day 7, the primary bone pillars in the condyles were oriented more perpendicular than those in the controls (Fig. 2).

Immunoreactivity for 3B3 increased in the proliferative, maturative and hypertrophic cell layers on days 1 and 2, and reduced to the control level on day 7. In contrast to the 3B3 epitopes, the epitopes for 2B6 and 5D4 in the interterritorial matrix were decreased on days 1 and 2 (Fig. 5). At the same time, immunoreaction for all of the GAGs disappeared in the residual calcified cartilage matrix surrounded by immature bone matrix of primary spongiosa. At the end of the experiment, calcified cartilage was again stained for 3B3 and 2B6. On days 1 and 2, immunoreactivity for 5D4 was greatly decreased in the maturative and hypertrophic cell layers.

2) Posterior area (Fig. 6, Table 2)

The cartilaginous layers were also composed of 5 layers, similar to the central area described above. The staining intensity and patterns of immunohistochemistry in this area changed during the experiment. Corresponding to attachment to the retrodiscal pads, cartilaginous tissue disappeared from the transitional cell layer on day 2, and was replaced by polygonal-shaped cells similar to the osteo-chondro progenitor cells in the proliferative cell layer, as

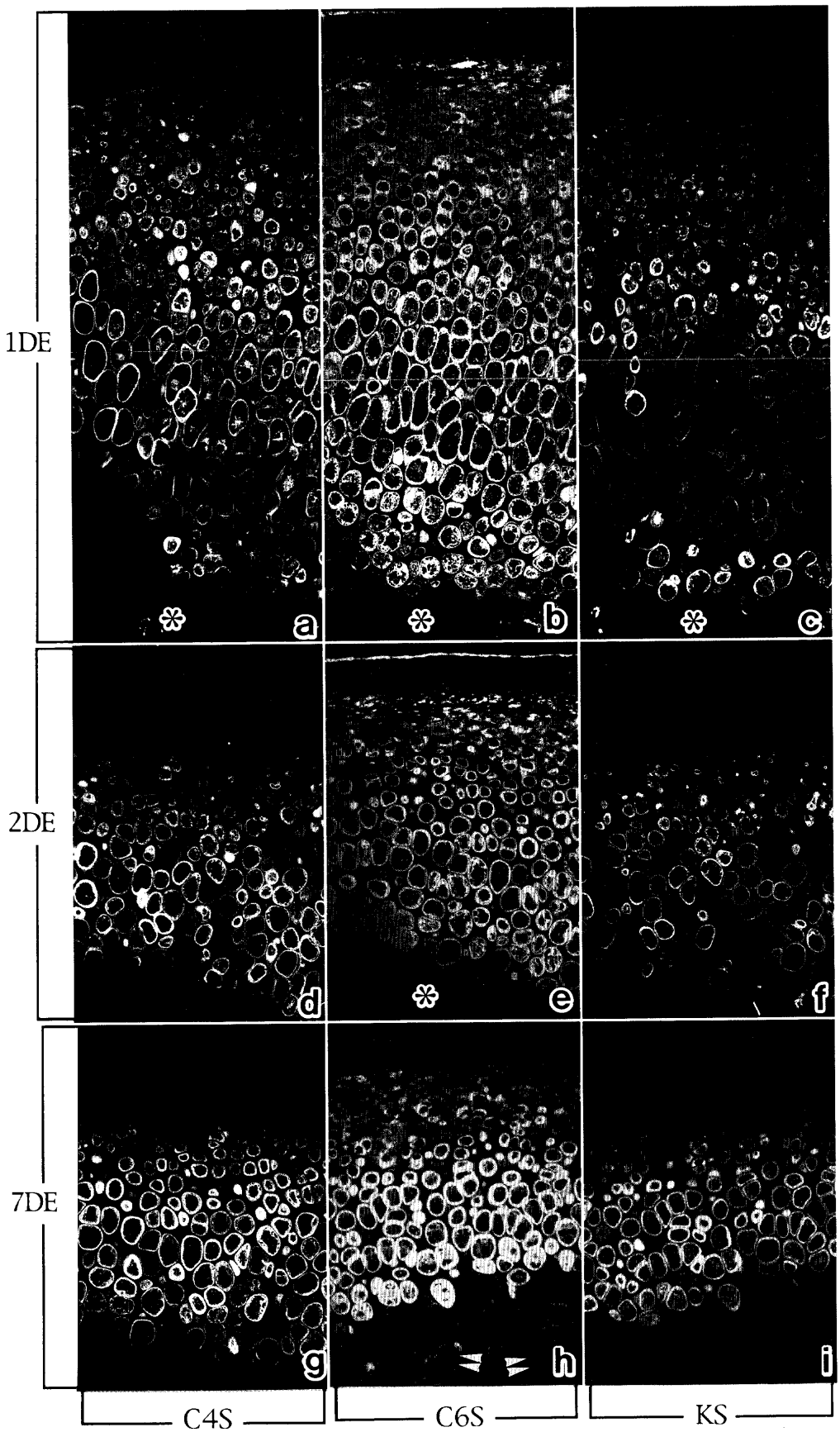

Fig. 5 Immunohistochemical localization in the central areas of the experimental groups, upper row : day 1, middle : day 2, and lower : day 7, C4S : chondroitin-4-sulfate (2B6) ; C6S : chondroitin-6-sulfate (3B3) ; and KS : keratan sulfate (5D4). Arrows indicate the area of increased immunoreactivity for 3B3 on day 1. "*" indicates the calcified cartilaginous matrix that was not positive for immunostaining. Arrowheads indicate the calcified cartilage that was positive for immunostaining of 3B3. (Original magnification : ×62.5)

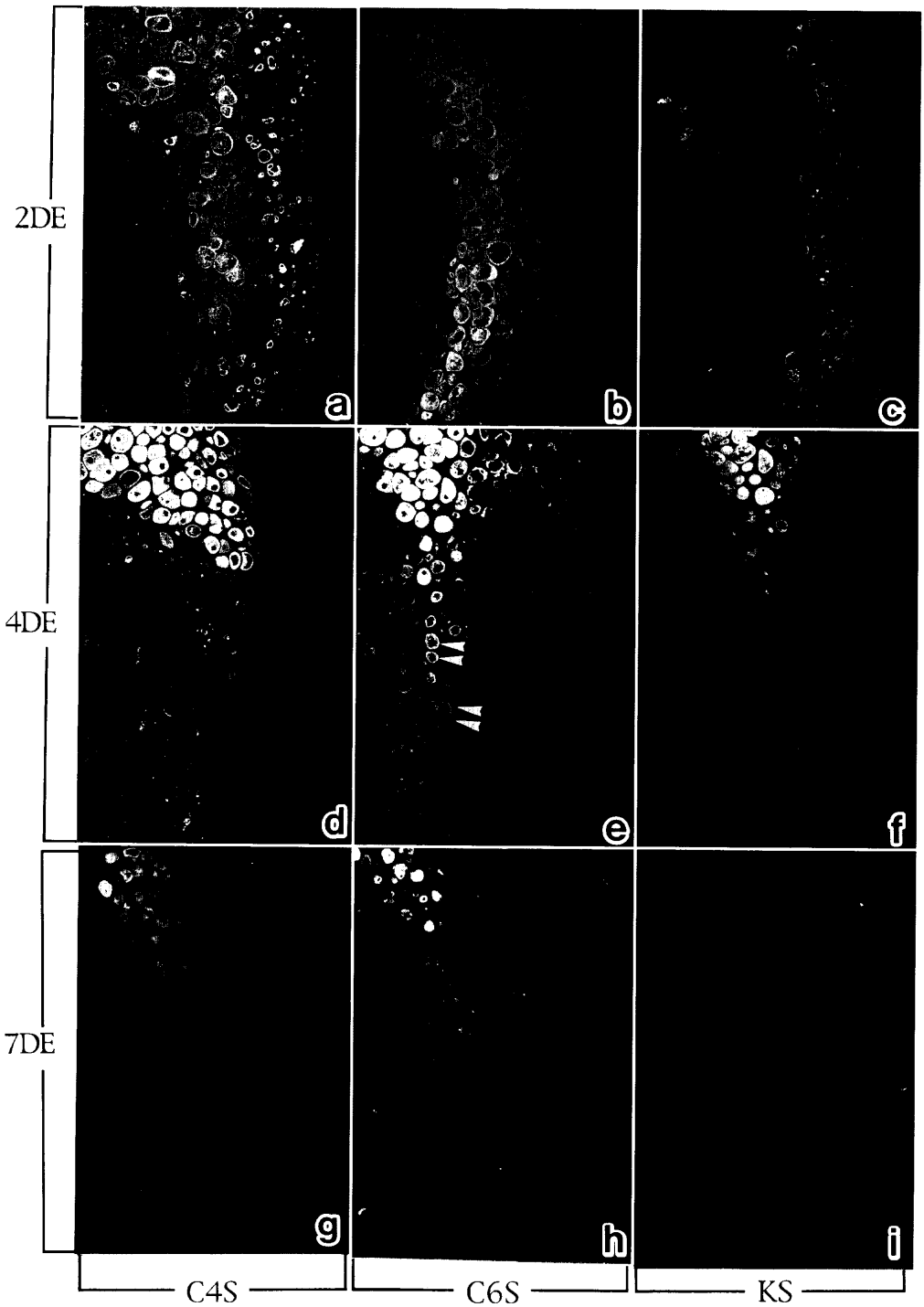

Fig. 6 Immunohistochemical localization of the posterior areas of the experimental groups, upper row : day 2, middle : day 4, and lower : day 7, C4S : chondroitin-4-sulfate (2B6) ; C6S : chondroitin-6-sulfate (3B3) ; and KS : keratan sulfate (5D4). Arrowheads indicate the ring-like staining pattern in the pericellular matrix of young osteocytes for 3B3. (Original magnification : ×62.5)

described previously[7,8].

The changes observed in sections stained with either 2B6 or 3B3 were similar to each other (Fig. 6). The immunoreactivity for both antibodies decreased in the interterritorial matrix during the early stages of the experiment, whereas territorial matrix maintained intense reactivity (Fig. 6a, b). However, immunoreactivity for 3B3 and 2B6 at the territorial matrix was also decreased after 4 days, and was seen only at the peripheral region of the

Table 2 Immunoreactivity and its changes in the posterior area

	Cell layers	Morphological features of cells	C4S	C6S	KS
Control	Fibrous	Perichondrium, Figroblastic cells	−	++	−
	Proliferative	Polygonal shaped progenitor cells	±	+	−
	Transitional	Spindle-shaped immature chondroblasts	++	++	−
	Maturative	Matured chondrocytes	+++	+++	++
	Hypertrophic	Hypertrophic chondrocytes	+++	+++	+++
Day 2	Fibrous	Perichondrium, Fibroblastic cells	−	++	−
	Proliferative	Polygonal shaped progenitor cells	±	+	−
	Transitional	Polygonal shaped progenitor cells	±↓	+↓	−
	Maturative	Matured chondrocytes	++↓	++↓	+↓
	Hypertrophic	Hypertrophic chondrocytes	++↓	++↓	+↓
Day 7	Fibrous	Perichondrium, Fibroblastic cells	−	+↓	−
	Proliferative	Polygonal shaped progenitor cells	−↓	−↓	−
	Transitional	Onteoblasts	+↓	+↓	−
	Maturative	Bone and small number of chondrocytes	+↓	+↓	+↓
	Hypertrophic	Bone	−↓	−↓	−↓

C4S : Chondroith-4-sulfate, C6S : Chondroitin-6-sulfate, KS : Keratan sulfate

lacunae. While staining with 5D4 showed changes similar to those with 2B6 and 3B3 (Fig. 6c, f, i), immunoreactivity for 5D4 decreased earlier than those for 2B6 and 3B3. By the end of the experiment, the immunoreactivity for 5D4 had disappeared from the bone matrix.

Discussion

It is well known that cartilage contains large amount of glycosaminoglycans of PG, which are ubiquitous components of connective tissue[16,17]. GAGs in cartilage include chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid, which are characterized by their specific repeating disacharide units[15~17].

Recently, immunohistochemical analysis with monoclonal antibodies which recognize specific determinants in GAGs has demonstrated that the various subspecies of GAGs have distinct and restricted patterns of distribution in connective tissues, including cartilage[20~22]. Using the monoclonal antibodies 2B6, 3B3 and 5D4, the present study determined the localization of the epitopes and how it changes under electrical stimulation of the LPM.

The results of this investigation confirmed that three types of GAGs, (chondroitin-6-sulfate, chondroitin-4-sulfate, keratan sulfate) are present in three layers of the condylar cartilage, i.e., in the transitional, maturative, and hypertrophic cell layers, and that the pericellular matrix contained more GAGs than the interterritorial matrix. Chondroitin-6-sulfate was also observed in the fibrous and the proliferative cell layers, whereas keratan sulfate epitope was not found to any significant extent in these layers. Like another ECM molecule, type II collagen, these epitopes persisted in cartilage trabeculae embedded within the new bone matrix of primary spongiosa. In these cartilage trabeculae, the 2B6 epitope was more intense than the 3B3 and 5D4 epitopes. These results are similar to those reported in other cartilage, such as growth plate[25,26], tracheal cartilage[27] and articular cartilage[28]. During the development and maturation of the articular cartilage, collagen types I and II are both expressed in the ECM, as in mandibular condylar cartilage[28].

The results of the present study also demonstrated the dynamic changes in the distribution of GAGs within mandibular condyles that are subjected to electrical stimulation of the LPM. These changes were observed in both the central and peripheral regions of the condyles.

In the central area, the 3B3 epitope increased on days 1 and 2, which was associated with an increase in the thickness of the cartilage, and returned to the control level at the end of the experiment when the thickness of the cartilage returned to the control level. In contrast, immunoreactivity for 2B6 and 5D4 decreased on days 1 and 2, and returned to the control level at the end of the experiment. Although the significance of the heterogeneity of the chemical structure of the GAGs is unclear, and no control

mechanism has yet been elucidated, the increased expression of the 3B3 epitope and the decreased expression of the 2B6 and 5D4 epitopes are particularly interesting because these differences suggest different roles and metabolic mechanisms for the GAGs.

As discussed in our previous report[7], the central area of mandibular condyles might be under compressive force[10~12,29,30]. Since GAGs of PG are highly anionic, and are believed to be related to tissue viscoelasticity, biochemical and histochemical studies have been performed to elucidate the relationship between biomechanical force and the content of GAGs[29,30]. An experiment in which cartilage was exposed to continuous and intermittent compressive force in vitro[30] demonstrated that a continuous force inhibits the synthesis of sulfated GAGs and collagen, whereas an intermittent compressive force stimulates their synthesis. Furthermore, studies on the flexor digitorum tendon have shown that the area under compressive stress contains more chondroitin sulfates than the area under tensional force[31]. In addition, freeing the tendon from compressive force results in a decrease in GAG content[32]. This evidence suggests that compressive force may be related to the increased synthesis of GAGs that was observed in the present study through immunolocalization of 3B3 in the central area of the mandibular condyles. However, the present study also revealed decreased immunoreactivity for 2B6 and 5D4 in the central area that was subjected to compressive stimuli. This suggests the possibility that only specific GAGs respond to biomechanical stress. Apparently, further studies are needed to elucidate this possibility.

In contrast to the central area, the posterior area showed a phenotypic change from cartilage to bone, as has been shown previously using anti type I and type II collagens[8]. Immunoreactivity for 2B6 and 3B3 decreased during the early period and disappeared at the end of the experiment. The distribution of GAGs in the posterior area at the end of the experiment was comparable to that in bone[33].

It has been widely accepted that cartilage ECM responds to compression, while tendon responds to tensile force. In vitro studies have demonstrated the unique activity of the progenitor cells which can differentiate into both osteoblasts and chondroblasts[5,6]. The differentiation of progenitor cells has been shown to depend on the adhesion of these cells to substrates such as type I collagen or fibronectin[34]. Some types of receptors for ECM proteins, such as integrin receptor, have been found in human chondrosarcoma cell lines[35]. In addition, hyaluronic acid inhibits cell adhesion[17]. Therefore, it is possible that the reduction in GAGs may be related to the transdifferentiation of progenitor cells from chondroblasts to osteoblasts. However, there are no data available concerning the relation between cell adhesion and GAGs other than hyaluronic acid. The mechanism by which progenitor cells differentiate into either chondroblasts or osteoblasts still remained to be elucidated.

In summary, the present results strongly suggest that the expression of GAGs is controlled by biomechanical stress on the tissue and may be related to the differentiation of progenitor cells in the mandibular condyles.

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Chief : Prof. Hideo Mitani

Corresponding address : Ichiro Takahashi
Department of Orthodontics, School of dentistry, Tohoku University 4-1, Seiryo-machi, Aoba-ku, Sendai, 980-77 Japan
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