SHORT COMMUNICATION

Ultrastructural demonstration of soybean agglutinin binding sites in mandibular condylar chondrocytes from normal, hypophysectomized, and growth hormone-treated hypophysectomized rats

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Introduction

Cartilage growth is mostly dependent upon cell division and matrix production¹⁾. In this respect, the matrix production is measured by the rate of proteoglycan synthesis. This parameter may be a substantial marker of cartilage tissue growth^{2,3)}. However, there is little information as to intra- and intercellular regulatory mechanisms of proteoglycan synthesis. The production of proteoglycans is known to be controlled hormonally⁴⁾, and one of the most striking examples of hormonal control of cartilage growth is the regulatory role of growth hormone (GH)⁵⁾.

Although GH has been shown to have a pronounced effect on the growth of cartilaginous tissue both *in vitro*^{6,7)} and *in vivo*^{8,9)}, the regulatory mechanism of the hormone on cartilage proteoglycan synthesis is not yet well defined. However, recent studies suggest that GH may play a central role in cartilage growth by stimulating the insulin-like growth factor I (IGF - I)^{10,11)}. Furthermore, receptors for GH were also identified in rabbit¹²⁾ and human¹³⁾ chondrocytes.

It is commonly thought that glycosylation is the most important event in the pathway of proteoglycan synthesis in cartilage^{14,15)}. The histochemical approach to studying of this process has been successfully

applied with the use of lectin probes¹⁶⁻¹⁸⁾. Takagi *et al.*¹⁷⁾ introduced soybean agglutinin (SBA), as well as peanut agglutinin, as a specific histochemical probe for localizing chondroitin sulfate-containing proteoglycans in rat epiphyseal cartilage.

Production of the chondroitin sulfate chains in chick embryo cartilage proteoglycan was shown to contain a N-acetyl-D-galactosamine (GalNAc) residue on their non-reducing end¹⁹⁾. Therefore, SBA lectin may be a particularly useful histochemical tool for the assessment of cartilage proteoglycan glycosylation. In this study, ultrastructural SBA lectin histochemistry was applied for the evaluation of the mode of proteoglycan glycosylation in chondrocytes of mandibular condylar cartilage, which is known to respond quickly to hormonal action²⁰⁾, from normal, hypophysectomized, and GH-treated hypophysectomized rats.

Materials and Methods

A total of 25 Wistar-strain rats were used throughout this experiment. Hypophysectomy was performed on twenty 8-week-old rats which had been deeply anesthetized with diethyl ether. Five weeks after the operation, half of the animals received a hypodermic injection of human GH at a dose of 1 IU/Kg of body

weight (Nikken Kagaku, Tokyo, Japan) twice a day for 1 week. Five age-matched unoperated rats served as the normal group.

Mandibular condylar cartilages from normal, hypophysectomized, and rats injected with GH after hypophysectomy were removed under diethyl ether anesthesia. The tissue blocks were fixed in 2% paraformaldehyde-2% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 6 h at 4°C. For immuno-electron microscopic observation, a post-embedding method was applied. After fixation, the tissue blocks were washed with PBS three times for 5 min each, and then dehydrated through a graded series of increasing concentrations of ethanol in distilled water (70, 80, 90, 95 and 100%) over a total period of 1 h at 4°C. After dehydration, the blocks were infiltrated with Lowicryl K4M at 4°C, and polymerization was carried out under UV light for 24 h at 4°C.

Ultra-thin sections were mounted on nickel grids and washed in PBS for 5 min. The grids were then incubated in 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature, and treated with pure SBA (2.5 µg/ml in 1% BSA-PBS, EY Lab., CA,

USA) for 6 h at 4°C. Thereafter, the grids were rinsed three times in PBS and incubated with anti-SBA antibody (1 : 5000 dilution, EY, Lab., CA, USA) in 1% BSA-PBS for 12 h at 4°C. Following this, the grids were rinsed three times in PBS and labeled with protein A-gold (15 nm ; ×20, Bio Cell Int., UK) for 45 min at room temperature. After incubation, the grids were rinsed several times in PBS, and replaced in distilled water. Finally, they were stained with uranyl acetate (3 min) and lead citrate (3 min) before being examined under a JEM 100-CX electron microscope.

The specificity of the lectin binding was checked by the following ultra-thin sections of negative controls : 1) sections incubated in 0.2 M GalNAc ; 2) sections incubated with anti-SBA antibody and protein A-gold complex without prior treatment with SBA ; and 3) sections incubated with protein A-gold complex without SBA and anti-SBA antibody.

Results

SBA binding sites could be demonstrated in the Golgi complexes of the proliferative chondrocytes

Fig. 1 Electron micrographs of Lowicryl K4M-embedded mandibular condylar chondrocytes from normal rats.

- a : Chondrocytes reacted with complete system for immunolabeling of SBA. Labeling is shown over the intermediate region of Golgi complex (arrows in '1a'), but not over the endoplasmic reticulum.
- b : Negative control section incubated with anti-lectin and protein A-gold complex without prior treatment with SBA, showing complete lack of binding. a, b × 8,000. Bars = 1 µm

Fig. 2 Electron micrographs of Lowicryl K4M-embedded mandibular condylar chondrocytes of hypophysectomized (a, b) and GH-treated hypophysectomized (c, d) rats labeled with SBA-gold complex. In the chondrocytes from hypophysectomized rats, labeling is present over the Golgi complex, which is extensively vacuolated, and is almost absent over the rough endoplasmic reticulum. Parts of chondrocytes from GH-treated hypophysectomized rats show greatly diminished labeling over aggrandized Golgi vacuoles. As seen in micrograph '2d', which represents the rectangular area seen in micrograph '2c', a small number of the gold particles can be detected over the intermediate region of the Golgi complex.

a, d $\times 10,000$; b $\times 15,000$; c $\times 6,000$. Bars = $1\ \mu\text{m}$

throughout this experiment. In unoperated normal rat chondrocytes, SBA binding sites were detected in the intermediate region of the Golgi complexes (Fig. 1a). There were no gold particles detected in the Golgi complexes or elsewhere when sections were reacted with SBA solution together with 0.2 M GalNAc, with anti-lectin antibody solution, and protein A-gold complex only (Fig. 1b), or with protein A-gold solution

only.

In the hypophysectomized rats, intense labeling was observed over an extensively vacuolated Golgi complex (Fig. 2a), but not over the rough endoplasmic reticulum, mitochondria, lysosomal bodies, or entire plasma membrane. Although the topography of Golgi subcompartments was not well defined due to this particular pathologic condition, SBA-labeled gold

Fig. 3 Diagrammatic summary of SBA binding sites in the Golgi complex from normal (a), hypophysectomized (b), and GH-treated hypophysectomized (c) rat mandibular condylar chondrocytes. SBA binding sites are demonstrated in the intermediate region of Golgi complex in an intact chondrocyte (a), while trans to transmost regions are irregularly labeled with numerous gold particles in a hypophysectomized chondrocyte (b). A remarkable diminution of the binding sites, remaining some gold particles in the intermediate region, is evident in a GH-treated hypophysectomized chondrocyte (c).

particles were specifically localized over the aggrandized vacuoles and in the intermediate region of the Golgi complex (Fig. 2b).

Following the injection of hypophysectomized rats with GH, SBA binding in the Golgi complexes was markedly reduced: only a small number of gold particles were found, and they were restricted to the intermediate region of the Golgi complexes (Fig. 2c); and no reaction could be detected in the disorganized Golgi vacuoles (Fig. 2d). Although the gold labeling in GH-treated hypophysectomized animals was comparatively scanty, their binding profiles disclosed a pattern similar to that seen in the chondrocytes of unoperated normal animals (Fig. 1a). Our schema shows the modifications of SBA binding sites in the Golgi complexes due to these experimental chondrocytes (Fig. 3).

Discussion

Much is known about the effects of hormones on cartilage growth and metabolism from both experimental and clinical studies⁴⁾. In the present study, the influence of GH on SBA binding was demonstrated in the Golgi complex of chondrocytes of mandibular condyle from hypophysectomized rats. Chondrocytes from hypophysectomized rats disclosed preferential SBA binding sites in the Golgi complexes with an increased number of SBA-labeled gold particles compared with those seen in unoperated, normal

animals. The administration of GH to hypophysectomized animals had a pronounced effect on the binding sites in Golgi complexes, *i. e.*, a marked reduction. The cytochemical alterations (or modifications) were interpreted as direct or indirect effects of GH on the chemical composition of glycoconjugates present in the Golgi complexes, *i. e.*, on the Golgi function.

In chondrocytes, cartilage proteoglycan consists of a repeating disaccharide structure of chondroitin sulfates and O-linked and N-linked oligosaccharides^{14,21)}. Takagi *et al.*¹⁷⁾ described that chondroitin sulfate-containing proteoglycans in epiphyseal cartilage can be stained by SBA, since a GalNAc residue occupies the non-reducing end of chondroitin sulfate¹⁹⁾. Although the GalNAc residue may be sulfated or non-sulfated at C-4, C-6, or both²¹⁾, biochemical study has suggested that an axial 4-OH group and a 6CH₂OH group of saccharides are necessary for the agglutination reaction of SBA²²⁾. In this context, Hoedt-Schmidt *et al.*¹⁶⁾ noted that specific breakdown of the chondroitin sulfate chain is required for binding of SBA. Therefore, SBA appears to be the most feasible lectin to analyze glycosylation prior to sulfation during the course of elaboration of chondroitin sulfates in cartilaginous tissues.

It is believed that the conformational properties of the oligosaccharide structure of cartilage proteoglycans are modified, to an extent, depending on the degree of glycosylation and sulfation, during the

course of proteoglycan metabolism and pathology¹⁴⁾. Hagiwara *et al.*²³⁾ described that an increase of sulfation in chondroitin sulfate chains might induce a concentration of calcium in the matrix or might be concerned with structural modifications of aggregated proteoglycans. From the results of the present study, and from the lectin binding affinity mentioned above, the following plausible explanation can be offered for the influence of GH on the mechanism of synthesis of cartilage specific disaccharides : GH affects the process of glycosylation and subsequent sulfation occurring in the Golgi complexes, in which exceeding sulfation (representing a reduction of SBA binding) can take place. In support of this assumption, a number of biochemical findings can be cited²⁴⁻²⁶⁾.

An inhibitory effect of hypophysectomy on SO₄ incorporation, and subsequent recovery from this inhibitory effect by GH administration, has been reported for cartilaginous tissues²⁴⁾. Incidentally, the stimulation of chondrocyte proliferation and matrix production are mediated by GH-dependent 'sulfation factor(s)' known as somatomedin (IGF-I)²⁷⁾. This cytokine, which stimulates incorporation of SO₄ into cartilage proteoglycans in normal rats, cannot be detected in the serum of hypophysectomized animals, but it returns to a normal level following treatment with GH⁴⁾. Previous histochemical²⁸⁾ and biochemical²⁹⁾ studies on brachymorphic cartilages revealed a property of undersulfation, compared with those cartilages in normal animals. Further, Fargu-harson *et al.*³⁰⁾ indicated that the sulfation of proteoglycans is more important than their concentration in chondrocyte maturation. In view of these findings together with our present results, it is conceivable that the rate of elaboration of cartilage specific repeating disaccharides in the Golgi regions may bear a close relationship to the mechanisms of cell growth and the matrix production.

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