Light Microscopic Autoradiography and Ultrastructural Cytochemistry of Sulfated Proteoglycans in Degenerated Chondrocytes of Rat Epiphyseal Cartilage

by

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Abstract

Degenerating chondrocytes are often localized in the lower hypertrophic and calcifying zone of rat epiphyseal cartilage. The ability of these degenerating chondrocytes to synthesize and secrete sulfated proteoglycans was investigated using the light microscopic radiosulfate labeling method and the high-iron diamine-thiocarbohydrazide-silver proteinate (HID-TCH-SP) method for sulfated complex carbohydrates at the ultrastructural level. At 30 min following intraperitoneal (IP) injection of $^{35}$SO$_4$, only intracellular autoradiographic labeling was observed, whereas both intracellular and extracellular radiosulfate labeling was seen at 1 h post-injection. A gradual increase in intracellular and extracellular labeling was seen at 3, 12 and 24 h following IP injection of $^{35}$SO$_4$. In ultrastructural cytochemical preparations, HID-TCH-SP weakly to moderately stained the chondrocyte Golgi vacuoles or immature and intermediate secretory granules but did not stain Golgi saccules. Mature secretory granules and extracellular matrix granules demonstrated strong staining. These results therefore indicate that proteoglycans are sulfated in the degenerating chondrocyte Golgi vacuoles, packaged in the secretory granules and then released into the extracellular matrix to form matrix granules.

Introduction

Proteoglycans (PGs) are closely associated with calcification of cartilage[1-3]. PGs in cartilage exist as high-molecular-weight PG aggregates and their constituent subunits. PG monomer is composed of glycosaminoglycans (GAGs) covalently bound to core protein. GAGs found in cartilage are chondroitin sulfates A and C, keratan sulfate and hyaluronic acid. The latter acid is not covalently bound to core protein but plays a role in the linking of PG monomers[4]. Epiphyseal car-
tilage is often used in research on the calcification of cartilage because synthesis and secretion of PGs are very active in its constituent chondrocytes, especially in the proliferative and upper part of the hypertrophic zones. On the other hand, active sites of appositional mineralization are observed in the longitudinal septum of the calcified and lower hypertrophic zones. There are two types of chondrocytes\(^5\). The first type is a hypertrophic cell with a clear cytoplasm and nucleoplasm, and the second type is a chondrocyte which shows signs of degeneration (degenerated chondrocytes). Previous studies on hypertrophic chondrocytes using \(^{35}\)SO\(_4\) light microscopic autoradiography and the high-diamine-thiocarbohydrazide-silver proteinate (HID-TCH-SP) method which detects sulfate groups in the tissue have revealed that sulfation of PGs occurs in the Golgi vacuoles and that sulfated PGs are secreted in the form of secretory granules which are closely related to calcification of the cartilage (Takagi et al.\(^8\)). However, no extensive examinations of degenerated chondrocytes have been done.

The purpose of the present study was to examine the synthesis and secretion of sulfated PGs by degenerated chondrocytes and the ultrastructural localization of sulfated PGs in these cells by use of \(^{35}\)SO\(_4\) light microscopic autoradiography and the HID-TCH-SP method.

**Materials and Methods**

A total of 25 Wistar rats, each weighing between 15 and 20 g, were used in the experiment.

1) \(^{35}\)S Autoradiography

\(^{35}\)S-Sulfate (a product of the Japan Atomic Power Research Institute, with a radioactivity level of 49 mCi/ml, specific activity, carrier-free) was dissolved in physiological saline at 37°C. The solution was injected into the peritoneal cavity of each test animal at a dose of 10 \(\mu\)Ci/g wt. The rats were then decapitated at various intervals after the injection: 30 min, and 1, 3, 24 and 48 h. Immediately after decapitation, the proximal epiphyseal cartilage was taken out and soaked for 2 h in a fixative solution consisting of 4% formaldehyde and 2.5% glutaraldehyde diluted in 0.1 M cacodylate buffer solution (pH 7.3). Samples were then washed with 0.1 M cacodylate buffer (pH 7.3) containing 7% sucrose and fixed for 2 h in 1% osmium tetroxide (OsO\(_4\)) dissolved in 0.1 M cacodylate buffer (pH 7.3). Each sample was dehydrated through a graded ethanol series and embedded in Spurr’s resin. Each embedded specimen was then sectioned (1 \(\mu\)m in thickness) with a glass knife. The sections were mounted on a slide glass for autoradiographic processing by the dipping method, exposed for 3 weeks in a cool dark room using NR-M2 emulsion for autoradiography, and then developed with Konidor X and fixed with Konifix. Light microscopic autoradiograms were produced after staining of the sections with toluidine blue.

2) HID-TCH-SP Method

Epiphyseal cartilages removed from rats under Nembutal anesthesia were cut into pieces and fixed for 2 h in 2.7% glutaraldehyde (pH 7.3). Each sample was then washed with 0.1 M cacodylate buffer (pH 7.3) containing 7% sucrose and immersed in Spicer’s HID solution for 18 h at room temperature. Magnesium
chloride solution\cite{10-12} was used as a control instead of Spicer's HID solution.

Half of the samples were fixed with 1\% OsO$_4$ solution in 0.1 M cacodylate buffer (pH 7.3), then embedded in Spurr's resin after dehydration. The remaining samples were similarly embedded without OsO$_4$ fixation.

These ultrathin sections were stained with TCH-SF\cite{8-12,15} before examination by electron microscopy.

**Results**

1) Light Microscopic Autoradiography

Examinations were made to determine how $^{35}$S permeated in each of the proliferative, hypertrophic and calcified zones of epiphyseal cartilage (Fig. 1) with passage of time. Particular attention was paid to the metabolism of $^{35}$S found in degenerated chondrocytes in the areas from the lower part of the hypertrophic zone to the calcified zone.

**30 minutes after injection**

Accumulation of silver grains was noticeable in chondrocytes in the areas from the proliferative zone to the upper hypertrophic zone. Small amounts of silver grains were also detected in the cellular matrix (Figs. 2 and 3). However, silver grains did not accumulate uniformly in all chondrocytes in these sections; some cells had only a very small amount of silver grains. Silver grains were detected in the Golgi area of degenerated chondrocytes (Fig. 4). This finding indicated that at 30 min after injection, the distribution of silver grains in each zone was characterized by accumulation within chondrocytes. The distribution of silver grains in the intercellular matrix was very slight in the areas from the proliferative zone to the middle of the hypertrophic zone. No distribution was found in either the lower part of the hypertrophic zone or the calcified zone.

**60 minutes after injection**

The chondrocytes and matrix in the areas from the proliferative zone to the middle of the hypertrophic zone showed more silver grain accumulation than in those observed 30 min after injection. An increase in the amount of silver grains was similarly observed in degenerated chondrocytes in both the lower hypertrophic zone and calcified zone. A small amount of silver grains had begun to appear in the matrix of these zones (Fig. 5).

**3, 24 and 48 hours after injection**

There was no difference in the ultrastructural distribution of silver grains at 3, 24 and 48 h after injection.

A further accumulation of silver grains was observed with lapse of time in the chondrocytes and the matrix in areas from the proliferative zone to the middle of the hypertrophic zone. Increased amounts of silver grains were also found in the degenerated chondrocytes, the matrix of the lower hypertrophic zone and the calcified zone (Figs. 6 and 7).

2) HID-TCH-SP Method

Many reports\cite{16-21} are already available on the ultrastructure of the chondrocytes in each zone of the epiphyseal cartilage. Therefore, in this article, we describe only the HID-TCH-SP staining properties of degenerated chondrocytes in the
Fig. 1  Light micrograph of rat epiphyseal cartilage
P: Proliferative zone
H: Hypertrophic zone
C: Calcified zone
D: Degenerated cells
Arrow: Hypertrophic cell

Fig. 2  Thirty minutes after injection: accumulation of silver grains in the proliferative zone

Fig. 3  Thirty minutes after injection: accumulation of silver grains in the hypertrophic zone

Fig. 4  Thirty minutes after injection: accumulation of silver grains in degenerated chondrocytes in the calcified zone
lower hypertrophic and calcified zones. HID-TCH-SP staining indicating the existence of sulfated GAG was observed in Golgi vacuoles, i.e., immature secretory granules, but not in Golgi sacculae (Fig. 8). The HID-TCH-SP stained Golgi vacuoles weakly to moderately (Fig. 8V) and mature secretory granules strongly (Fig. 8SG), but the nucleus, rough endoplasmic reticulum and mitochondria were unstained.

On the other hand, strong reactions were observed in extracellular matrix granules\(^{12,21,22}\) (Fig. 8; arrow) which were considered to contain PG monomer. Reactions in the cell organelles and matrix granules were not affected after OsO\(_4\) postfixation.

In the control sections, no TCH-SP staining was observed in cell organelles and matrix granules.

**Discussion**

We examined the synthesis, secretion and ultrastructural localization of PGs in degenerated chondrocytes present in the lower hypertrophic zone and the calcified area of rat epiphyseal cartilage utilizing \(^{35}\)SO\(_4\) light microscopic autoradiography and the HID-TCH-SP method at the ultrastructural level. \(^{35}\)SO\(_4\) autoradiography for cartilage has been used by many researchers\(^{25–28}\) since DZIEWIATKOWSKI\(^{23,24}\) tested it to observe the metabolism of sulfated GAGs. However, the synthesis and secretion of PGs in degenerated chondrocytes have not previously been revealed by means of a combination of electron microscopic cytochemistry and light microscopic autoradiography. In the present study, \(^{35}\)SO\(_4\) autoradiography revealed that silver grains appeared in the degenerated chondrocytes 30 min after injection and were observed outside the cell 60 min after injection. The numbers of silver grains increased both inside and outside the cells as time passed, when observed at 3, 12 and 24 h after injection. This indicated that sulfated PGs are synthesized and secreted in degenerated chondrocytes.

Spicer’s HID method\(^{13,14}\) has been used to test for the presence of sulfate groups in various types of cells and extracellular matrices histochemically. It has been reported that during the HID and HID-TCH-SP staining processes, diamine oxide in the HID solution specifically reacts with sulfate radicals to form an HID reaction product\(^{13,14,20}\). TCH then combines with the HID reaction product and HID-TCH complex formed has a high electron density after reduction of SP\(^{9–12}\).

In the present study on degenerated chondrocytes, HID-TCH-SP staining was observed in Golgi vacuoles, secretory granules and matrix granules. In previous studies of chondrocytes and other cell types utilizing \(^{35}\)SO\(_4\) autoradiography\(^{11,30,31}\), the Golgi complex has been shown to be the site where sulfation of secretory ma-

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Fig. 5 State of developing silver grains appearing in the degenerative cartilaginous cells of the calcified lamina 60 minutes after injection: accumulation of silver grains in the degenerated chondrocytes and the matrix of the calcified zone

Fig. 6 Three hours after injection: accumulation of silver grains in the degenerated chondrocytes and matrix of the calcified zone

Fig. 7 Twenty-four hours after injection: accumulation of silver grains in the degenerated chondrocytes and matrix of the lower hypertrophic zone
Fig. 8  HID-TCH-SP staining reaction of degenerated chondrocytes and matrix in the calcified
zone of rat epiphyseal cartilage
HID-TCH-SP staining reaction is recognizable in the Golgi vacuoles (V) of the degenera-
tive cartilaginous cells, but not in the Golgi sacules (S).
SG: Mature secretory granule
N: Nucleus
ER: Endoplasmic reticulum
Mt: Mitochondria
EM: Extracellular matrix
Arrow: Matrix granules
Inset: Magnified view of Golgi apparatus
terials occurs. In addition, Takagi et al.\cite{11} have reported that Golgi vacuoles of hypertrophic chondrocytes develop into secretory granules, and that their contents, sulfated PGs, are secreted to form extracellular matrix granules. Therefore, the HID-TCH-SP staining seen in Golgi vacuoles and secretory granules would represent the sites of sulfation and transport of PGs, respectively, while matrix granules stained with HID-TCH-SP are presumed to be extracellularly secreted PGs.

Moreover, the results obtained in the present study on degenerated chondrocytes are very similar to those of the previous examination of hypertrophic chondrocytes by Takagi et al.\cite{8}, so that it can be concluded that degenerated chondrocytes synthesize and secrete PGs in the same way as hypertrophic chondrocytes.

**Conclusion**

$^{35}$SO$_4$ light microscopic autoradiography and the HID-TCH-SP method capable of revealing the ultrastructural localization of sulfated GAGs were used to examine the fine structure of degenerated chondrocytes present in the hypertrophic zone of the epiphyseal cartilage and the calcified area of rat tibia. In addition, the synthesis and secretion of sulfated PGs as well as their ultrastructural localization were examined.

Upon $^{35}$SO$_4$ autoradiography, silver grains appeared only in degenerated chondrocytes 30 min after injection and were observed 60 min after injection both inside and outside the chondrocytes. The numbers of silver grains inside and outside the chondrocytes increased with the passage of time, as seen at 3, 12, 24 and 48 h after injection.

Reactions to the HID-TCH-SP method were observed in Golgi vacuoles, i.e., immature secretory granules, of degenerated chondrocytes, but not in Golgi sacculae. Golgi vacuoles were stained weakly to moderately with HID-TCH-SP, while mature secretory granules were strongly stained. On the other hand, strong HID-TCH-SP staining was observed in extracellular matrix granules which were considered to contain PG monomer.

Therefore it is suggested that degenerated chondrocytes produce sulfated PGs in the Golgi vacuoles, which is then secreted via secretory granules at a relatively slower pace than in chondrocytes in other zones.

**References**


