Temporal Dynamic Changes in Synthesis of Chondroitin Sulfate Isomers in Canine Articular Chondrocyte Culture

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ABSTRACT. To investigate temporal dynamic changes in the synthesis of chondroitin 6-sulfate (CS6) and chondroitin 4-sulfate (CS4) in vitro, normal articular cartilage of femoral heads was harvested from three dogs. Chondrocytes were isolated and cultured in alginate microspheres for 21 days. On days 7, 14 and 21, DNA content was quantified by fluorometric assay using Hoechst 33258. On days 14 and 21, proteoglycans were extracted, and the amounts of CS6 and CS4 were quantified after chondroitinase ABC digestion using capillary electrophoresis. The DNA content and amounts of CS6 and CS4 increased during the culture period. The amounts of CS6 and CS4 divided by DNA content revealed that the synthesis of CS6 was more up-regulated than CS4.

KEY WORDS: capillary electrophoresis, chondrocyte, chondroitin sulfate.

Chondrocytes play an important role in maintaining the extracellular matrix in articular cartilage (AC) [19]. Chondrocytes produce proteoglycans (PGs) and collagen, the major components of the extracellular matrix. One constituent of PGs is chondroitin sulfate (CS), which is the most abundant glycosaminoglycan (GAG) and consists of glucuronic acid and N-acetylgalactosamine. Chondroitin sulfate is further classified based on the sulfated portion of N-acetylgalactosamine, for example, chondroitin 6-sulfate (CS6) and chondroitin 4-sulfate (CS4).

Ratio of CS6 to CS4 alters with aging [1, 3, 14, 18]. In human newborns, CS6 and CS4 were present in equimolar amounts, while the ratio of CS6 to CS4 increased to between 19:1 (95%) and 26:1 (96%) in adults [18]. In the human distal femoral AC, the ratio of CS6 to CS4 was 1:1 (50%) in infants (0 to 2 years old) and 49:1 (98%) in adults [14]. In equine AC, similar results were seen in the relative concentration of CS6 and CS4 [1]. In canine AC, a similar pattern of change in the ratio of CS6 to CS4 was reported using agarose gel electrophoresis [3]. The average estimated amounts of CS6 and CS4 in newborns were 2.55 ± 0.11 (57%) and 1.92 ± 0.09 (43%) µg of hexuronic acid/mg dry weight, while those in adults were 2.59 ± 0.25 (75%) and 0.86 ± 0.08 (25%) µg of hexuronic acid/mg, respectively [3]. Although the methods of extraction of PGs and digestion of GAGs varied among these studies, there was a pattern of change in the ratio of CS6 to CS4 in AC tissues.

In vitro studies are useful to document the metabolic activities of chondrocytes. Using a three-dimensional chondrocyte culture system, the ratio of CS6 to CS4 in newly synthesized extracellular matrix was investigated in canine intervertebral disk [9, 10] and human AC [7]. In these studies the ratio of CS6 to CS4 was reported as ranging from 50 to 70%. Since chondrocytes undergo active cell division maintaining a differentiated phenotype in the three-dimensional culture, CS synthesis, especially sulfation pattern of CS may be dynamically altered during the culture period. It was reported that the ratio of CS6 to CS4 was gradually increased in culture of chondrocytes isolated from human AC [8]. In this study, however, the ratio of CS6 to CS4 was evaluated based on relative amounts of each CS isomers but not on their absolute amounts. Thus, it is difficult to determine from the study if the increased the ratio of CS6 to CS4 results from increased synthesis of CS6 or decreased synthesis of CS4.

Recently, we have developed the use of capillary electrophoresis for the quantification of CS6 and CS4 in three-dimensional chondrocyte culture [9]. The purpose of this paper was to report temporal dynamic change in the pattern of synthesis of CS6 and CS4 in three-dimensional chondrocyte culture of canine AC based on absolute amounts of CS isomers determined by capillary electrophoresis.

Three-dimensional chondrocyte culture, using alginate microspheres: Three female hound dogs of 12 (dog 1), 18 (dog 2) and 48 (dog 3) months of age were used in this study which was approved by the Institutional Animal Care and Use Committee at University of Florida. Articular cartilage tissues were aseptically collected from femoral heads. Upon gross examination, there was no sign of degenerative joint disease. Chondrocytes were isolated from the femoral heads and cultured as previously described [12]. Briefly, the isolated cells were mixed with Dulbecco’s phosphate-buffered saline (DPBS, Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.) containing alginate (Kelton LV®, Kelco Company, Chicago, IL, U.S.A.) with a viscosity of 1.2% at
a density of approximately 10^5 cells/ml. The alginate cell suspension was dropped into a 102 mM CaCl_2 solution (Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.), 1.5 to 2 cm from the surface using a 22-gauge needle. Slightly more than 400 microspheres were made from each dog. The microspheres were hardened in 102 mM CaCl_2 for 10 min. After 3 washes Ham’s F-12 medium (Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.), the microspheres were incubated in Ham’s F-12 medium containing 10% FBS (Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.) at 37° C for 3 hr [9]. The digestion was terminated by heating the tube in a boiling water bath for 1 min [5].

**Quantitative analysis of CS isomers, using capillary electrophoresis:** Capillary electrophoresis was carried out, using a fused silica capillary column (50 μm i.d., 37 μm o.d.) as previously described [9]. Peak areas of both CS4 and CS6 were divided by the peak area of a standard marker (CA). This normalized value was defined as a corrected value. The corrected values of CS4 and CS6 were compared to a standard curve that had been established using serially diluted and commercially available, highly purified unsaturated CS disaccharides, ΔDi-6S and ΔDi-4S (Seikagaku America, Inc., Ijamsville, MD, U.S.A.). The corrected values were then used to estimate synthesized amounts of CS6 and CS4.

**Statistical analysis:** The estimated amounts of CS6 and CS4 on days 14 and 21 were divided by the corresponding DNA content. The divided values for CS6 and CS4 were compared between day 14 and 21 using a paired t-test. Statistical analysis was performed using a computer program (JMP version 3.0, SAS institute Inc., Cary, NC, U.S.A.).

Active cell proliferation was confirmed based on microscopic observations and an increase in DNA content. The cell number was also estimated based on DNA content (1 x 10^6 ± 7 μg of DNA) [10]. The amounts of DNA in dogs 1, 2 and 3 were 767 (1.1 x 10^5 cells) and 672 (9.6 x 10^5 cells) ng on day 7, 2,438 (3.4 x 10^6 cells), 2,137 (3.0 x 10^6 cells) and 3,027 (4.3 x 10^6 cells) ng on day 14 and 2,863 (4.1 x 10^6 cells), 2,795 (4.0 x 10^6 cells) and 2,713 (3.9 x 10^6 cells) ng on day 21, respectively. The average of DNA content (mean ± standard deviation) were 694 ± 48.4 (9.9 x 10^5 ± 6.9 x 10^5 cells) ng on day 7, 2,534 ± 328.7 (3.6 x 10^6 ± 4.7 x 10^6 cells) ng on day 14 and 2,790 ± 51.6 (4.0 x 10^6 ± 7.4 x 10^6 cells) ng on day 21. The DNA content of dog 3 declined from days 14 to 21.

Relatively large and clear peaks of CS6 and CS4 were detected on electrophorograms on days 14 and 21. Peak areas of CS6 and CS4 increased proportionally with the duration of culture. The estimated amounts and proportions of CS6 and CS4 are summarized in Table 1. The average rates of increase (mean ± standard deviation) in CS6 and CS4 from days 14 to 21 were 2.5 ± 0.9 times and 2.2 ± 0.9 times, respectively. The average and standard deviation of CS6 and CS4 per DNA contents (CS isomer/total CS) were 13.1 (59.8%) ± 3.8 (1.4%) and 8.9 (40.2%) ± 3.0 (1.4%) ng/ng on day 14 and 28.4 (63.8%) ± 7.7 (1.6%) and 16.1 (36.2%) ± 3.7 (1.6%) ng/ng on day 21. The increase between day 14 and 21 was significant for both CS6 (P=0.016) and CS4 (P=0.016). Per DNA content, more CS6 (59.8% to 63.8%) than CS4 (40.2% to 36.2%) was synthesized between days 14 and 21.

Previously, relative proportions of CS6 and CS4 were investigated in canine intervertebral disk chondrocyte cultures [10]. However, from relative proportions, it is impossible to know if more CS6 has been produced than CS4. In the present study, quantification of CS6 and CS4 was based
on a normalized value that was measured by capillary electrophoresis and established by a series of dilutions of commercially available CS disaccharides [9]. Thus, the changes in the ratio of CS6 to CS4 could be precisely analyzed. The total amounts of both CS6 and CS4 at days 14 and 21 increased more than two-fold, confirming the vigorous metabolic activity of proliferating chondrocytes. Furthermore, the ratio of CS6 to CS4 in CS isomers that had been divided by DNA content increased from 59.8% to 63.8% as seen with aging in vivo [1, 3, 18] and in human AC tissue culture [13]. Presents results indicated that this increased ratio was due to the synthesis of more CS6 than CS4 from days 14 to 21.

We explain dynamic changes of CS synthesis by the different metabolic functions of chondrocytes during differentiation. The profile of receptors and associated cell signals might differ at various stages of differentiation. In human newborn femoral heads, the ratio of CS6 to CS4 gradually changed with depth from the articular surface to the hypertrophy zone [14]. At the surface where resting chondrocytes were abundant, the ratio was nearly 10:0 (almost entirely CS6). Near the bone-cartilage interface where actively proliferating cells were seen, the ratio became 1:1. Chondrocytes in deeper areas act as growth cartilage for epiphysis. Thus, actively proliferating chondrocytes produce more CS4 than CS6. In three-dimensional cultures, chondrocytes may undergo de-differentiation, mimicking the growth during newborn epiphysis. Due to their accelerated growth or proliferation in the culture system, chondrocytes may reach maturity between days 14 and 21. This is still speculation, since we did not obtain data on day 7, when the chondrocyte proliferation was at its peak.

Although cartilage tissues of adults are metabolically inactive, chondrocytes may proliferate in an attempt to repair damage [4]. In osteoarthritis, a lower proportion of CS6 was accompanied by the formation of clusters of chondrocytes [5]. The proliferation of chondrocytes is most likely initiated by the increasing activity of cytokines such as TNF-α and IL-1β [11] that might have caused increased production of CS4 compared to CS6. In osteoarthritis, however, degradation of PGs occurs due to increased activity of metalloproteinase [16]. Thus, the net effect is a reduction in GAG. In a recent report, chondrocytes exposed to IL-1β from the beginning of three-dimensional culture in agarose gel synthesized significantly less GAG than a control. In addition, the amount of matrix metalloproteinase-3 in the culture medium increased significantly [2].

In the present study, a small sample volume was one advantage but also a limitation. We have used 1×10⁵ chondrocytes in 100 microparticles. On days 14 and 21, based on the DNA content, the estimated number of chondrocytes was 3.6×10⁵ and 4.0×10⁵, respectively (1×10⁶ = 7 µg of DNA) [10]. It would have been ideal to have at least twice the concentration of CS6 and CS4 early in the culture such as on day 7. In future studies, it is recommended to use a high initial chondrocyte concentration such as 2 to 5×10⁶ in 100 microparticles to confirm the temporal change in the ratio of CS6 to CS4.

Long-term cell culture, more than 21 days will be necessary to investigate the stable expression of CS synthesis by chondrocytes. In a previous report, the chondrocytes needed a period of gradual stabilization for the phenotypic expression of PG synthesis in the three-dimensional culture [8]. De-differentiated chondrocytes re-gained their ability to synthesize cartilage-specific PGs after 25 days of culture [8]. However, the synthesized CS isomers were not fully quantified. In the future, the up-regulation of CS6 synthesis by de-differentiating chondrocytes should be investigated by extending the culture period.

In conclusion, we have documented a temporal change in the amounts of CS isomers synthesized by proliferating chondrocytes in vitro. Between days 14 and 21, the chondrocytes synthesize more CS6 than CS4 resulting in an increase in the ratio of CS6 to CS4. This culture system may be useful to investigate effects of various pharmaceuticals, nutraceuticals and hormones on synthesis of CS isomers.

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REFERENCES

Table 1. Absolute amounts (proportions) of chondroitin sulfate isomers synthesized in microparticles (µg / 100 microparticles)

<table>
<thead>
<tr>
<th>Dog number</th>
<th>CS6</th>
<th>CS4</th>
<th>CS6</th>
<th>CS4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>41.2 (58.3%)</td>
<td>29.5 (41.7%)</td>
<td>106.8 (64.9%)</td>
<td>57.7 (35.1%)</td>
</tr>
<tr>
<td>Dog 2</td>
<td>20.1 (60.2%)</td>
<td>13.3 (39.8%)</td>
<td>68.7 (62.0%)</td>
<td>42.1 (38.0%)</td>
</tr>
<tr>
<td>Dog 3</td>
<td>39.0 (60.9%)</td>
<td>25.0 (39.1%)</td>
<td>63.6 (64.4%)</td>
<td>35.1 (35.6%)</td>
</tr>
<tr>
<td>AVG</td>
<td>33.4 (59.8%)</td>
<td>22.6 (40.2%)</td>
<td>79.7 (63.8%)</td>
<td>44.9 (36.2%)</td>
</tr>
<tr>
<td>STD</td>
<td>11.6 (1.4%)</td>
<td>8.4 (1.4%)</td>
<td>23.6 (1.6%)</td>
<td>11.6 (1.6%)</td>
</tr>
</tbody>
</table>

CS6=chondroitin 6-sulfate, CS4=chondroitin 4-sulfate, AVG=average, STD=standard deviation.


