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RESEARCH ARTICLE

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Histological analysis of nucleus pulposus tissue from patients with lumbar disc herniation after condoliase administration

Yuka Minamisawa[1] | Taiichi Shirogane[2] | Ippei Watanabe[2] | Akira Dezawa[3]

[1]Central Research Laboratories, Seikagaku Corporation, Tokyo, Japan

[2]Medical Affairs, Seikagaku Corporation, Tokyo, Japan

[3]Dezawa Akira PED Clinic, Kanagawa, Japan

Correspondence
Taiichi Shirogane, Medical Affairs, Seikagaku Corporation, 1-6-1 Marunouchi, Chiyoda-ku, Tokyo 100-0005, Japan.
Email: taiichi.shirogane@seikagaku.co.jp

Abstract

Background: Condoliase is an enzyme used as a treatment for lumbar disc herniation (LDH). This enzyme degrades chondroitin sulfate (CS) in the nucleus pulposus of the intervertebral disc (IVD). However, there are cases in which symptoms do not improve, despite condoliase administration. This study reports histological analysis of lumbar disc tissue of LDH patients who underwent surgery because condoliase had no therapeutic effect.

Methods: Between March 2019 and August 2019, 12 LDH patients who underwent full endoscopic spine surgery (FESS) discectomy at the Dezawa Akira PED Clinic were the subjects of the study. There are two study groups: six cases underwent FESS after condoliase administration, while six underwent FESS without condoliase administration. The average duration from drug administration to surgery was 152 days. Herniated disc removed at surgery was evaluated by histological staining including immunohistochemistry by anti-CS antibodies.

Results: Multiple large clusters (40–120 μm in diameter) were observed in the nucleus pulposus of those who received condoliase, but no clusters were observed in those who did not. The lumbar disc tissues, including the nucleus pulposus of recipients, were stained with anti-CS antibodies that recognize the CS unsaturated disaccharide, but non-administration tissue was not stained. These findings suggest that the enzyme acted on the nucleus pulposus, even in cases where symptoms were not improved by condoliase administration. Furthermore, there was no histological difference between stained images of the extracellular matrix in those who did or did not receive condoliase, suggesting that condoliase acted specifically on CS in the nucleus pulposus.

Conclusions: We demonstrated that CS in the nucleus pulposus was degraded in patients in whom condoliase did not have a therapeutic effect. Moreover, condoliase acts in human IVD without causing necrosis of chondrocytes and surrounding tissues.

KEYWORDS

chemonucleolysis, chondroitin sulfate, condoliase, immunohistochemistry, lumbar disc herniation

1 | INTRODUCTION

Lumbar disc herniation (LDH) causes back and leg pain by extrusion of the nucleus pulposus through the posterior annulus fibrosus into the spinal canal leading to compression of the spinal nerve roots. In the 1960s, Smith reported that chymopapain, a proteolytic enzyme, was administered to patients with LDH as a therapeutic method termed "chemonucleolysis," to reduce intervertebral disc (IVD) pressure.[1] The therapeutic effect is based on a mechanism in which chymopapain lyses the nucleus pulposus to reduce the internal pressure of IVD, alleviating pressure on the nerve roots. Chemonucleolysis using chymopapain was used mainly in Europe and the United States because it is minimally invasive and shows therapeutic effects equivalent to surgery if the patient is appropriately selected.[2–4] However, chymopapain caused various adverse events including anaphylaxis, paraplegia, and back spasm.[5,6] Chymopapain is no longer used.

Condoliase is a chondroitin sulfate (CS)-degrading enzyme isolated and purified from *Proteus vulgaris*, a Gram-negative rod-shaped bacterium, and has been used as a research reagent for over 50 years.[7] CS is covalently linked to a core protein to form proteoglycans (PGs) in living organisms. PGs are present in connective tissues as the extracellular matrix, providing hydration and oncotic pressure to the tissue, allowing it to withstand compressional forces.[8–10] CS is abundant in the nucleus pulposus in the form of PGs and contributes to water retention in the nucleus pulposus. Therefore, the development of a condoliase with the property of specifically degrading CS was promoted as an LDH therapeutic agent.[11,12] The following mechanism of action of condoliase was anticipated. When administered into the IVD, condoliase effectively reduces intradiscal pressure and the volume of the herniated mass, ameliorating nerve root compression, because it degrades CS in the nucleus pulposus and attenuates water retention by the nucleus pulposus. Unlike chymopapain, condoliase completely lacks proteolytic activity and acts specifically on the CS of the nucleus pulposus. It was anticipated that condoliase might be a therapeutic drug with less effect on surrounding nerves and vessels.[11,12] As a preclinical step, the efficacy of condoliase on LDH was demonstrated in various animal studies.[13–15]

Condoliase was approved as a novel chemonucleolytic agent for LDH by the Japanese authority in 2018, and its efficacy and safety have been reported.[16,17] Currently (in 2023), a phase III trial is taking place in the United States to obtain FDA approval.[18] Patients with subligamentous disc herniation whose symptoms do not improve after at least 6 weeks of conservative treatment are optimal candidates for intradiscal administration of condoliase. However, in LDH patients, condoliase is administered only once in their lifetime. LDH patients whose symptoms have not improved despite administration of condoliase cannot have a repeat administration, and are treated by surgery, for example, full endoscopic spine surgery (FESS). Banno et al. reported that 12.5% of the patients who administered condoliase for LDH subsequently required surgical treatment within 1 year.[19] However, the reason why condoliase showed no therapeutic effect on these LDH patients has not been elucidated.

As shown in Figure 1, condoliase cleaves the glycosidic bond between D-glucuronic acid (GlcA) and *N*-acetyl galactosamine (GalNAc) in the CS molecule, generating a CS unsaturated disaccharide (ΔDi: ΔGlcA-GalNAc) with a double bond between C4 and C5 of the GlcA residue at the non-reducing end.[7,20] CS is classified into several isomers based on the binding position and the number of sulfate groups attached to GlcA or GalNAc, for example, non-sulfated chondroitin (CH), chondroitin 4-sulfate (CSA), and chondroitin 6-sulfate (CSC). CSA is mainly composed of A-units [GlcAβ1-3GalNAc(4S)], and CSC is mainly composed of C-units [GlcAβ1-3GalNAc(6S)]. CS contained in the nucleus pulposus is mainly composed of A and C units, with the latter accounting for more than 80% of the composition.[21] As condoliase shows high reactivity to CSA and CSC, among the CS isomers,[22–24] it is reasonable to impute that condoliase is an effective drug against LDH. However, as described above, in some cases symptoms caused by LDH do not improve despite the administration of condoliase. In this study, we evaluated histological staining of the nucleus pulposus of LDH patients who underwent FESS at the Dezawa Akira PED Clinic because no therapeutic effect was observed after administration of condoliase, compared with that of nucleus pulposus specimens where condoliase was not administered. Ishibashi et al. and Yoshida et al. reported histological analysis of human nucleus pulposus after administration of condoliase by hematoxylin–eosin (H&E) staining or toluidine blue staining[25,26]; however, there are no prior reports of histological analysis by anti-CS antibodies. To evaluate human lumbar disc tissue after condoliase administration, we used histological staining that included immunohistochemistry with anti-CS antibodies.

2 | MATERIALS AND METHODS

2.1 | Tissue samples

Herniated discs from 12 patients who underwent FESS at the Dezawa Akira PED Clinic between March 2019 and August 2019 were subjected to histological analysis. The treatment process that culminated in the removal of the herniated disc is shown in a flow chart (Figure 2). All patients had subligamentous disc herniation. Furthermore, all patients had symptoms that did not improve after 6 weeks of conservative treatment, and were Pfirrmann grade III or IV, without previous surgical intervention. Patient background is summarized in Table 1. The average age of patients was 44.3 ± 17.0, with five men and seven women. Before treatment with condoliase or FESS, the Pfirrmann classification was grade III in 11 cases and grade IV in one case. The site of surgery was L3/L4 in two cases, L4/L5 in five cases, and L5/S1 in five cases. Six patients (H1–H6) who had previously undergone chemonucleolysis with condoliase (Seikagaku Corp., Tokyo, Japan) underwent FESS because condoliase did not improve their symptoms (CA group). Condoliase (1.25 U), dissolved in 1 mL of saline, was injected if patients met the following three criteria: (i) agreed to condoliase administration, (ii) aged over 20 years, and (iii) there was good correlation between imaging findings and

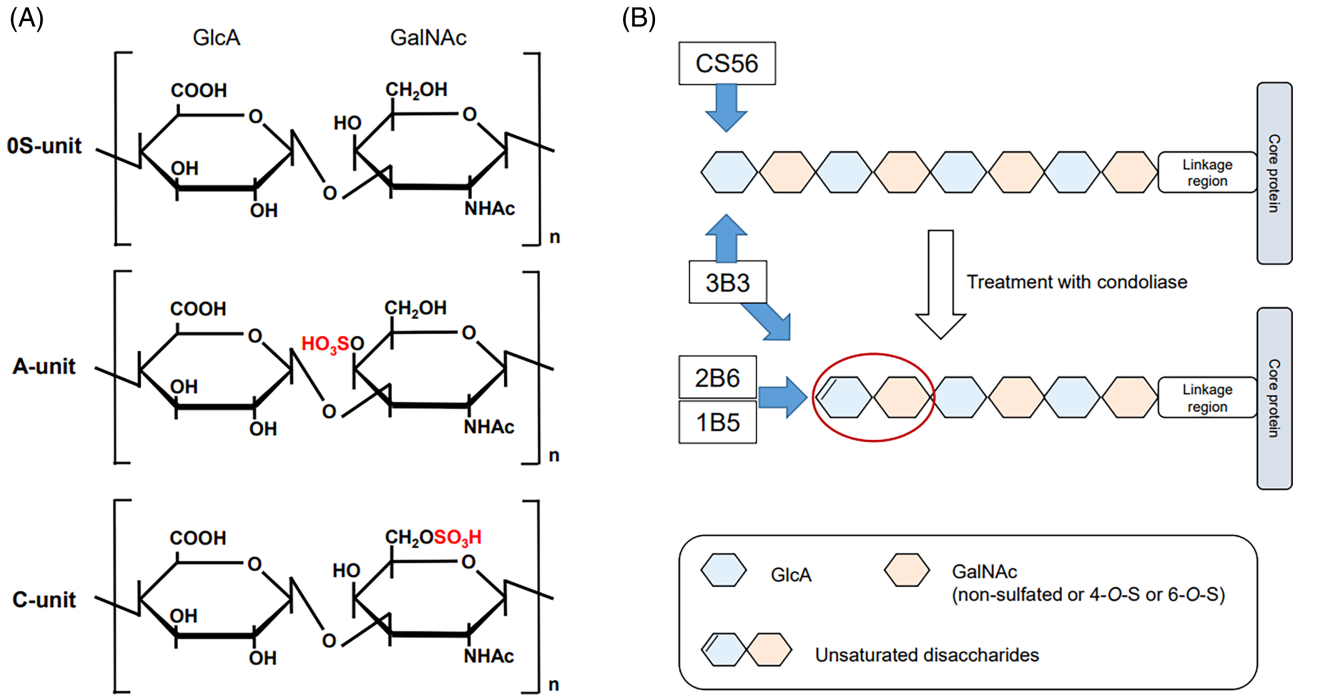
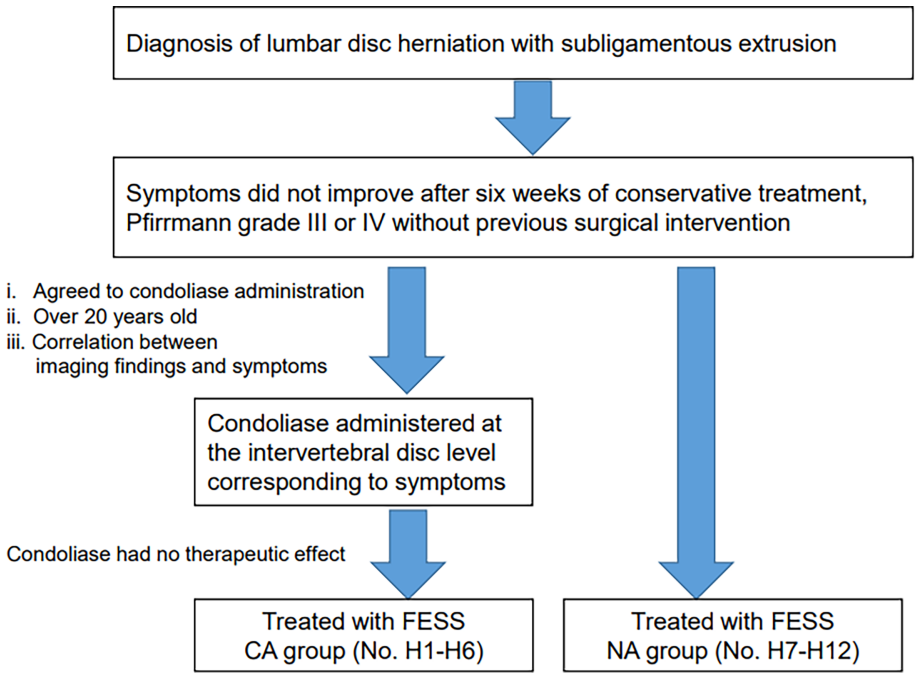


FIGURE 1 Three CS isoforms and CS structure recognizable by anti-CS antibodies. (A) Structural formulas of CS isomers. Non-sulfated chondroitin (CH) contains 0S-units [GlcAβ1-3GalNAc]; chondroitin 4-sulfate (CSA) contains mainly A-units [GlcAβ1-3GalNAc (4S)] with hydroxyl groups at the 4-positions of GalNAc; chondroitin 6-sulfate (CSC) contains C-units [GlcAβ1-3GalNAc (6S)] with hydroxyl groups at the 6-positions of GalNAc. (B) Antigen-recognition sites by each anti-CS antibody: CS56; undigested CS containing A- or C- units, 3B3; CS with ΔDi-6S at non-reducing end and undigested CS containing C-units, 2B6; CS with ΔDi-4S at the non-reducing end, 1B5; CS with ΔDi-0S at the non-reducing end. CS, chondroitin sulfate.

FIGURE 2 Treatment flow chart, culminating in removal of herniated disc.



symptoms. CA group underwent FESS following an observation period of at least 2 months, after administration of condoliase. In the other group, six patients (nos. H7–H12) were treated with FESS without condoliase administration (NA group). The average age of CA group was 52.8 ± 6.8 years (1 man and 5 women), and that of NA group was 35.8 ± 18.4 years (2 men and 4 women). The site of

TABLE 1 Patient background.

Condoliase	No.	Age	Sex	Site of surgery[a]	Pfirrmann grade	Duration[b]	Duration[c]
Administered (CA group)	H-1	63	F	L4/L5	IV	15 months	154 days
	H-2	43	F	L4/L5	III	4 years	56 days
	H-3	56	F	L3/L4	III	-	179 days
	H-4	49	M	L4/L5	III	2 years	135 days
	H-5	58	F	L4/L5	III	4 years	205 days
	H-6	48	F	L5/S1	III	3 years	184 days
Non-administered (NA group)	H-7	24	M	L5/S1	III	1 month	-
	H-8	72	F	L5/S1	III	1 year	-
	H-9	41	M	L5/S1	III	1 year	-
	H-10	39	M	L3/L4	III	5 months	-
	H-11	22	M	L5/S1	III	1 year	-
	H-12	17	F	L4/L5	III	5 months	-

Abbreviation: LDH, lumbar disc herniation.
[a]The site of condoliase administration is the same as the site of surgery.
[b]Duration of LDH.
[c]Duration from drug administration to surgery.

surgery in the CA group was L3/L4 and L5/S1 in one case each, and L4/L5 in four cases, while that in the NA group was L3/L4 or L4/L5 in one case each, and L5/S1 in four cases. Pfirrmann's grade was III in all cases, except for one case in the CA group. The present study was conducted in accordance with the principles embodied in the Declaration of Helsinki, 2013, and all experiments were approved by the ethics committee of Seikagaku Corp. (permit no. Kenrin-74-22) and Dezawa Akira PED Clinic (permit no. 2020H).

2.2 | Histological studies

The herniated disc removed during surgical treatment was fixed with 10% neutral buffered formalin, and paraffin tissue sections were prepared (hereinafter referred to as "lumbar disc tissue" or just "tissue"). Tissues were mainly analyzed with two staining methods: (i) tissue morphology was evaluated by the following staining methods: H&E stain, Masson's trichrome (MT) stain, safranin O/fast green (SO/FG) stain, and toluidine blue (TB) stain. Each staining was carried out according to a conventional method. (ii) In addition, to evaluate the expression levels of CS in the lumbar disc tissue, the tissue was immunostained with four monoclonal antibodies that recognize CS. To compare the staining intensity with these anti-CS antibodies, the tissues were treated with 100 U/mL chondroitinase ABC reagent (cABC, Seikagaku Corp., Tokyo, Japan) for 2 h at 37°C. Before and after treatment of each tissue with cABC, hereinafter referred to as pre-cABC and post-cABC, respectively. Immunostaining for tissues with anti-CS antibodies was carried out according to a conventional method.[27] Anti-CS antibodies, CS56 (mouse IgM), 1B5 (mouse IgG1), 3B3 (mouse IgM), and 2B6 (mouse IgG1), were purchased from Abcam plc. (Cambridge, UK) or Cosmo Bio Co., Ltd. (Tokyo, Japan). Antigen-recognition sites by each anti-CS antibody are shown in Figure 1B. For immunostaining, after blocking with 0.1% casein in PBS (phosphate-buffered saline) for 1 h at room temperature, the tissues were incubated with each anti-CS antibody diluted 100- to 200-fold with 0.1% casein in PBS overnight at 4°C, without antigen retrieval. As a negative control, mouse IgM or IgG isotypes (Abcam plc. or GeneTex International Corp., CA, USA) were used. Immunostaining with these antibodies was evaluated in two cases (H1 and H4), although not in all cases. After washing with PBS, a peroxidase-conjugated anti-mouse secondary antibody (Nichirei Biosciences Inc., Tokyo, Japan) was added, and the tissue was then incubated for 30 min at room temperature, and washed with PBS. CS-antigen on the tissue sections was visualized by adding 3,3′-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. Tissues were observed using a biological microscope (Olympus Corp., Tokyo, Japan) with an objective lens range of 4× to 20×.

2.3 | Evaluation of immunohistochemistry

Immunohistochemical staining intensity was quantified using ImageJ Fiji software.[28] The procedure is briefly described in Figure S1. Images are opened in the ImageJ. "H DAB" is selected in the color deconvolution popup window, and the original image is then automatically split into three single-color images. To evaluate the intensity of the brown-stained areas of the image, "invert" is applied to the red-colored image (R, 0.268; G, 0.570; and B, 0.776). The positively stained areas with brown staining are selected with an area of 4 mm^2 or less using the "create selection" option. Once this selection had been applied to the image, staining intensities per unit area were obtained for each tissue. Images of each staining intensity are shown in Figure 3. Staining intensities were measured using a masked methodology so that patient numbers were not identified. After measurement of

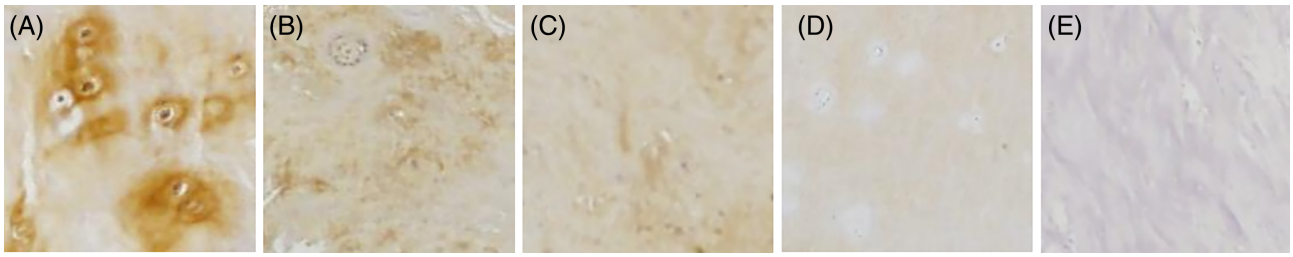


FIGURE 3 Exemplary images depicting the range of staining intensity. The values of staining intensity are as follows: (A) 60 or more, (B) 50 or more and less than 60, (C) 40 or more and less than 50, (D) 30 or more and less than 40, (E) 20 or less.

immunohistochemical staining intensity with each anti-CS antibody, Student's *t* test was used to compare the CA group and NA group, or the same group pre-cABC and post-cABC. *p* Values of less than 0.05 were considered to be statistically significant. In addition, the number of clusters per 1 mm^2 and cluster size were evaluated using the ImageJ software.

3 | RESULTS

H&E staining showed that the lumbar disc tissue removed by FESS contained not only the nucleus pulposus but also the annulus fibrosus and cartilaginous endplates (Figure S2). The nucleus pulposus tissue had a homogeneous configuration, and was composed of an extracellular matrix containing collagen fibers and chondrocytes (Figure 4). In the nucleus pulposus tissues of the CA group (H1-6), chondrocytes and their surroundings were stained by SO/FG and TB, and collagen fibers in the extracellular matrix were stained by MT (Figure 4A). The tissues of NA group (H7–H12) also exhibited similar staining characteristics to those of the CA group (Figure 4B). In other words, there was no clear difference in the staining image of the tissue morphology between the CA and NA groups (Figures 4 and S3). The difference between the two cases is that multiple large clusters were observed in the nucleus pulposus tissue in four (H1, H3, H4, and H5) of six cases treated with condoliase (Figure 4A). The average large cluster size was 7×10^{-3} mm^2 (40–120 μm diameter), and there were approximately four clusters per 1 mm^2. Conversely, no large cluster was observed in the nucleus pulposus tissues of the NA group. Such clusters were not observed in the annulus fibrosus and cartilaginous endplates even in the CA group (Figure S3), suggesting that these findings are specific to the nucleus pulposus treated with condoliase.

Immunostaining images of all cases are shown in Figure 5 and S4. The staining intensity of tissues with each anti-CS antibody is shown in Figure 6. The pre-cABC tissue of the CA group exposed to 3B3 and 2B6 showed stronger staining than that of the NA group ($p < 0.01$ or $p < 0.001$, Student's *t* test, Figure 6). In the NA group, the value of the pre-cABC staining intensity for 3B3 or 2B6 was less than 20, comparable to that with control IgM and IgG (Figure S5). These findings suggest that condoliase degraded CS in the nucleus pulposus of those patients in whom condoliase had an insufficient therapeutic effect. The post-cABC tissue of both groups showed the following: staining with CS56 is lost, and staining intensity of 3B3, 2B6, and 1B5 are unchanged or enhanced compared to that of pre-cABC tissue. With tissue from the CA group, the staining intensity post-cABC for 2B6 was higher than that of pre-cABC tissue ($p < 0.05$, Student's *t* test). In particular, staining around clusters was enhanced by 2B6 after cABC treatment in the four cases where clusters were observed (Figures 6 and 7).

4 | DISCUSSION

A scenario can be constructed in which the pressure in the IVD suddenly decreases due to chemonucleolysis, and a rapid narrowing of the disc-space causing loading of the facet joints. As a result, the lumbar hernia may be temporarily extruded, causing temporary worsening of symptoms such as lower limb pain and lower back pain. In most cases, symptoms improve within a few hours to a few days after administration, but surgery may be required in some cases.[12] Six patients who underwent surgery after the administration of condoliase, the subjects of this study, showed no improvement in symptoms from around 50 to 200 days following the administration of condoliase. In other words, the symptoms of these six cases did not represent a temporary exacerbation after the administration of condoliase, but it was possible that the symptoms persisted due to a lack of therapeutic effect. Severe degeneration of IVD in the elderly may contain the annulus fibrosus and cartilaginous endplates in the lumbar disc tissue, suggesting the possibility of a poor outcome with condoliase treatment.[29] The herniated disc tissues of the subjects treated with condoliase in this study included not only the nucleus pulposus but also the annulus fibrosus and cartilaginous endplates. However, some subjects in this study had less advanced disc degeneration. Therefore, we could not find if a relationship existed between the severity of disc degeneration and the lack of improvement in symptoms after condoliase treatment.

The CS composition ratio in the nucleus pulposus of human changes with age: the amount of A-unit decreases, and the C-unit to A-unit ratio increases with increasing age, so that eventually the C-unit accounts for more than 80% of the total CS composition.[11,21] The CS content in the nucleus pulposus decreases linearly with age. Furthermore, the nucleus pulposus of a prolapsed disc contained less CS in comparison to normal tissue.[21] Since the amount of A-unit is

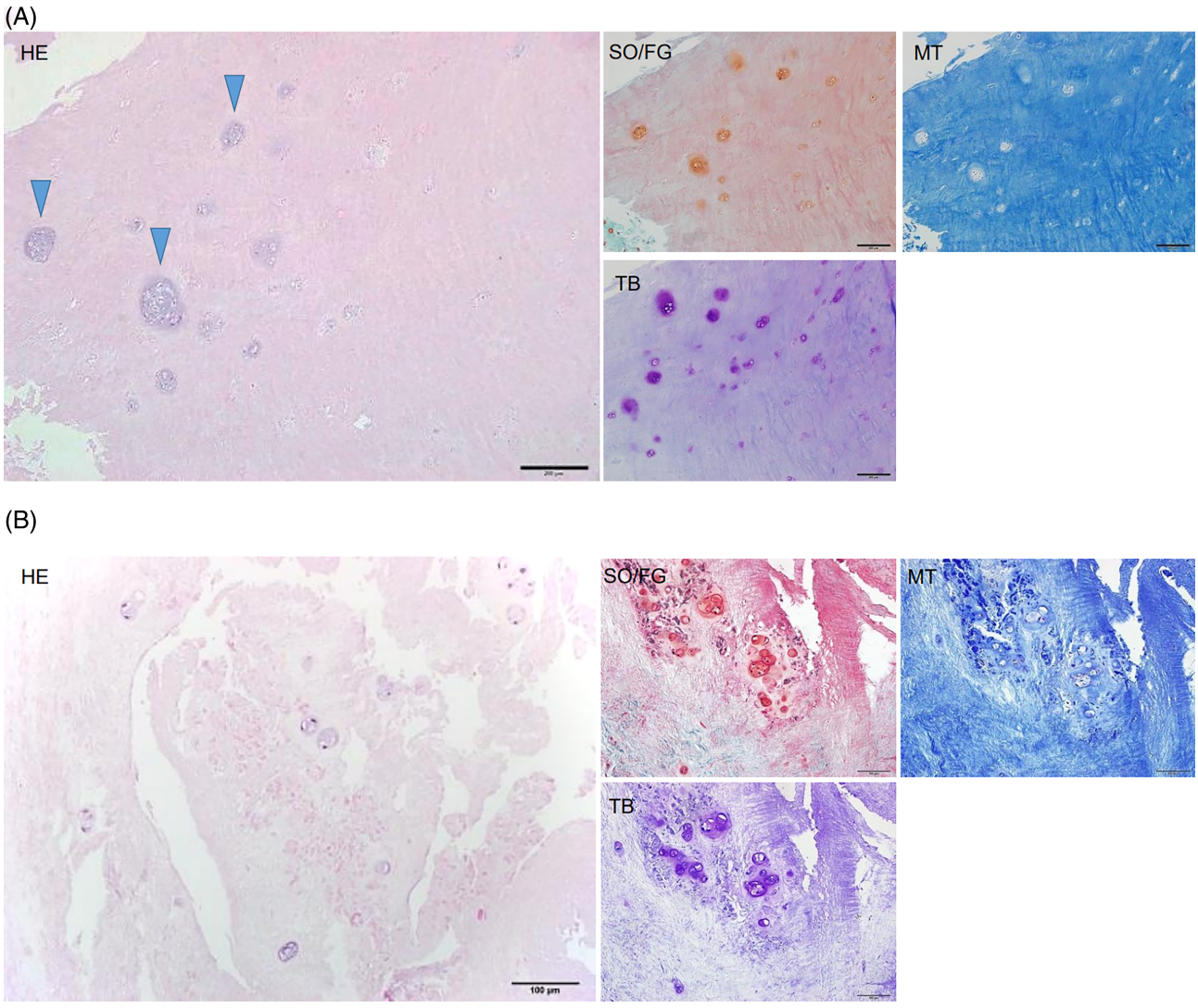


FIGURE 4 Representative histological images of lumbar disc tissue including the nucleus pulposus (A) Condoliase administration (H5). Arrows in the figure indicate clusters (40–120 μm in diameter). Scale bar = 200 μm. Tissues were observed using a biological microscope with a 10× objective lens. (B) Condoliase non-administration (H7). Scale bar = 100 μm. Tissues were observed using a biological microscope with a 20× objective lens.

small compared to that of C-unit, tissue staining by 2B6 (that recognizes CS containing ΔDi-4S) was assumed to be weak or non-staining. Contrary to our expectations, the antibody gave good tissue staining images. The findings suggest that immunostaining by anti-CS antibody is a useful method to detect small amounts of CS in tissues. To confirm whether or not the administered condoliase acted on the nucleus pulposus, immunostaining of the lumbar disc tissue including the nucleus pulposus was performed with an anti-CS antibody. The preCABC tissue of the CA group was stained with antibodies (3B3 and 2B6) that recognize CS containing unsaturated disaccharides. Unsaturated disaccharides derived from CS are generated after enzyme action. It was suggested that the condoliase acted on the nucleus pulposus and degraded CS in patients whose symptoms were not improved by condoliase. We were able to rule out the possibility that condoliase did not act on the nucleus pulposus of patients whose symptoms did not improve. According to Yoshiiwa et al., patients whose hernia size decreased after condoliase administration were Pfirrmann grade III before administration.[30] While it has been reported that higher rates of improvement for lower Pfirrmann classifications,[30,31] Oshita et al. reported that condoliase appeared more effective for patients with Pfirrmann grade IV compared to grade III patients.[32–34] Regardless of the therapeutic effect of condoliase, we selected patients with Pfirrmann grade III, apart from one of the 12 patients in this study. However, we did not evaluate the action of condoliase on the nucleus pulposus clinically, for example, measurement of the hernia size before and after condoliase administration.

The immunostaining images in this study also showed no correlation between CS degradation and symptom improvement after administration of condoliase. Roggendorf et al. disproved the hypothesis that failure of chemonucleolysis with chymopapain is due to failure of enzyme activity because tissues from patients

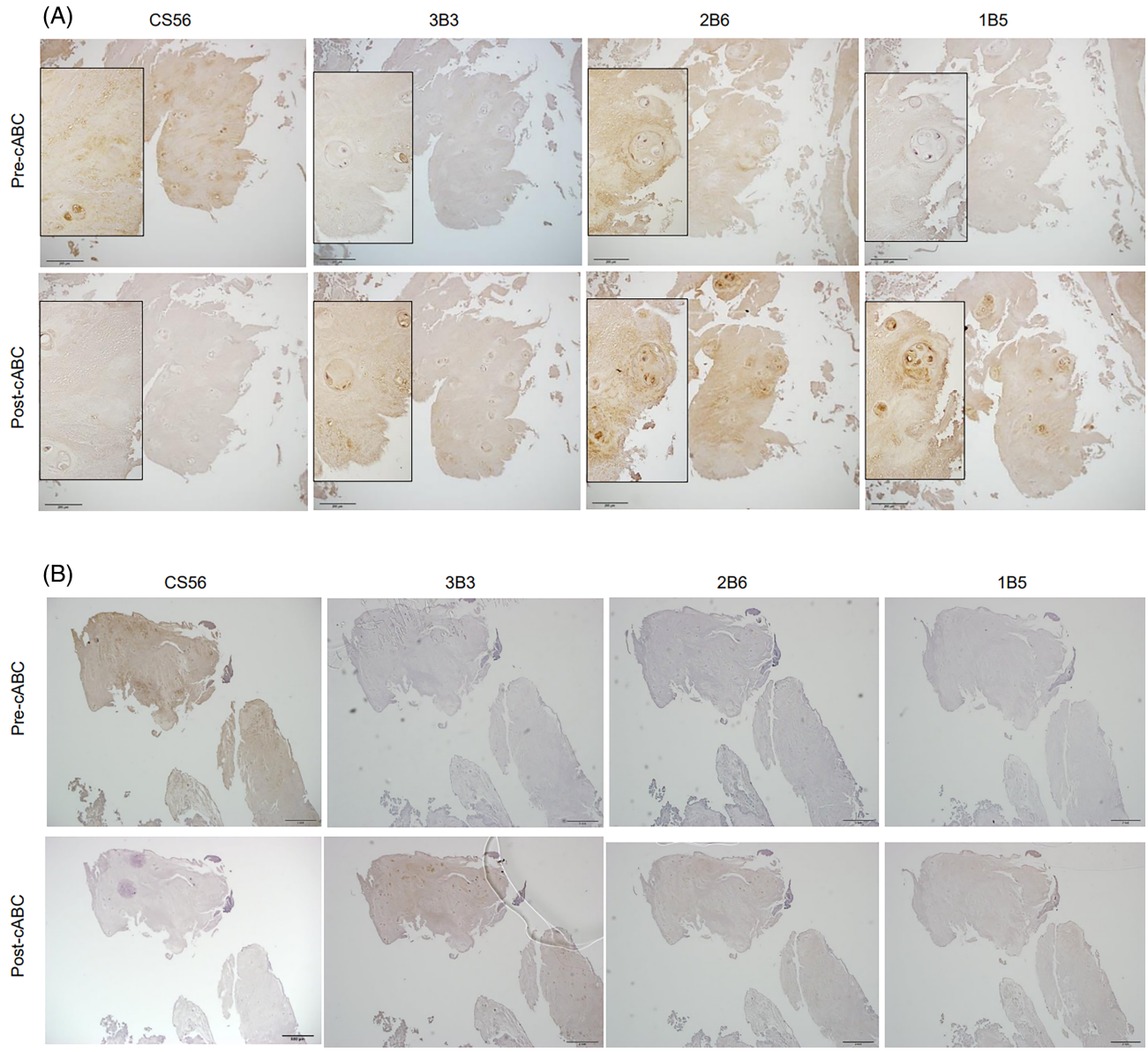


FIGURE 5 Representative immunostaining images of lumbar disc tissue including the nucleus pulposus with anti-CS antibodies
(A) Condoliase administration (H4). Scale bar = 200 μm. Tissues were observed using a biological microscope with a 10× objective lens.
(B) Condoliase non-administration (H7). Scale bar = 500 μm. Tissues were observed using a biological microscope with a 4× objective lens. CS, chondroitin sulfate.

without successful treatment with chymopapain showed distinct signs of tissue digestion based on their histological results.[35] Banno et al. have assessed the clinical outcome of condoliase therapy in patients with LDH, as well as factors affecting outcomes. They reported that condoliase therapy was less effective in patients with a history of discectomy, spondylolisthesis, or those with a posterior intervertebral angle (≥5°), while trans-ligamentous type and high T2 herniation were associated with increased efficacy.[36] Since the CS in the nucleus pulposus of the patients treated with condoliase in this study was digested, the lack of therapeutic effect may be due to factors other than the action of condoliase. Multiple factors may exert an influence on the lack of symptomatic improvement reported by Banno et al. To discuss the relationship between the effects of condoliase and symptom improvement, we believe that a multifaceted evaluation is necessary.

Tissues treated with chymopapain showed markedly reduced or absent SO/FG and TB staining, whereas condoliase-treated tissues retained their stainability indicating the presence of PGs.[14,37] In this study, the nucleus pulposus was stained with SO/FG, TB, and MT. In addition, there was no clear difference in staining characteristics between CA and NA groups. Although collagen was not evaluated in this study, type I and II collagen are known to be another major component of the extracellular matrix of IVD tissue. Nemoto et al. reported that there was no clear difference in staining characteristics of both collagens in rabbit nucleus pulposus, with or without

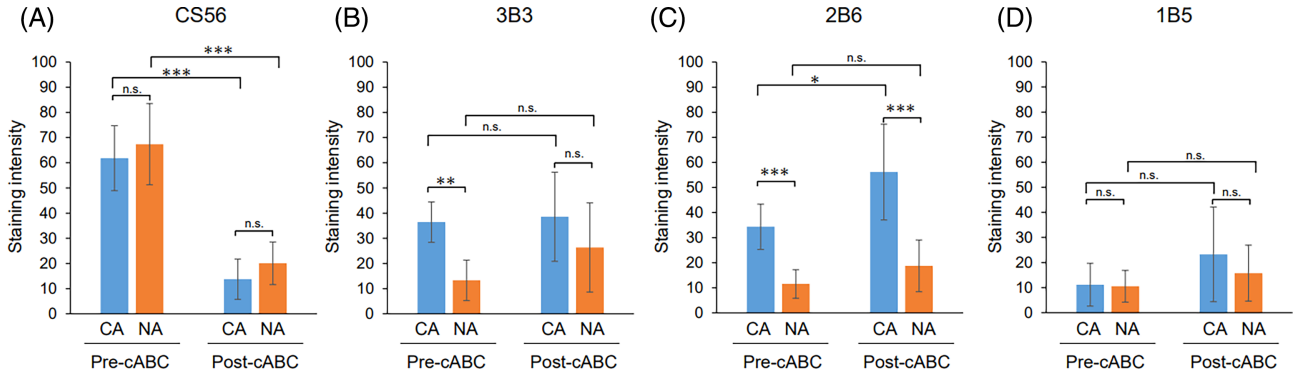


FIGURE 6 Quantitative assessment of immunohistochemical staining intensity. The results show immunohistochemical staining intensity of lumbar disc tissue including the nucleus pulposus with each anti-CS antibody. (A) CS56, (B) 3B3, (C) 2B6, and (D) 1B5. CA; condoliase administered group (H1-6), NA; non-administered group (H7-12). Post-cABC; tissue treated with cABC, Pre-cABC; tissue untreated with cABC. The values represent the mean ± standard deviations ($n = 6$). The statistical analysis was performed by Student's t test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. CS, chondroitin sulfate; n.s., not significant.

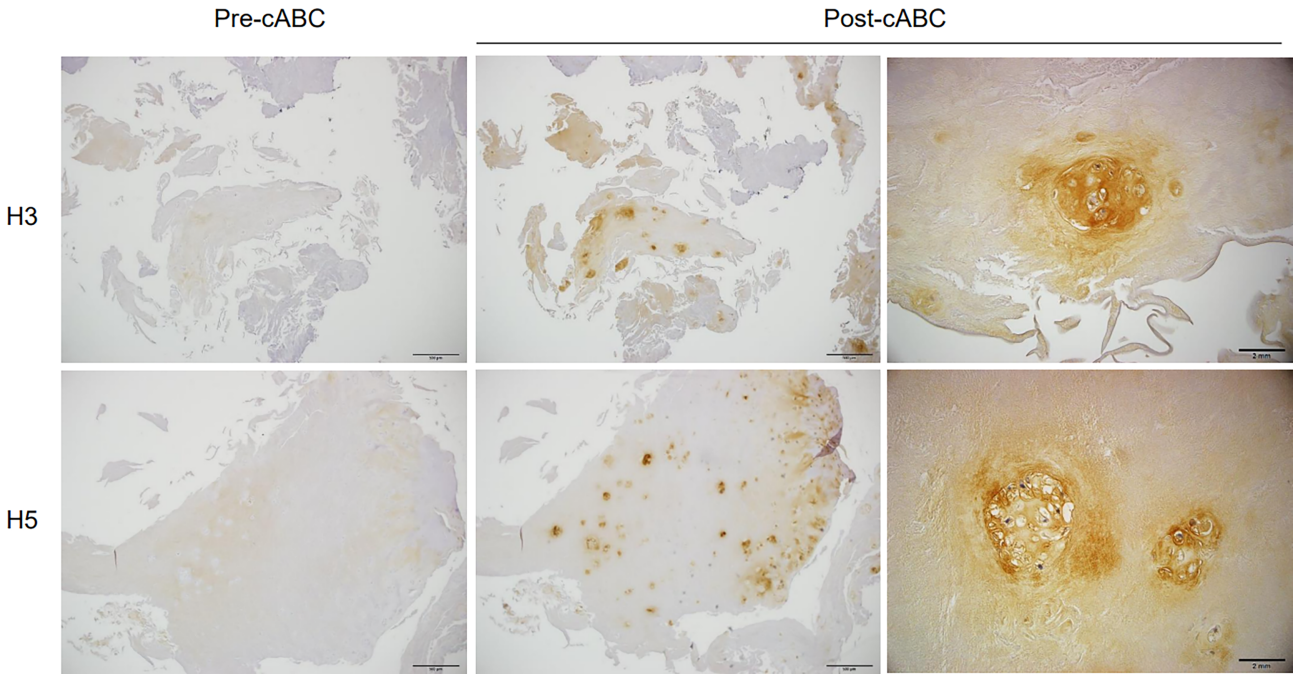


FIGURE 7 Large cluster stained by 2B6 after cABC treatment. Condoliase administration (H3 and H5). Scale bar = 500 μm or 2 mm. Tissues were observed using a biological microscope with a 4× or 40× objective lens.

administration of condoliase.[38] Considering both conventional knowledge and our evidence in this study, it appears that condoliase acts in humans with low tissue toxicity that does not cause necrosis of chondrocytes and surrounding tissues, because condoliase acts specifically on the CS of the nucleus pulposus.

As shown in Table 2, many in vitro or in vivo studies have reported that condoliase-treated tissue temporarily depletes extracellular matrix and CS, but regenerates after condoliase exposure.[13,14,37–40] In vivo studies show that the amount of CS increases approximately 12–24 weeks after administration of condoliase.[13,14,37,38] The lumbar disc tissues evaluated in this study were acquired 14–51 weeks after administration of condoliase, which was consistent with the observation time points of previous in vivo studies. According to Nemoto et al., the number of CS-positive cells increased over time in rabbit nucleus pulposus after administration of condoliase. However, the CS-positive cells that appeared after 24 weeks comprised numerous chondroid cells with a spherical morphology, which was different from normal morphology.[38] In this study, large clusters appeared in the tissues of the condoliase-treated cases, which were not observed in the NA group. Staining around clusters was enhanced by anti-CS antibody may indicate the possibility that CS regeneration (Figure 7). The large clusters had a peculiar tissue morphology that appeared after the administration of condoliase, similar to the report by Nemoto et al. It has been reported that

TABLE 2 Regeneration of CS and extracellular matrix in tissues after condoliase treatment.

Author	Animal or cells	Summary	References
Kato et al., 1990	Rabbit	The concentration of uronic acid decreased up until 4 weeks following condoliase administration, but it recovered by 12 weeks, and resynthesis of the matrix by the remaining nucleus pulposus cells was observed/Chymopapain-treated intervertebral discs, the entire intervertebral disc including the annulus fibrosus became necrotic; fibrosis occurred at 8–12 weeks.	13
Kato et al., 1993	Rabbit	Condoliase administration reduced staining with toluidine blue after 2 weeks, but restored staining after 12 weeks indicating matrix replenishment/Chymopapain administration to the knee joint caused necrosis of chondrocytes and loss of staining with toluidine blue.	37
Sugimura et al., 1996	Rhesus monkey	After 6 weeks of administration, the chymopapain-treated intervertebral discs showed decreased safranin O staining, while the condoliase-treated intervertebral discs remained stainable/ Although the amount of CS decreased immediately after condoliase administration, the amount of CS recovered after 24 weeks.	14
Nemoto et al., 1998	Rabbit	In the nucleus pulposus after condoliase administration, the number of CS-positive cells disappeared after 2 weeks, but the number of CS-positive cells increased over time, and reaching about 50% of that in normal saline after 24 weeks/CS-positive cells after 24 weeks comprising many chondroid cells exhibiting a spherical morphology, which is different from the normal morphology/ Chymopapain administration showed no tissue regeneration even after 24 weeks.	38
Chiba et al., 2007	Rabbit nucleus pulposus or annulus fibrosus cells	The nucleus pulposus cells and annulus fibrosus cells treated with condoliase showed higher PG production and matrix replenishment than those treated with chymopapain.	39
Muramatsu et al., 2020	Cynomolgus monkey	Cellular regeneration in the lumbar intervertebral disc was observed as a recovery change after 4 weeks of condoliase administration.	40

Abbreviations: CS, chondroitin sulfate; PG, proteoglycan.

chondrocyte clusters are expressed in osteoarthritis of the human knee.[41,42] Fibroblast growth factor 2, which increases with mechanical stress, induces clustering of chondrocytes.[43,44] Chondrocyte clusters are known to undergo marked proliferation and produce cartilaginous nodules.[45] The clusters observed in this study may also be aggregates of chondrocytes, but the details of cluster formation in lumbar disc tissue and the mechanism of CS regeneration in tissues after condoliase administration remain unknown. Banno et al. reported that disc height decreased 3 months after condoliase administration, but recovered after 1 year in younger patients. Moreover, patients with disc height recovery had a higher incidence of recovery based on Pfirrmann grading, compared to those without disc height recovery.[19]

The current study has several limitations. (i) We have been able to obtain useful information by evaluating human-derived tissues in this study, but there are limits to investigating the relationship between patient background and clinical or histological findings. This is because patient factors varied apart from the Pfirrmann grade, and the number of subjects was small. To draw more accurate conclusions, it is necessary to conduct studies with uniform patient characteristics and using an increased number of subjects. Furthermore, the following are unclear: (ii) why symptoms failed to improve despite the action of condoliase on the nucleus pulposus, and (iii) how clusters arise and CS regeneration occurs in lumbar disc tissues after condoliase administration. For more detailed investigation of the relationship between cluster expression and CS regeneration in lumbar disc tissue, and disc recovery in patients after condoliase administration, clinical studies as well as in vivo and ex vivo evaluations will be needed.

5 | CONCLUSION

This study is the first to use histological staining including immunohistochemistry by anti-CS antibodies to evaluate human lumbar disc tissue after condoliase administration. Although it is possible to quantitatively detect CS in biological samples by disaccharide analysis and mass spectrometry, analysis requires a large amount of raw material and complicated work. On the other hand, an immunostaining method using an anti-CS antibody can identify the localization of a minute amount of CS in a biological sample. In this study, we demonstrated by histological staining that CS in the nucleus pulposus was degraded even in patients who did not show therapeutic effects after condoliase administration. Moreover, condoliase acts in humans without necrosis of chondrocytes and surrounding tissues, as reported in in vivo studies.

AUTHOR CONTRIBUTIONS

Yuka Minamisawa: Designed the experiments, performed the experiments, data curation, and data interpretation. **Taiichi Shirogane**: Project administration, supervision, and reviewing the manuscript from clinical and scientific standpoints. **Ippei Watanabe**: Writing—original draft preparation, investigation, data curation, data interpretation, and writing—reviewing and editing. **Akira Dezawa**: Conceptualization, resources, supervision, and reviewing the manuscript from clinical and scientific standpoints. All authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

Yuka Minamisawa, Taiichi Shirogane, and Ippei Watanabe are employees of Seikagaku Corp.

ORCID

Ippei Watanabe https://orcid.org/0000-0002-7862-5655

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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