Research Article

Antiadhesive Property of Photoreactive Azidophenyl Low-Molecular-Weight Chitosan in Rabbit Laminotomy Model

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Newly developed photoreactive azidophenyl chitosan (P-ALMC) has characteristics of a transformable gel type and its outer layer could be sealed up like a film after UV radiation. We aim to evaluate the antiadhesive properties of P-ALMC through comparing it with hyaluronic acid-carboxymethylcellulose membrane (HA-CMC) in a rabbit laminotomy model. Laminotomies were performed at the L3-4, L4-5, and L5-6 levels in 41 rabbits and each level was randomly assigned to either receive saline (group I), HA-CMC (group II), or P-ALMC (group III). The extent of peridural fibrosis, density of fibroblasts and inflammatory cells, and dural thickness were evaluated at 6 and 12 weeks postoperatively. In the groups II and III, the extents of peridural fibrosis and dural thickness were significantly smaller than those in group I ($P < 0.001$) and no differences between groups II and III were found at the postoperative 6 and 12 weeks. There were no differences of cell density among groups. P-ALMC showed effective antiadhesive properties comparable to HA-CMC and could be one of the candidates as an anti-adhesive agent for spine surgery even further study is required to identify the effectiveness of its unique characteristics as mechanical barrier.

1. Introduction

The formation of scar tissue over the epidural space, after surgical exploration of the spinal canal, has been a cause for concern because it significantly promotes surgical hazards to subsequent spinal surgeries. Additionally, it can result in leg and back pain caused by the tethering of the neural tissue [1, 2]. One inevitably hazardous consequence of spinal surgery is the formation of scar tissue designated as the "postlaminectomy membrane" by LaRocca and Macnab [2] and Gill et al. [3].

Various efforts to prevent scar formation after laminectomy have been evaluated over the years [1, 3–10]. Pathoetiologically, it has been reported that the formation of a hematoma, after laminectomy, constitutes a scaffold for the migration of fibroblasts from the periosteum and paraspinal muscles. This migration of fibroblasts is considered to be one of the main causes of postlaminectomy peridural fibrosis [2, 5, 6]. However, if materials are used to limit this contact, scar tissue formation may be prevented. In order to prevent scar tissue formation with this strategy, the material must have proper biocompatibility, biodegradability, space-occupying properties, and optimal mechanical properties.

Chitosan is one of the most abundant marine-based biopolymers [11]. Low-molecular-weight chitosan (LMC) is a water-soluble hydrolysate of chitosan that has been shown to have a wide range of biological activities and industrial applications [12–17].

Photoreactive azidophenyl low-molecular-weight chitosan (P-ALMC) used in our study was formed by conjugating LMC with a photoreactive azidophenyl group [18]. The compound can be applied in the form of a gel/film-type condition. As it is applied to the laminotomy site, according to shape, this material could then serve as a more complete mechanical barrier for the posterior surface. Since the material's posterior outer layer can seal up like a film following treatment with ultraviolet (UV) radiation, the material will ultimately form a sealed mechanical barrier to prevent the invasion of the hematoma into the peridural space posteriorly at the laminotomy site. Based on these mechanical characteristics of P-ALMC, we hypothesized that the application of P-ALMC to a laminotomy defect would be helpful to effectively reduce peridural scar formation as a mechanical barrier. To verify the hypothesis, we applied A-LMC in rabbit spine laminotomy model and compared its results with hyaluronic acid-carboxymethylcellulose membrane (HA-CMC).

2. Methods

2.1. Synthesis of Azidophenyl Chitosan Derivative. P-ALMC was synthesized with the use of the method described in the previous study [18]. Briefly, LMC was prepared using sodium nitrite depolymerization. Then, depolymerized chitosan in deionized water was ultrafiltered using YM10 (molecular weight cut-off 10,000 Da) and YM3 (molecular weight cut-off 3,000 Da) membranes (Amicon, USA). To prepare azidophenyl chitosan, a single chitosan fraction of about Mw 10,000 was used and the chitosan oligomer (0.20 g, Mw = 10,000) was dissolved in 5 mL of distilled water. N-(4-azidobenzoyloxy) succinimide was prepared according to a previously reported method. The synthesized 0.11 g of N-(4-azidobenzoyloxy) succinimide was dissolved in as small amount of dioxane as possible and then added to the chitosan oligomer solution. The reaction products were washed with acetone to remove the remaining N-(4-azidobenzoyloxy) succinimide. The main macromolecular structure of the azidophenyl chitosan derivative was identified using FT-IR and 1H-NMR analysis. The cytotoxicity test confirmed that azidophenyl chitosan derivative irradiated with UV light was noncytotoxic to the proliferation of 3T3 cells (mouse embryonic fibroblast cell line) cultured for 48 hours.

2.2. Synthesis and Photoreactivity Assay of Azidophenyl Chitosan Derivative. The photoreactivity of azidophenyl chitosan was determined using micropatterning. 30 μL of 20 wt/wt % Azidophenyl chitosan solution was cast onto a polypropylene plate, and the cast azdidophenyl chitosan was dried in the dark. Photomask was placed on the dried azidophenyl chitosan. With a photomask, azidophenyl chitosan was irradiated with UV light for 3 minutes using a UV lamp (model: spot cure-9, Tokyo, Japan). During UV irradiation, the UV light lamp and sample were 5 cm apart, resulting in an intensity of 48 mW/cm^3. After UV light irradiation, the polypropylene plate was washed three times with distilled water for five minutes each time.

The micropattern illustrated the same pattern as that of the photomask (Figure 1). The azidophenyl chitosan reacted after irradiation with UV light became insoluble in water and remained on plate, in contrast to the soluble nonirradiated azidophenyl chitosan washed out.

2.3. Animal Preparation. This study was performed in accordance with guidelines from the Animal Care and Use Committee at our institute. Forty-one New Zealand white female rabbits weighing 2.5 to 3.8 kg were used, and all animals were housed individually seven days before the procedures to ensure proper adaptation and the inner surface of the rabbits' ears were tattooed. Anesthesia was induced by intramuscular injection of a mixture (0.8 mg/kg) of Rompun 2% (5 mL, Virbac, France) and Zoletil 50 (5 mL, Bayer, Germany). Under adequate anesthesia, the surgery was performed following strict aseptic techniques. After dissecting the subcutaneous tissue, fascia, and muscles to expose L3 to L6, three separate laminotomies (6 × 8 mm size) at the L3-4, L4-5, and L5-6 levels were performed with the aid of an electrical drill, and the ligamentum flavum and peridural fat tissue were cleared. After meticulous hemostasis around the exposed dural area, each of the three laminotomy sites was treated. Each laminotomy level was randomly assigned to either receive nothing, HA-CMC, or P-ALMC. Groups were divided as follows: group I: the defect was irrigated with saline alone, group II: the defect was covered by a HA-CMC (300 ug hyaluronic acid-carboxymethylcellulose membrane, Guardix, Seoul Korea), and group III: the defect was covered by P-ALMC (300 ug) followed by radiation with UV light (48 mW/cm^2, model: spot cure-9, Tokyo, Japan) for 15 seconds to consolidate the outer layer of covered materials (Figure 2). The fascia was then closed with 2-0 vicryl, and the skin was closed with 3-0 nylon sutures. All animals, except for three that died perioperatively, recovered from the surgical procedure without complications.

2.4. Histological Evaluation. The animals were killed at one week ($n = 3$), six weeks ($n = 16$), or 12 weeks ($n = 19$) postoperatively by means of an overdose of intravenous sodium pentobarbital (60 mg/kg). The spinal column, including the surrounding muscle tissue, was totally removed between L3 and L6. The sections were fixed with 10% formalin for 24 hours and were decalcified in formic acid for two weeks. After fixation, the spines were further sawed axially at each laminotomy site, and the axial sections (thickness, 6 μm) of the laminotomy site were stained with hematoxylin and eosin (H&E). The information of injected materials at each spine level can be identified by rabbits' ear tattoo. Prepared histological slides were evaluated in a blinded fashion by a pathologist who had 15 years of experience.

The extent of peridural fibrosis, the cell densities of fibroblasts and inflammatory cells, and the thickness of the dura were evaluated and measured at 6 and 12 weeks postoperatively. The extent of peridural fibrosis was evaluated according to the five-point modified criteria devised by He et al. [19]. Grading from 0 to 4 was applied as follows: G0 = no adhesion, G1 = adhesion was observed but included less than
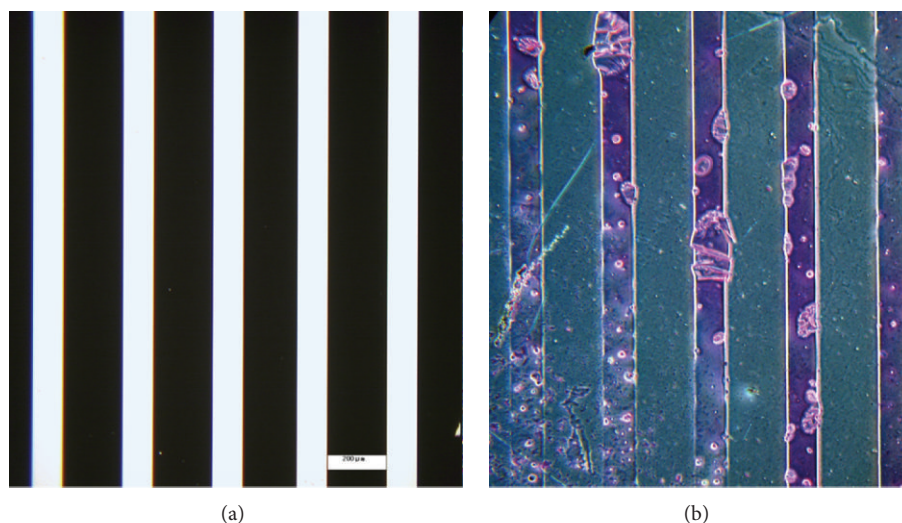
(a)   (b)

FIGURE 1: Photomask (a) and micropatterned azidophenyl low-molecular-weight chitosan (P-ALMC) after UV irradiation (b). The pattern of P-ALMC shows that P-ALMC reacted after irradiation with UV light and became insoluble in water, in contrast to the soluble nonirradiated azidophenyl chitosan, is the same as the photomask.
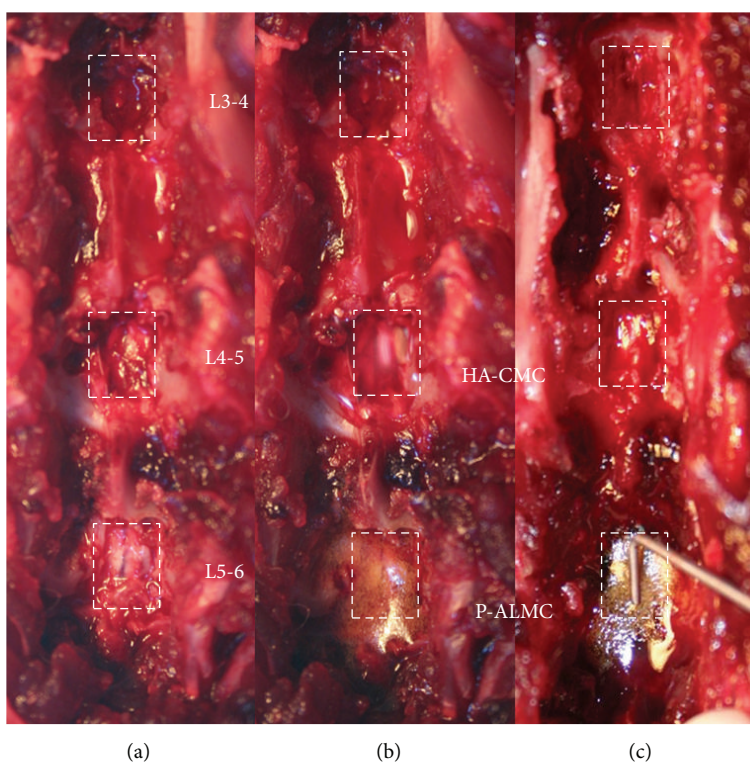

(a)   (b)   (c)

FIGURE 2: Operative fields in a rabbit model. (a) Pretreatment laminotomy windows. (b) After conditioning according to randomization, at L3-4 (control, group I), L4-5 (HA-CMC, group II), and L5-6 (P-ALMC, group III). (c) The consolidation at only outer surface of the covered P-ALMC at L5-6 following UV radiation for 15 seconds was observed and its consistency was soft such like muscle.

one-third of the laminotomy defect, G2 = adhesion was more than one-third and less than two-thirds, G3 = adhesion was more than two-thirds, and G4 = G3 and severely distorted dura due to severe scar tissue. If G0 was observed, the shortest distance from the dura to the posterior scar tissue within the range of the laminotomy site was measured.

Cell densities using fibroblast and inflammatory cell counts were also evaluated, following the classification under 400 times magnification by Hinton et al. [8] grade 1 for 100 or fewer cells in every region; grade 2 for 100–150 cells in every region; and grade 3 for 150 or more cells in every region. Fibroblasts and inflammatory cells were counted in
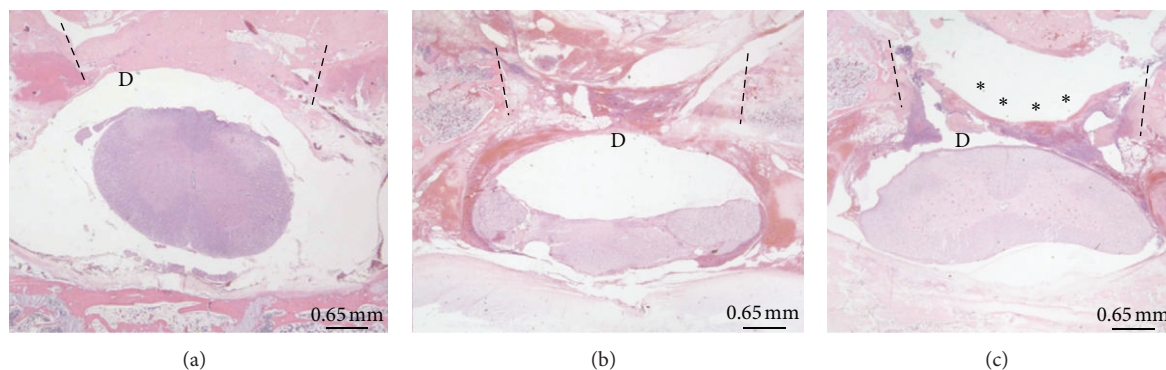
FIGURE 3: Histological sections of the laminotomy sites (between dotted lines) at one week ((a) control, group I; (b) HA-CMC, group II; (c) P-ALMC, group III). Group III showed that the clear space (asterisks) formed by the interpositional barrier of P-ALMC between the dura (D) and the posterior soft tissue is maintained in a comparison of groups I and II (H&E, ×1.25).

three fields located in the middle and at each margin of the laminotomy.

Dural thicknesses were measured at three different points, where the dura was the thickest within the margins of the laminotomy area based on the judgment of the pathologist.

All evaluations were performed twice with 2-week interval by one pathologist. The above measurements of distance from dura to posterior scar tissue and dural thickness are presented as average values.

2.4.1. Statistical Analysis.
Adhesion, fibrosis score, and dural thickness were analyzed using the Kruskal-Wallis test followed by Boneferroni's posttest. For categorical variable, Chi-squared test was used to correct for multiple comparisons. Data are reported as mean values, with variability expressed as standard deviations in the table and standard errors in the figure. Interclass correlation coefficients (ICCs) and kappa reliability coefficient analyses were used to determine the intraobserver agreements. Statistical analyses were performed with SPSS version 15.0 (SPSS Inc., Chicago, IL, USA), and significance was defined as $P < 0.05$.

3. Results

Intraobserver agreements for grading of peridural fibrosis and the densities of cells were $k = 0.911$ and $k = 0.874$, respectively, and we considered evaluation at the second time as the data. Intraobserver ICCs for measurement of distance from dura to scar tissue and dural thickness were 0.795 and 0.784, respectively.

3.1. One Week Postoperatively.
In three groups, hemorrhage, inflammatory cells, and some fibrous tissues were markedly observed on the posterior side of the dura. It was difficult to objectively compare those findings at the laminotomy site among the groups. However, there were tendency that the less infiltration of the hematoma into the spinal canal in groups II and III were observed compared to group I. All three samples in group III demonstrated the clear space considered as the

effects of mechanical barrier of P-ALMC between dura and posterior hematoma (Figure 3).

3.2. Six Weeks Postoperative (Table 1).
In group I, no grade 0 (G0) of peridural fibrosis was found, and all specimens were graded from G2 to G4, with an average of 2.24 grade. In groups II and III, almost all of the peridural fibrosis was graded as G0 except for two cases graded as G1 and G2 in group II and two cases graded as G1 in group III. The average grades were 0.25 in group II and 0.25 in group III. There were significant differences ($P < 0.001$) in terms of the extent of peridural fibrosis between groups I and II and between groups I and III. However, no difference was found between group II and group III ($P = 0.832$) (Figures 4(a) and 5). There were no differences in terms of distance from the dura to the posterior scar between groups II and III on the specimens graded as G0 ($P = 0.778$) (Figures 4(b) and 5).

No statistical differences in grading of the cell densities of fibroblasts and inflammatory cells were found among the three groups ($P = 0.161$) (Figure 4(c)). The average values for the dural thicknesses in groups II and III were significantly lower than the values in group I ($P < 0.001$), and there were no differences observed between groups II and III ($P = 0.672$) (Figure 4(d)).

3.3. Twelve Weeks Postoperatively (Table 1).
Group I showed no G0 of peridural fibrosis. The degree of grading from G1 to G4 was significantly higher in group I, with an average grading of 2.68 compared to the average grading of 0.53 in group II and 0.26 in group III ($P < 0.001$) (Figures 4(a) and 5), but there was no difference between group II and group III ($P = 0.583$). The distance from dura to posterior scar in specimens showing G0 did not differ between group II and group III ($P = 0.583$) (Figures 4(b) and 5). No differences in the grading for cell density ($P = 0.288$) were found among the three groups (Figure 4(c)). In measuring the dural thickness, those of groups II and III were significantly lower than dural thickness of group I ($P < 0.001$) and there was no observed difference between groups II and III ($P = 0.672$) (Figure 4(d)).
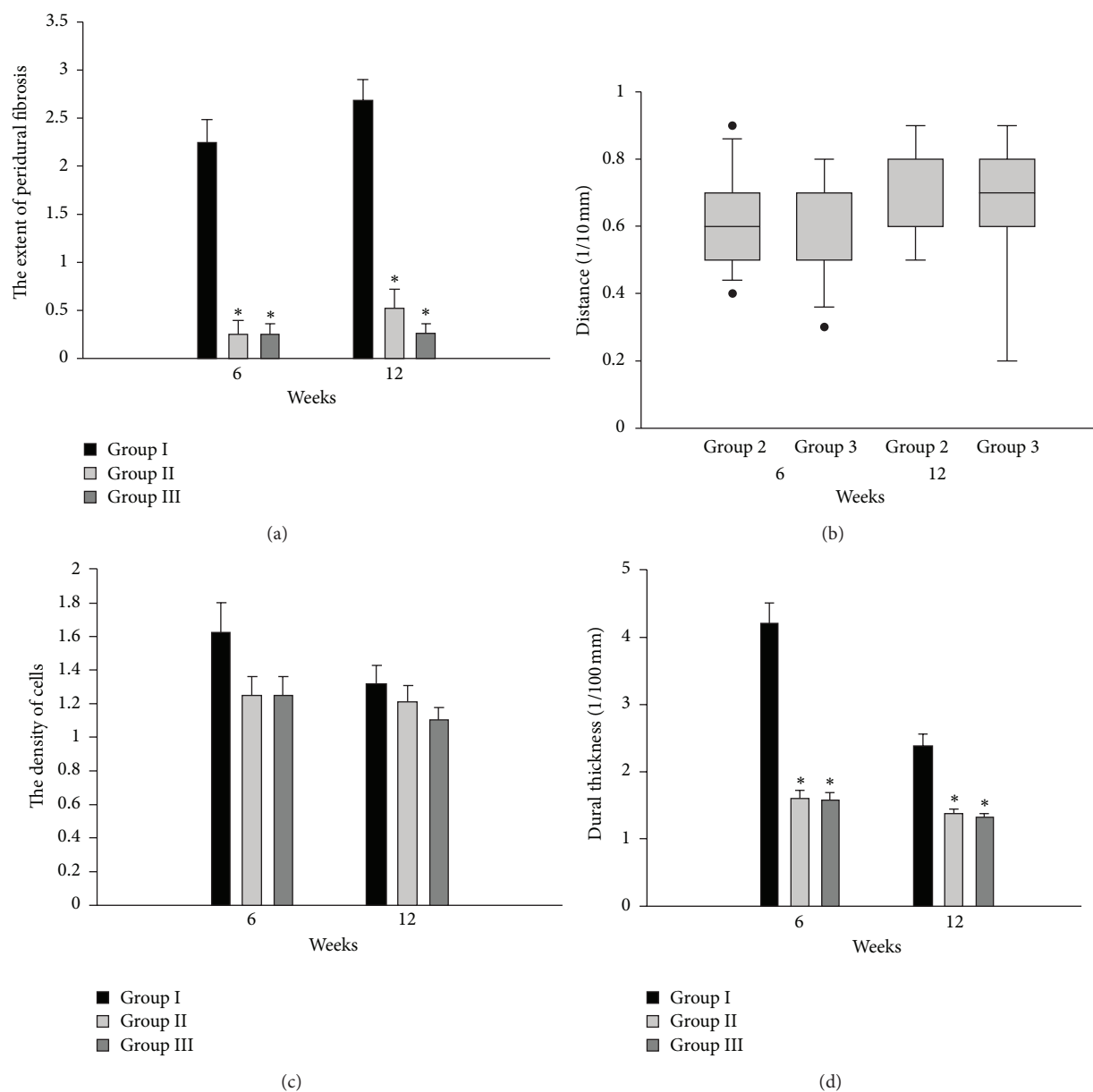
FIGURE 4: (a) The extents of peridural fibrosis, (b) distances from the dura to the posterior scar tissue in the specimens graded G0, (c) the densities of cells, and (d) dural thickness. Group I: control, group II: HA-CMC, group III: P-ALMC. Groups II and III showed significantly lower extent of peridural fibrosis and lower dural thickness than group I at weeks 6 and 12 ($P < 0.001$). $^{*}P < 0.05$ (Kruskal-Wallis test).

4. Discussion

Postoperative epidural adhesion is one of the most common problems associated with spinal surgery; there are few effective treatments currently available for this [5]. Occurrences of epidural adhesion depend on surgical technique, such as meticulous hemostasis and gentle soft tissue handling. Nonetheless, the source of fibrosis inevitably comes from either the disruption of the intervertebral disc anteriorly in the surgical field or the disruption of the bone and muscle posteriorly in the surgical field. Regardless of various kinds of causes, it seems that the key feature preventing peridural fibrosis is to limit invasion of fibroblasts into peridural space in the early healing stage. For the reason, a variety of materials as mechanical barrier on the exposed dura has been proposed to limit the migration of fibroblasts mainly posteriorly. Historically, Gelfoam or free fat grafts were used after laminectomy for the purpose, but the results are not promising [3, 20]. After then, the biomaterials composed of carboxymethylcellulose, polyethylene oxide, and so forth. have been introduced to reduce peridural fibrosis [5, 6, 21].

The mechanical characteristics of P-ALMC as a gel-/film-based anti-adhesive barrier could be ideal to limit the invasion of blood into the epidural space in laminotomy or laminectomy model because inner space facing dura is maintained as a soft gel type, and outer surface posteriorly is hardened and sealed up after exposure to UV radiation. In terms of biological properties, LMC is a fascinating candidate for
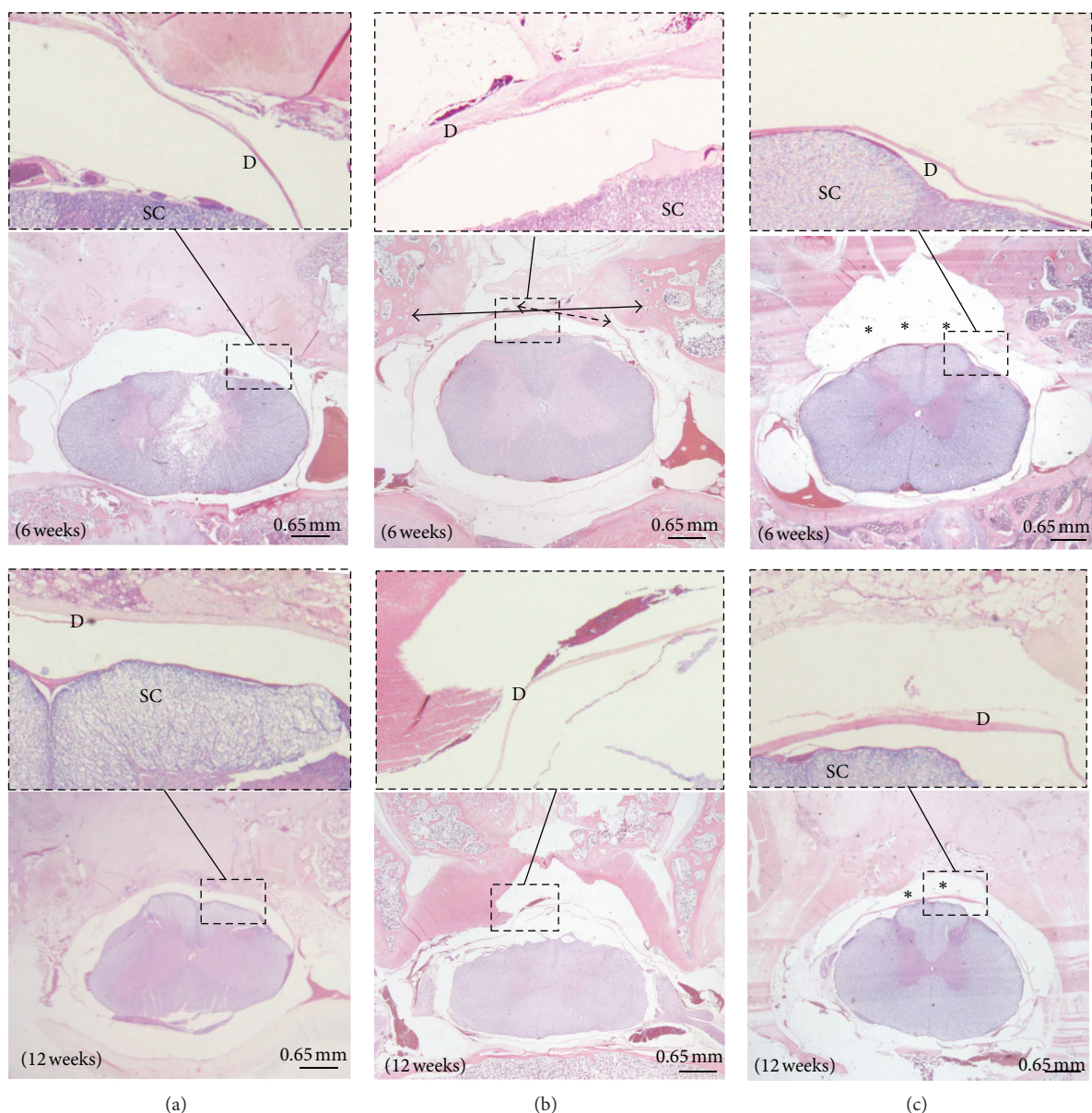
Figure 5: Histological sections (H&E staining, ×1.25) and magnified views (×40, dotted boxes) at weeks 6 and 12 after the operation ((a) control, group I; (b) HA-CMC, group II; (c) P-ALMC, group III). Group I at both 6 and 12 weeks showed entire scar tissue adhesion to the entire exposed dura (D), graded 4. Group II showed a nondistorted dural contour, but the extent of adhesion (adhesion (dotted line)/laminotomy defect (solid line)) was graded 2 at week 6 and graded 0 at week 12. Group III showed a maintained clear area (asterisks) from the dura to the scar tissue at weeks 6 and 12, graded 0.

a broad spectrum of applications given its unique biological properties including biocompatibility, biodegradability into harmless products, lack of toxicity, physiological inertness, and remarkable affinity to proteins [14–17]. P-ALMC also showed suitable biological properties with no toxicity [18].

We compared the results of P-ALMC as an anti-adhesive agent with HA-CMC (Guardix, Hanmi Pharmacy, Republic of Korea). It is well known that HA-CMC has been reported to be useful in preventing adhesions in experimental studies [1, 6, 7, 20, 22] and is in clinical use in the field of spine surgery [23]. Our newly developed P-ALMC demonstrated

similar results to those observed in HA-CMC group in a rabbit model. Both P-ALMC and HA-CMC groups showed that the extents of peridural fibrosis were significantly smaller than those in control group at 6 and 12 weeks. These results could be promising for the possibility of using P-ALMC as an anti-adhesive agent.

We presumed that the sealed-up outer layer of P-ALMC by treatment of UV could be a more complete interpositional mechanical barrier to prevent invasion of blood into the epidural space. However, there were no significant differences between HA-CMC groups and P-ALMC group at six weeks

TABLE 1: Histological results.

		Group I	Group II	Group III	P value
6 weeks (n = 16 at each group)	Grade of peridural adhesion	2.24 (0.93)	0.25 (0.58)*	0.25 (0.45)*	<0.001
	Distance (1/10 mm)	0	6.23 (1.42)	6.25 (1.49)	0.778
	Grade of cell density	1.63 (0.72)	1.25 (0.45)	1.25 (0.45)	0.161
	Dural thickness (1/100 mm)	4.21 (1.20)	1.60 (0.52)*	1.58 (0.45)*	<0.001
	No. of new bone formation at laminotomy site†	4	3	2	0.557
12 weeks (n = 19 at each group)	Grade of peridural adhesion	2.68 (0.95)	0.53 (0.84)*	0.26 (0.45)*	<0.001
	Distance (1/10 mm)	0	67.7 (14.2)	67.1 (22.0)	0.518
	Grade of cell density	1.32 (0.48)	1.21 (0.42)	1.11 (0.32)	0.288
	Dural thickness (1/100 mm)	2.39 (0.78)	1.39 (0.25)*	1.33 (0.27)*	<0.001
	No. of new bone formation at laminotomy site†	6	3	4	0.478

Data are given as mean with SD in parentheses.
*$P < 0.05$ (Kruskal-Wallis test).
†Chi-squared test.

($P = 0.832$) and 12 weeks ($P = 0.583$). This led us to question the role of the sealed outer barrier. This suggests that hematoma originates from the anterior epidural space to cause peridural fibrosis, despite efforts of preventing posterior hematoma invasion. It could be supported by histological findings at one week in the A-LMC group, in which the considerable hematoma and inflammatory cells were observed in the peridural space despite the well-sustained outer interpositional barrier (Figure 3).

In considering another suggestion, P-ALMC could be degraded by lysozymes more rapidly than expected. The exact degradation time P-ALMC in vivo needs to be evaluated in future studies.

In measuring dural thickness, both P-ALMC and HA-CMC groups were significantly different ($P < 0.001$) from control group, but there were also no differences between P-ALMC and HA-CMC groups. These results indicate that gel-type portion of P-ALMC contacting dura would have a biocompatible barrier property with less irritability, which can be comparable to HA-CMC.

Although HA-CMC and P-ALMC groups showed excellent results in regard to the extent of peridural fibrosis and dural thickness, the grading for cell densities of fibroblasts and inflammatory cells did not differ among the three groups at six weeks ($P = 0.161$) or 12 weeks ($P = 0.288$). Kurt et al. [21] experimented with mitomycin C, aprotinin, and Adcon-L groups at four weeks in rabbits and demonstrated the results of cell density in three groups were better than the results of the control group. The grading methods would be more appropriate for short follow-up period, at least within four weeks, after surgery. With the longer period after surgery, there would be no differences in the inflammatory cell or fibroblast counts.

In our study, a rabbit laminotomy model was adopted. Although this animal model can be handled with regular human surgical tools, it has been found that the formation of newly formed lamina is significant 24 weeks postoperatively [7]. In some cases of the present study, new bone formations at laminotomy site were observed during the evaluation periods. However, the incidences did not differ among the three groups (Table 1).

In the first application of the photoreactive A-LMC to the laminotomy experimental animal model, P-ALMC showed effective anti-adhesive properties comparable to HA-CMC in the rabbit postlaminotomy model. Differences between the HA-CMC group and P-ALMC group were not significant. Thus, further studies will be required to identify the effectiveness of the unique mechanical barrier of P-ALMC. P-ALMC may potentially be an effective candidate as an anti-adhesive agent, especially for a suction drainage system since the sealed outer layer can act as a barrier with long in situ residence time.

5. Conclusion

P-ALMC showed effective anti-adhesive properties, comparable to HA-CMC. With further studies elucidating P-ALMC effectiveness as a mechanical barrier, P-ALMC may become a potential anti-adhesive agent for spine surgery to prevent fibrosis.

Acknowledgment

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