(immediately after mixture of PMBV and PVA solutions).

To evaluate the in vivo stability of the MPC hydrogel, a diffusion chamber containing the MPC hydrogel above was subcutaneously implanted into the back of an 8-week-old Slc:Wistar rat (Sankyo Labo Service Co. Inc., Tokyo, Japan) under general anesthesia with tribromoethanol (400 mg/kg body weight). After 1 and 3 weeks, the chamber was extracted, and the elastic modulus and the viscous modulus of the hydrogel were measured by a rheometer (Rheolograph-Micro, Toyoseiki, Tokyo)[19]. Higher elastic modulus than viscous modulus indicated gel properties[20]. A scanning electron microscope (SM-200; Topcon Co., Tokyo) was used to evaluate the microstructure. All animal experiments were performed according to the protocol approved by the Animal Care and Use Committee of Central Research Laboratories of Kaken Pharmaceutical Corporation.

Rat Achilles tendon model

Under general anesthesia with pentobarbiturate (50 g/kg body weight), the Achilles tendon of a 7-week-old Slc:Wistar rat was exposed through a posterior approach. After cutting the tendon with a scalpel at 5 mm above its insertion to the calcaneus, it was sutured using a Kessler stitch with 6-0 braided polyester and a continuous adaptation stitch with 8-0 nylon monofilament for circumferential epitenon repair[21]. The animals were then randomly divided into 3 groups, subjected to local applications of the MPC hydrogels formed from 2.5% PMBV and 5.0% PMBV, and the distilled water control. After the skin closure, the leg was immobilized in a cast to limit movement of the talocrural joint. The rats were allowed unrestricted activity and received food and water *ad libitum*.

Rabbit flexor digitorum profundus (FDP) tendon model

Under general anesthesia with intramuscular injections of ketamine (33.3 mg/kg body weight)

and xylazine (6.7 mg/kg body weight), the zone II area of the FDP tendon of 10-week-old Japanese white rabbits (Kitayama Co., Ltd., Nagano, Japan) was exposed, released from the tendon sheath, divided, cut with a scalpel just distal to the chiasm and proximal to the vincula, and sutured by a Kessler stitch with 6-0 braided polyester and a continuous adaptation stitch with 8-0 nylon monofilament for circumferential epitenon repair[22]. The animals were then randomly divided into 2 groups, subjected to local applications of the MPC hydrogel formed from 5.0% PMBV and the distilled water control. After the skin closure, the operated leg was immobilized in a cast to limit the movement of the interphalangeal joints. The animals were allowed unrestricted activity and received food and water *ad libitum*.

Macroscopic evaluation

Before sacrificing the animals, the skin incision site was visually examined to check for any sign of inflammation and to confirm wound healing. To evaluate peritendinous adhesions, we counted the number of fibrous adhesions around the sutured tendon which required sharp dissection for release, as previously reported[23,24]. This parameter was evaluated by a single observer who was blinded to the experimental group and the period after surgery.

Histological evaluation

The second toes of the rabbits were fixed in 4% paraformaldehyde buffered with PBS, sectioned into 4-μm slices, and subjected to hematoxylin-eosin (HE) staining according to standard protocol. To categorize the severity of peritendinous adhesions, we used a histological grading system that evaluates the adhesions by adding quantity points (0, no apparent adhesions; 1, several scattered filaments; 2, a number of filaments; 3, countless filaments) and quality points (0, no apparent adhesions; 1, regular, elongated, fine filamentous; 2, irregular, mixed, shortened, filamentous; 3, dense, not filamentous), as previously reported[25,26].

Mechanical evaluation

To evaluate peritendinous adhesions and tendon repair, the work of flexion and maximal tensile strength, respectively, were measured using a rheometer (CR-500-DX-LII; Sun Scientific, Tokyo). The work of flexion represents the work necessary to overcome the resistant forces from within the tendon sheath. One, 3 and 6 weeks after surgery, the rabbit fourth toes were harvested with the flexor tendon and sheath intact. The proximal end of the FDP tendon was attached to the crosshead nonslip clamp of the rheometer, and the actuator pulled the tendon at 20 mm/min up to 120 degrees flexion. The force and excursion of the clamp were measured directly, and the work of flexion was calculated by the curve integration[27,28]. To measure the maximal tensile strength, the sutured rat Achilles tendon (3 weeks after surgery) and rabbit FDP tendon (1, 3 or 6 weeks after surgery) were harvested, and the proximal and distal ends of the tendon were fixed with nonslip clamps attached to the rheometer. The actuator pulled the tendon at 600 mm/min until the terminal rupture, and maximal tensile strength was recorded as the maximum tension force[27,29].

Cell culture in double chambers

To learn the underlying mechanism, we evaluated the effects of MPC polymer on cell migration and viability using mouse fibroblastic NIH3T3 cells (RIKEN Cell Bank, Tsukuba, Japan) cultured in D-MEM (Wako Pure Chemical Industries, Ltd., Osaka) containing 2% or 10% fetal bovine serum (FBS) in a double chamber dish divided by a porous membrane (Transwell™ culture insert; Corning, NY). The cells were inoculated at a density of 5.0×10^5/well in the upper chamber containing 2% FBS medium with and without the MPC polymer coating at the bottom. After 24 h of the culture, cell migration was determined by the number of cells that moved to the lower chamber containing 10% FBS medium due to the difference in serum

concentration[30]. The lower surface of the division membrane was stained with Giemsa (Sigma)[31]. Cell viability or MPC biocompatibility was evaluated by MTT assay using a cell counting kit-8 (Dojindo, Kumamoto, Japan) on NIH3T3 cells seeded at a density of 5.0×10^5 cells/well in the upper chamber containing 10% FBS medium in the absence and presence of the MPC polymer coating in the lower chamber containing the same concentration of FBS[30].

Statistical analysis

The data were analyzed by Welch's *t* test or Mann-Whitney's *U* test, and P-values less than 0.05 were considered significant.

Source of Funding

This study was supported by Health and Welfare Research Grant for Translational Research (H19-006) from the Japanese Ministry of Health, Labor and Welfare. The sponsor had no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript.

Results

Preparation of MPC hydrogel

We initially sought to determine the concentration of PMBV that forms the MPC hydrogel with optimal viscoelasticity. After mixture of several concentrations (1.25, 2.5, 5.0, and 10.0%) of PMBV with 2.5% PVA in a vial, 2.5 and 5.0% PMBV successfully formed hydrogel properties, while 1.25 and 10.0% PMBV formed liquid and concrete properties, respectively (Figure 1B). To compare the degradation of the MPC hydrogel formed from 2.5 and 5.0% PMBV, we examined the time course of in vitro degradation of the MPC hydrogel in a diffusion chamber which was immersed and gently stirred in PBS, and found comparable degradation rates between the two concentrations (Figure 1C).

We then subcutaneously implanted a diffusion chamber containing MPC hydrogel formed from a mixture of 2.5 or 5.0% PMBV and 2.5% PVA in rats, and looked at changes of the hydrogel properties for 3 weeks after the implantation (Figure 2). Macroscopic findings of the MPC hydrogel extracted 1 and 3 weeks after the implantation confirmed the comparable degradation between the two hydrogels containing 2.5 and 5.0% PMBV (Figure 2A). Measurements of elastic and viscous moduli revealed that the two MPC hydrogels similarly kept higher levels of elastic modulus than viscous modulus, indicating that both maintained the gel properties (Figure 2B). Scanning electron microscopic analyses showed that both MPC hydrogels maintained a honeycomb microstructure with pores of 400-800 nm in diameter, which are assumed to block passage of cells but to allow that of cytokines and growth factors, even 3 weeks after the implantation (Figure 2C).

Effects of a local application of MPC hydrogel formed from 2.5 or 5.0% PMBV in the rat Achilles tendon model

To examine the effects of a local application of MPC hydrogel formed from 2.5 or 5.0% PMBV, we initially used the rat Achilles tendon model. Immediately after cutting and suturing the tendon, two aqueous solutions, PMBV (2.5 or 5.0%) and PVA (2.5%), were locally injected around the sutured tendon to form the hydrogel. After 3 weeks, the peritendinous adhesions and tendon healing were compared with those by the distilled water control application. Although there were severe peritendinous adhesions that prevented the passage of a spatula under the sutured tendon in the control group after 3 weeks, a spatula could go through under the tendon in the MPC hydrogel groups (Figure 3A). The passage was easier in the 5.0% PMBV hydrogel group than in the 2.5% PMBV group. Peritendinous adhesions determined by fibrous adhesion

number were more than 60% reduced by the 5.0% PMBV hydrogel (P=0.031), although not significantly by the 2.5% PMBV hydrogel (P=0.349) (Figure 3B, upper graph). Contrarily, the tendon healing determined by the maximal tensile strength measured with a rheometer was comparable in the three groups (Figure 3B, lower graph). Hence, we decided to use 5.0% PMBV for the hydrogel formation for further studies.

Effects of a local application of MPC hydrogel containing 5.0% PMBV in the rabbit FDP tendon model

To confirm the effects of the MPC hydrogel formed from 5.0% PMBV, we used the rabbit FDP tendon model because the flexor mechanism is analogous to that of the human digit[32]. Immediately after cutting and suturing the tendon at zone II, two aqueous solutions of 5.0% PMBV and 2.5% PVA were locally injected to form the hydrogel in situ. After 1, 3, and 6 weeks, the peritendinous adhesions and tendon healing were compared with those by the distilled water-application. Three weeks after the surgery, there were severe peritendinous adhesions that prevented the passage of a vessel loop under the sutured site in the control group while few or no peritendinous adhesions were observed in the MPC hydrogel group, so that a vessel loop could go through under the tendon even after 6 weeks (Figure 4A). Histological analyses of the interfaces between tendon and subcutaneous tissue (Figure 4B, upper three panels) and between tendon and bone (Figure 4B, lower three panels) showed that peritendinous adhesion tissues appeared at 1 week and increased thereafter in both interfaces of the control group. In the MPC group, however, the formed MPC hydrogel remained in both interfaces up to 3 weeks, and disappeared at 6 weeks. Neither inflammatory reaction nor peritendinous adhesion tissue formation was observed around the MPC hydrogel throughout the observation periods.

Histological adhesion grade confirmed that peritendinous adhesions were 57% and 32% lower in the MPC group than in the control group (P=0.043 and 0.013) at 3 and 6 weeks, respectively (Figure 4C). In the mechanical analyses using the rheometer, peritendinous adhesions determined by the work of flexion were 27% decreased by the MPC hydrogel at 3 weeks (P=0.046) (Figure 4D, upper graph). Interestingly, the maximal tensile strength representing the tendon healing was not impaired but instead was 55% enhanced by the MPC hydrogel at 6 weeks (P=0.048) (Figure 4D, lower graph).

Taken together, the results using the two animal models indicate that the local application of the MPC hydrogel significantly reduced peritendinous adhesions without impairing the tendon healing.

Mechanism underlying the anti-adhesion effect of MPC hydrogel using cell cultures

To learn the underlying mechanism, we examined migration and viability of mouse fibroblastic NIH3T3 cells cultured in a double chamber dish divided by a porous membrane. The cell migration determined by the number of cells that moved from the upper chamber to the lower chamber due to the difference of the serum concentration was decreased to 1/10 by the MPC hydrogel (5.0% PMBV and 2.5% PVA) that covered the bottom of the upper chamber (P=0.015) (Figure 5A). Contrarily, the cell viability determined by MTT assay in the upper chamber was not affected by the MPC hydrogel that covered the bottom of the lower chamber containing the same serum concentration (Figure 5B). These results indicate that the inhibition of the cell migration was not due to cytotoxicity of the MPC hydrogel, but probably due to the microstructure which physically blocked passage of the cells.

Discussion

In the present two animal models, the MPC hydrogel efficiently reduced peritendinous adhesions without impairing healing of the sutured tendon (Figures 3 & 4). Compared to conventional anti-adhesion agents, the MPC hydrogel has several advantages for clinical use. First, the hydrogel properties that tightly cover the sutured tendon were achieved immediately after a local injection and mixture of two aqueous solutions PMBV and PVA, providing an easy procedure in the clinical scene. The degradation rate of the formed hydrogel was controllable by the PMBV concentration, and the optimal concentration of PMBV for the tendon healing was determined to be 5.0% using in vitro and in vivo assay systems (Figures 1-3). The MPC hydrogel formed from 5.0% PMBV was shown to remain over 3 weeks (Figure 4B), which covers critical phases of the tendon healing process[2-4].

A more important mechanism underlying the anti-adhesion effect is the honeycomb microstructure with nanometer-scaled pores (400-800 nm in diameter) of the MPC hydrogel; these are assumed to block passage of extrinsic fibroblastic cells (more than 8-10 μm in diameter) for the peritendinous adhesions but allow passage of cytokines and growth factors for the tendon healing (Figure 2C)[17,33,34]. This may explain the results of the cell culture showing the physical prevention of cell migration by the MPC hydrogel without affecting the cell viability (Figure 5). In fact, several biologic and synthetic materials have been developed as physical barriers to prevent adhesion formation[35]. Although biologic materials like paratenon, periosteum, and tendon sheath transplantation have met with variable success, they still have limitations in donor site morbidity and surgical complexity to the procedure[36]. To overcome these problems, cellophane[37], polyethylene[38], and silicone sheeting[39] have been tested. These materials, however, failed because they prevented nutrient diffusion to the healing tendon

leading to tendon necrosis[38,40,41]. Although the focus has recently turned to diffusible membranes of polytetrafluoroethylene[42], hyaluronic acid membrane[43], polyvinyl alcohol hydrogel (PVA-H)[41], and hydrogel sealant (FocalSeal-L)[27], most of them are cumbersome to use and difficult to prepare.

In addition to its role as a physical barrier, we believe that the strongest and the most characteristic advantage of the MPC hydrogel is its excellent biocompatibility without foreign body reaction, since the MPC polymer is composed of phosphorylcholine mimicking the neutral phospholipids of biomembranes[11]. Although many synthetic materials for adhesion resistance so far have failed because they stimulated inflammatory responses or allowed ingrowth of adhesions around the materials[36-40,42], the bioinert MPC hydrogel will overcome this problem even in clinical settings. In fact, the MPC polymer has already been clinically used on the surface of other medical devices to suppress biological reactions[11-16]. Unexpected enhancement of the maximal tensile strength by the MPC hydrogel at 6 weeks in the rabbit FDP tendon model (Figure 4D, lower graph) might possibly be due to the continuous passage of cytokines and growth factors without being trapped on the surface.

Taken together, MPC hydrogel seems to be a promising material for reduction of peritendinous adhesions without theoretical drawbacks or risk for clinical use. Considering the urgent need for successful healing of severed tendons with smooth digital motion, this nanotechnology would possibly improve the quality of care of patients with tendon injury, especially at the zone II digital flexor tendon. However, since many drugs, materials and biomaterials which were proven to be effective in experimental studies have not entered clinical practice[26,28,32,36-39,41-43], we should note that it remains unknown whether the improvements associated with the MPC hydrogel are clinically relevant. The use of rodents may not be directly

translatable to human clinical medicine, as the physical and anatomical features of their tendons do not match the human anatomy[44]. The results would need to be confirmed in other models that are more similar to humans, such as dogs, chicken, and non-human primates, before considering that this compound has anything more than potential at this point.

Acknowledgments

We thank Akio Terashima, Toshimitsu Hori, Takashi Maeda, Yuji Ogata, Katsuhiko Ase and Reiko Yamaguchi for their excellent technical assistance.

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Figure Legends

Figure 1. In vitro findings of the MPC hydrogel. (A) Chemical structure of poly(MPC-*co*-*n*-butyl methacrylate-*co*-*p*-vinylphenylboronic acid) (PMBV). The PMBV became an aqueous solution when it contained the hydrophilic MPC polymer. (B) Finding of MPC hydrogel formed from a mixture of several concentrations (1.25, 2.5, 5.0, and 10.0%) of PMBV and 2.5% PVA in a vial. Immediately after mixture of the two aqueous solutions of polymers, the vial was held upside down. Arrowheads indicate the mixture. (C) Time course of degradation of the MPC hydrogel formed from a mixture of PMBV (2.5 or 5.0%) and 2.5% PVA in a diffusion chamber that was immersed and gently stirred in PBS. The degradation was determined by percent weight change as compared to time 0 (immediately after mixture of PMBV and PVA solutions). Data are expressed as means (symbols) ± S.E. (error bars) for 6 hydrogels / group.

Figure 2. Time course of changes of the MPC hydrogel (300 μL) formed from a mixture of PMBV (2.5 or 5.0%) and 2.5% PVA in the diffusion chamber before (0) and after (1 and 3 weeks) subcutaneous implantation in rats. (A) Macroscopic findings of the implanted MPC hydrogel. Scale bars, 5 mm. (B) Viscoelasticity of the implanted MPC hydrogel. The elastic and viscous moduli were measured using a rheometer. Higher elastic modulus than viscous modulus indicates gel properties. Data are expressed as means (bars) ± S.E. (error bars) for 3 hydrogels / group. (C) Scanning electron microscopic images of the implanted MPC hydrogel. Scale bars, 30 μm.

Figure 3. Effects of a local application of the MPC hydrogel (50 μL) formed from a

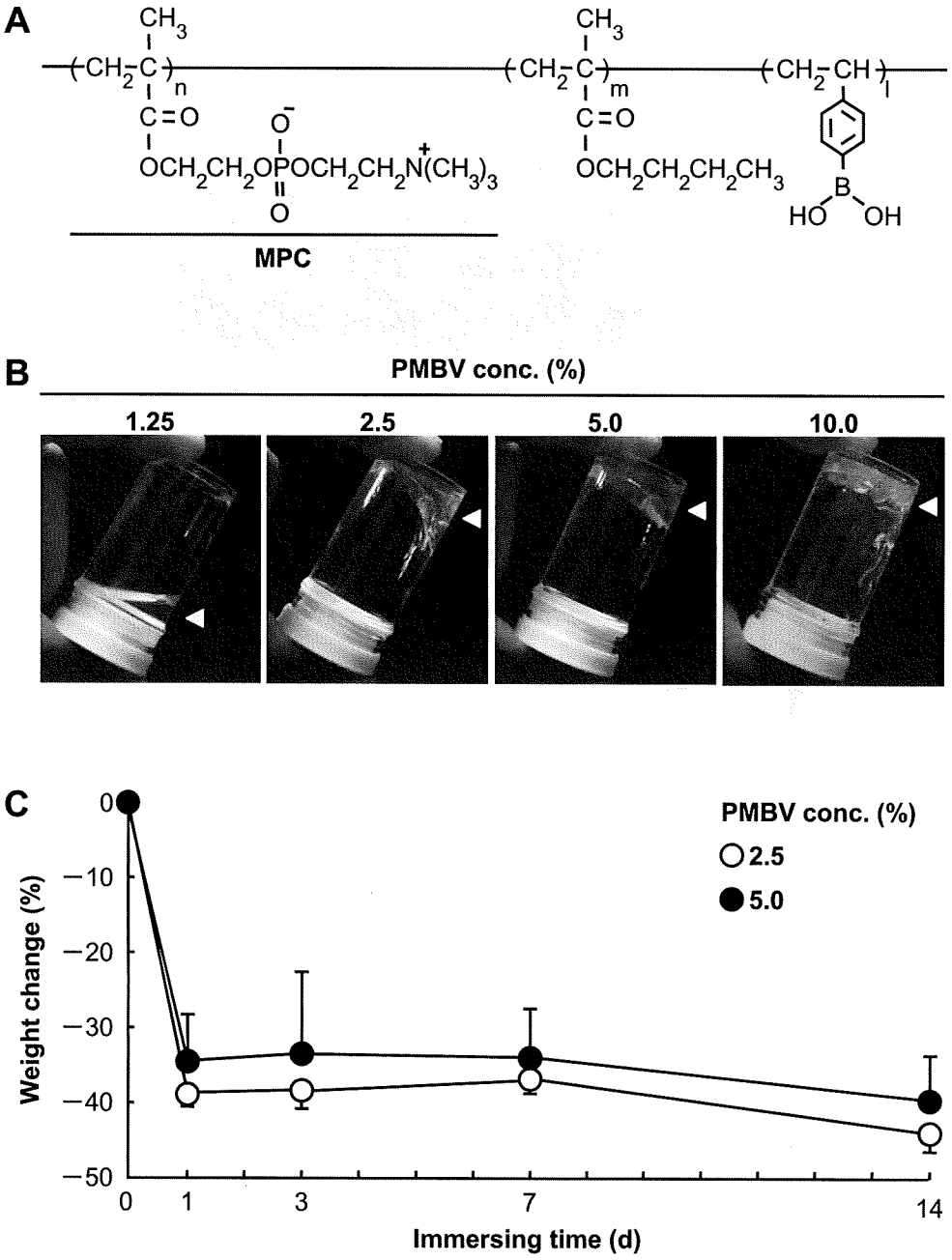
mixture of PMBV (2.5 or 5.0%) and 2.5% PVA on peritendinous adhesions and tendon healing in the rat Achilles tendon model. (A) Passage of a spatula under the sutured tendon 3 weeks after the local application of distilled water (control) or the MPC hydrogel. Scale bars, 1 mm. (B) Fibrous adhesion number and maximal tensile strength, representing peritendinous adhesions and tendon healing, respectively, 3 weeks after the local application of distilled water (control) or the MPC hydrogels. Data are expressed as means (bars) ± S.E. (error bars) for 6 tendons / group. *P<0.05 vs. control.

Figure 4. Effects of a local application of MPC hydrogel (50 μL) formed from a mixture of 5.0% PMBV and 2.5% PVA on peritendinous adhesions and tendon healing in the rabbit FDP tendon model. (A) Passage of a vessel loop under the sutured tendons 1, 3, and 6 weeks after the local application of distilled water (control) or the MPC hydrogel. Scale bars, 1 mm. (B) Histological findings by HE staining of the interfaces between tendon (T) and subcutaneous tissue (SC) (upper three panels) and between tendon (T) and bone (B) (lower three panels) at 1, 3, and 6 weeks. A, peritendinous adhesion tissue; M, MPC hydrogel. Arrowheads indicate the interface without peritendinous adhesions. Scale bars, 50 μm. (C) Histological adhesion grade (quantity + quality points = 0-6) of the sutured tendons at 1, 3, and 6 weeks. Data are expressed as means (bars) ± S.E. (error bars) for 5 tendons / group. *P<0.05 vs. control. (D) Mechanical properties. Work of flexion for evaluation of peritendinous adhesions (upper graph) and maximal tensile strength for the tendon healing (lower graph) at 1, 3, and 6 weeks. Data are expressed as means (bars) ± S.E. (error bars) for 6-10 tendons / group. *P<0.05 vs. control.

Figure 5. Assays for cell migration and viability using fibroblastic NIH3T3 cells cultured

in a double chamber dish divided by a porous membrane. (A) Cell migration assay. The cells were seeded in the upper chamber containing 2% FBS with and without the MPC hydrogel (mixture of 5.0% PMBV and 2.5% PVA) coating at the bottom. After 24 h of the culture, cell migration was determined by the number of cells that moved to the lower chamber containing 10% FBS due to the difference in serum concentration. Middle panel: Giemsa staining of the lower surface of the division membrane. Scale bars, 100 μm. Bottom graph: cell number in the lower chamber. Data are expressed as means (bars) ± S.E. (error bars) for 3 wells / group. *$P<0.05$ vs. control. (B) Cell viability assay. The cells were seeded in the upper chamber containing 10% FBS with and without the MPC hydrogel coating in the lower chamber containing the same concentration of FBS. Middle panel: Microscopic findings of the cells on upper surface of the division membrane after 24h cultivation. Scale bars, 100 μm. Bottom graph: viability of the cells in the upper chamber determined by the absorbance using MTT assay. Data are expressed as means (bars) ± S.E. (error bars) for 3 wells / group. No significant difference between the two groups.

Figure 1


A
MPC
B
PMBV conc. (%)
1.25
2.5
5.0
10.0
C
PMBV conc. (%)
2.5
5.0
Weight change (%)
0
−10
−20
−30
−40
−50
0
1
3
7
14
Immersing time (d)