Blocking Collagen Fibril Formation in Injured Knees Reduces Flexion Contracture in a Rabbit Model

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ABSTRACT: Post-traumatic joint contracture is a frequent orthopaedic complication that limits the movement of injured joints, thereby severely impairing affected patients. Non-surgical and surgical treatments for joint contracture often fail to improve the range of motion. In this study, we tested a hypothesis that limiting the formation of collagen-rich tissue in the capsules of injured joints would reduce the consequences of the fibrotic response and improve joint mobility. We targeted the formation of collagen fibrils, the main component of fibrotic deposits formed within the tissues of injured joints, by employing a relevant rabbit model to test the utility of a custom-engineered antibody. The antibody was delivered directly to the cavities of injured knees in order to block the formation of collagen fibrils produced in response to injury. In comparison to the non-treated control, mechanical tests of the antibody-treated knees demonstrated a significant reduction of flexion contracture. Detailed microscopic and biochemical studies verified that this reduction resulted from the antibody-mediated blocking of the assembly of collagen fibrils. These findings indicate that extracellular processes associated with excessive formation of fibrotic tissue represent a valid target for limiting post-traumatic joint stiffness. © 2016 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 35:1038–1046, 2017.

Keywords: joint contracture; posterior capsule collagen; collagen fibrils; arthrofibrosis; antibody

The fibrotic deposits in stiff joints consist mainly of fibril-forming collagen I and collagen III.[1,2] The formation of these collagen fibrils is needed for proper wound healing in response to joint injury, but excessive fibrillogenesis causes unwanted fibrosis and results in post-traumatic joint contracture.[3] Non-surgical treatments to improve range of motion (ROM), including physical therapy, continuous passive motion, splinting or casting, as well as anti-inflammatory drugs, often fail to improve joint mobility. In cases where conservative management fails, surgical intervention can be attempted to correct joint function. Procedures used to improve ROM include manipulation under anesthesia, open or arthroscopic debridement, capsular release, lysis of adhesions, and tenolysis.

The biologic prevention or treatment of arthrofibrosis may provide alternative therapies to improve ROM. Experimental approaches to limit the formation of fibrotic tissue in injured joints have included blocking the profibrotic activity of tissue growth factor β1 (TGF-β1) with neutralizing antibodies or decorin, inhibiting vascular endothelial growth factor and fibroblast growth factor 2, and applying ketotifen to reduce profibrotic processes in mast cells that invade the injured joint tissues.[4–9]

In this study, we tested the feasibility of limiting post-traumatic joint contracture by reducing the formation of collagen-rich deposits in the capsules of injured knee joints in rabbits. We aimed to directly interfere with the extracellular process of collagen fibril formation by blocking the critical collagen–collagen interaction mediated by the C-terminal telopeptide region of collagen I molecules.[10] Prior studies have shown that this strategy reduces collagen fibril formation in vitro, in ex vivo cell culture systems, and in organotypic tissue constructs.[11–13] Here, employing a rabbit model of post-traumatic joint contracture, we tested the utility of a recombinant antibody that targets the C-terminal telopeptide of the α2 chain of collagen I (α2Ct) to reduce the formation of fibrotic tissue. We hypothesized that, with the intraarticular utilization of the recombinant antibody, we would see improved biomechanical characteristics in the form of decreased severity of flexion contracture compared to control, as well as a beneficial pattern of collagen organization and morphology compared to controls. To determine the effectiveness of the proposed strategy, we conducted mechanical measurements of the flexion contracture that developed in injured rabbit knee joints in the presence or absence of the therapeutic antibodies. We also performed microscopic and biochemical analyses of collagen deposits and examined the organization and morphology of collagen fibrils formed in the posterior capsules of these two groups of rabbits.

MATERIALS AND METHODS

Antibodies

We employed two recombinant antibody variants of IgG type: (i) non-modified anti-α2Ct antibody (ACA) and (ii) PEGylated ACA (P-ACA).[13] The PEGylated variant was generated by binding a 12-mer polyethylene glycol chains

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(PEG; ThermoFisher Sci., Rockford, IL) to random lysine residues present in the ACA. Since binding the PEG molecules increases the mass of the ACA chains, we monitored the outcomes of the PEGylation by gel electrophoresis (Supplementary Material).

Binding Characteristics of the P-ACA

We have described the specificity and the affinity of the ACA-collagen I binding elsewhere.[13] Here, we analyzed the binding characteristics of the P-ACA (Supplementary Material).

Stability of Antibodies

Prior to employing the antibodies for their pump-controlled delivery into the injured knees, we analyzed whether their stability, solubility, and the binding characteristics remained unchanged despite the antibody-filled pumps being kept constantly under the skin of the rabbits (Supplementary Material).

Animals

We employed female New Zealand White rabbits, 8- to 12-months old (Covance, Inc., Princeton, NJ). All animal studies were approved by the TJU Institutional Animal Care and Use Committee. The following attributes make rabbits a relevant model to study post-traumatic joint contracture: (i) the critical α2Ct epitope recognized by the ACA in rabbits is identical with its human counterpart and (ii) the rabbit model employed here adequately represents clinical features associated with post-traumatic joint contractures in human patients.[2,14–16]

Experimental Groups

We created the following experimental groups: (i) 2- and 16-week recovery control groups treated in the injured knees with phosphate buffered saline (PBS) only (PBS-2, PBS-16); (ii) 2- and 16-week recovery groups treated in the injured knees with a 2-mg/ml solution of the ACA (ACA-2, ACA-16); and (iii) 2- and 16-week recovery groups treated in the injured knees with a 2-mg/ml solution of the P-ACA (P-ACA-2, P-ACA-16). In these groups, the PBS and antibodies were delivered continuously for 8 weeks.

Surgical Procedures

We employed an established rabbit model of post-traumatic joint contracture.[2,15–17] In brief, knee injury is caused by intra-articular fracture and mechanical disruption of the posterior capsule (PC). The injured knees are maintained in a flexed position for 8 weeks with the use of Kirschner (K)-wires. The unoperated contralateral leg serves as control. Following 8 weeks of immobilization, the K-wires are removed and the rabbits are allowed free cage activity for 2 or 16 weeks. Specific modification to this model, namely installation of a peristaltic pump for direct antibody delivery into articular cavity, is described in Supplementary Material (Figs. S1 and S2). This modification is based on earlier studies utilizing pumps for direct drug delivery into joints of model rabbits.[6,8,18]

Antibody Delivery

The delivery of the ACA and P-ACA started immediately after surgery and continued for 8 weeks at 1 μl/h. This 8-week time frame is referred to as the treatment period. At the mid-point of this period, that is, 4 weeks after surgery, the pumps' reservoirs were refilled through a subdermal port (Fig. S2).

We randomly selected a group of rabbits to confirm a correct flow path at the end of the treatment period. In those tests we utilized a contrast agent (Hexabrix, donated by Mallinckrodt Inc., St. Louis, MO) and X-ray imaging. Moreover, immunostaining was employed to analyze the presence of the ACA and P-ACA within the PCs collected from rabbits that had to be sacrificed within the initial 8-week treatment period (Table 1).

Joint Angle Measurements

Following euthanasia, flexion contracture was measured using a custom-made instrument (Test Resources, Inc., Shakopee, MN), as described (Fig. S3).[2] Flexion contracture was defined as the difference between the angles recorded at 0.2 Nm torque for the control limb and for the injured limb.[2,17] Accordingly, a larger difference between the maximum extension angles of control and operated limbs indicates a more severe joint contracture (Supplementary Material).

Tissue Collection

Since the PC is the main contributor to the knee contracture in the rabbit model employed here, the PCs were dissected from the limbs, as described (Fig. S4).[1,2] While one part of the PC was processed for histology, another part was processed for assays of collagen content and composition.

Quantitative Microscopy of Collagen Fibrils

Three-micrometer-thick cross-sections and longitudinal sections of the PCs were stained with collagen-specific picrosirius red to visualize collagen fibrils (Polysciences, Inc., Warrington, PA).[2] Polarized-light microscopy of the fibrils made it possible to describe their thickness and the organization.[2,19] As the thickness of fibers increases, their birefringence color changes from green to yellow to orange to red, that is, from shorter to longer wavelengths.[19] Employing a polarizing microscope (Eclipse LV100POL, Nikon Inc., Melville, NY) and the NIS Elements software (Nikon Inc.), the following groups of colors were defined: (i) green, (ii) yellow, and (iii) orange-red.[2,20] Utilizing the NIS Elements software, we collected data from the entire image, and then we calculated the surface areas occupied by pixels corresponding to the defined colors. Considering the sum of all pixels to be 100%, the percentage of each color group in the analyzed images was determined. Subsequently, we calculated changes in the injured PC, within each color category, as the injured-PC/uninjured-PC ratios. A minimum of three histological sections for each PC sample was examined per each analyzed rabbit. Employing the longitudinal sections of the PCs, the organization of collagen fibrils, expressed as theoretical anisotropy score, was evaluated by ImageJ software that included the FibrilTool plug-in, as described.[2,21,22]

Relative Collagen Content

The PC samples were frozen in liquid nitrogen and then pulverized; each sample was then divided for assays of total collagen content and for extraction of fibrillar collagens. Following fat removal, the samples used for total collagen assays were lyophilized and weighed. Finally, a hydroxyproline assay was employed to determine the collagen content per unit of dry mass.[2,23]

Table 1. Surgery Outcomes

	Number of rabbits in specific groups					
Status	PBS-2	ACA-2	P-ACA-2	PBS-16	ACA-16	P-ACA-16
[a]A	13	14	7	6	6	6
B	2	1	0	0	0	0
C	2	0	0	0	0	0
D	0	0	1/3 wks 1/6 wks 1/7 wks	0	1/4 wks	0
E	0	0	1/4 wks	0	0	0
F	2/2 h 1/5 wks	0	0	0	0	0
G	0	0	0	0	1	1
H	0	0	0	0	1/4 days	0
I	0	1/4 wks	0	0	0	1/3 wks

[a]A, a number of rabbits that reached the end of study; all rabbits were used in described assays. B, intraoperative tibia fracture due to drilling during initial surgery. C, intraoperative femur fracture during initial surgery. D, post-operative tibia fracture/weeks after initial surgery; fracture occurred during normal cage activities. E, post-operative tibia fracture/weeks after initial surgery; fracture occurred at K-wire entry during handling of rabbit during pump refill. F, peri- and post-operative failure of a k-wire/time after k-wire installation. G, stopped breathing during preparation for the second surgery. H, autopsy revealed pre-existing congenital heart problems. Significant findings included chronic active myocardial degeneration with fibrosis of older lesions, inflammatory infiltrates associated with degenerated myocytes, and acute injury with swelling and hyalinization of myocytes. Multifocal hepatic centrilobular necrosis and congestion was observed and these lesions can be associated with impaired heart function. Moderate pulmonary edema was also observed which also is suggestive of impaired cardiac function. I, euthanized due to complications associated with self-mutilation/weeks after initial surgery; chewing/biting of toes.

Collagen III:Collagen I Ratio

Collagen was extracted from the PC samples with the use of 0.5 M acetic acid containing pepsin added at 0.1 mg/1 mg of wet tissue. The extraction was done by placing the samples in a shaker for 48 h at 4°C. Following extraction, the samples were centrifuged and then filtered to remove insoluble debris. Finally, the concentration of pepsin-soluble collagen was determined by a hydroxyproline assay. Employing an interrupted electrophoresis in 6% polyacrylamide gels, we separated the monomeric, that is, not cross-linked collagen I chains, from monomeric forms of collagen III chains.[2] Subsequently, protein bands were stained with Coomassie blue and then pixel intensities of protein bands corresponding to the monomeric collagen I and collagen III α-chains were measured by densitometry (EZQuant Ltd., Tel-Aviv, Israel). Then, the relative amounts of collagen III in injured PC and in uninjured PC were expressed as the collagen III:collagen I ratio. Finally, the proportion of the collagen III:collagen I ratios calculated for the injured PC and the uninjured PC was determined for each rabbit.[2]

Collagen Cross-Linking

Using electrophoresis, the relative amount of the cross-linked collagen chains that persisted after limited pepsin extraction in the pool of collagen molecules was analyzed for each PC. In brief, following electrophoretic separation in reducing conditions, the relative content of the cross-linked α-chains forming the β and the γ oligomers was calculated by comparing their amount to the amount of the monomeric α-chains.[2] Based on these measurements the relative amount of cross-linked chains was expressed as the cross-linked chains:non-cross-linked chains ratio. Finally, the proportion of these ratios calculated for injured PC and uninjured PC was determined for each rabbit.

Statistical Analyses

Upon arrival, the rabbits were randomly divided to specific groups. We analyzed each outcome in a context of measurements obtained for the injured side versus the measurement obtained for the healthy side, in effect, using each animal as its own control. Consequently, ratio = 1 indicates no change in injured PC versus uninjured PC, ratio > 1 indicates increase in a measured parameter, and ratio < 1 indicates decrease in the measured parameter. The main comparisons of the means were done between the PBS and the ACA groups and between the PBS and the P-ACA groups. The Student's *t*-test was employed to determine the statistical significance of differences between the control group mean and the experimental group mean (GraphPad Prism v. 5.03, GraphPad Software, Inc., La Jolla, CA).

Considering the number of rabbits needed for each group, we focused on a parameter directly influenced by the ACA (i.e., the amount of collagen fibrils formed de novo). Employing quantitative microscopic techniques, we calculated the change in the amount of new fibrils formed in response to injury as the function of the presence or the absence of a tested agent.[2] Considering a 1.5-fold increase in newly formed fibrils in the PBS-2 weeks group, a 1.14-fold increase in the ACA-2 weeks group, and the standard deviation (SD) of about 0.2, we estimated we will need approximately six animals per group to have 80% power to detect such a difference using a two-sided *t*-test, with an alpha of 0.05 (StatMate 2, GraphPad Software, Inc.). Specific numbers (n) of animals analyzed in each group are indicated in Table 1.

RESULTS

Antibody Variants

Although the presence of PEG molecules on the surface of the P-ACA variant caused some steric hindrance, assays indicated that this antibody variant

retains its binding specificity (Fig. S5). Biosensor-based binding assays demonstrated that the affinity for the P-ACA-collagen I binding compares to that of the ACA (data not shown).[13]

Stability of Antibodies

Assays of antibodies stored at 37°C in an incubator and those recovered from pumps implanted for 8 weeks in the rabbits indicated that long-term incubation changed neither their structural integrity nor their binding specificity nor their binding affinity (not shown). Analysis of the size exclusion chromatography (SEC) elution profiles of the antibody variants did not show formation of antibody aggregates (not shown).

Surgery Outcomes

We processed 70 rabbits divided into experimental and control groups (Table 1). While 52 rabbits (74%, Table 1, group A) reached the experimental end point, 18 rabbits (26%) had to be eliminated from the study (Table 1). The most notable single problem (5.7%) was post-operative fracture of tibia (Table 1, group D). In this group, all tibia fractures occurred at the point of entry of K-wires during normal cage activities 3–7 weeks post-surgery. The majority of the losses of rabbits in categories B (4.3%), C (2.9%), and F (4.3%) were associated with perioperative bone fractures and K-wire failures. Losses in groups E (1.4%), G (2.9%), H (1.4%), and I (2.9%) occurred due to tibia fracture during handling a rabbit to refill a pump, complications due to anesthesia, other non-orthopaedic medical problems, and complications associated with self-mutilation, respectively.

Antibody Delivery

Examinations of the rabbits during the K-wire removal surgery showed neither infection nor inflammation reactions to the 8-week presence of the pumps. All the pumps operated, as programmed, and no tubes failed or became dislodged from the articular cavities of the operated knee joints (Figs. S2 and S4). We demonstrated an unobstructed flow path from the pump to the injured knee at the end of the 8-week treatment period (Fig. 1). Moreover, immunohistological assays done on the PCs of rabbits that had to be sacrificed during the treatment period (Table 1) have shown ACA- and P-ACA-positive staining in antibody-treated knees. In contrast, the antibody-specific staining was not seen in PCs isolated from untreated healthy joints (Fig. S6).

Effects of ACA and P-ACA on Joint Contracture

Compared to the PBS-2 group, the flexion contracture was significantly reduced in the ACA-2 group ($p = 0.05$) and P-ACA-2 group ($p = 0.003$) (Fig. 2 and Table S1). Rabbits treated with the P-ACA showed a trend toward greater reduction of the flexion contracture compared to those treated with the ACA-2 ($p = 0.1$). The flexion contractures for the PBS-16 and ACA-16 groups were similar ($p = 0.7$). In comparison to the PBS-16 or ACA-16 groups, the flexion contracture of the P-ACA-treated rabbits trended toward lower values ($p = 0.2$ and 0.1, respectively).

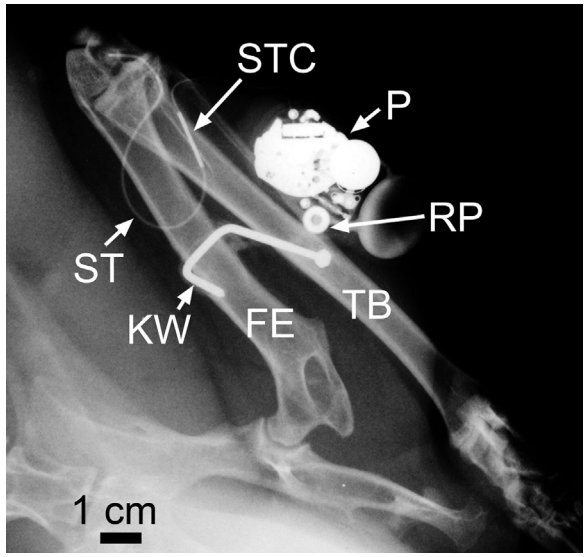

Figure 1. An X-ray depicting an uninterrupted flow path of a contrast agent from a pump to the knee cavity at the end of the 8-week delivery period. STC—stainless steel connector; P—pump; RP—refill port; TB—tibia; FE—femur; KW—K-wire; ST—silicone tube.

Comparing the flexion contractures between corresponding groups of rabbits from the 2- and 16-week recovery groups (i.e., PBS-2 vs. PBS-16, ACA-2 vs. ACA-16, and P-ACA-2 vs. P-ACA-16), we observed a statistically significant reduction of the flexion contracture had occurred in the PBS-16 group ($p = 0.005$).

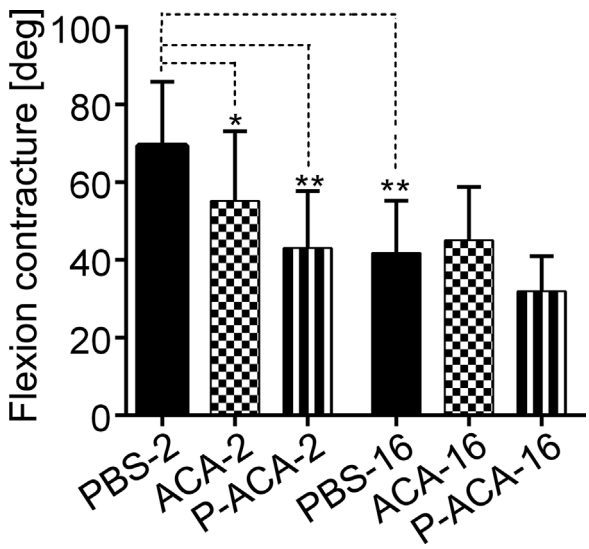

Figure 2. A graphic representation of the measurements of the flexion contracture of the injured knees. The flexion contracture was calculated as the difference between the flexion contracture of the non-injured leg and the flexion contracture of the injured leg. Note that a larger difference between the maximum extension angles of control and operated limbs indicates a more severe joint contracture. Asterisks indicate statistically significant differences between analyzed groups: $^{*}p < 0.05$, $^{**}p < 0.01$.

Although the flexion contractures trended toward lower values in the ACA-16 and P-ACA-16 groups compared to the 2-week recovery counterparts, these changes were not statistically significant ($p = 0.2$ and 0.1, respectively).

Subpopulations of Collagen Fibrils

Consistent with earlier observations, the relative content of green-colored thin fibrils in the PBS-2 group increased in the injured knees (Fig. 3A).[2] Compared to the PBS-2 group, the increase in the relative content of thin fibrils was significantly smaller in the ACA-2 group ($p = 0.01$) and P-ACA-2 groups ($p = 0.04$) (Fig. 3).

A similar trend was observed in the 16-week recovery groups, but the differences were not statistically significant between the PBS-16 and ACA-16 groups ($p = 0.3$) and between the PBS-16 and P-ACA-16 groups ($p = 0.4$) (Fig. 3B). Figure 4 depicts representative images of collagen fibrils seen in cross sections of analyzed in the PCs.

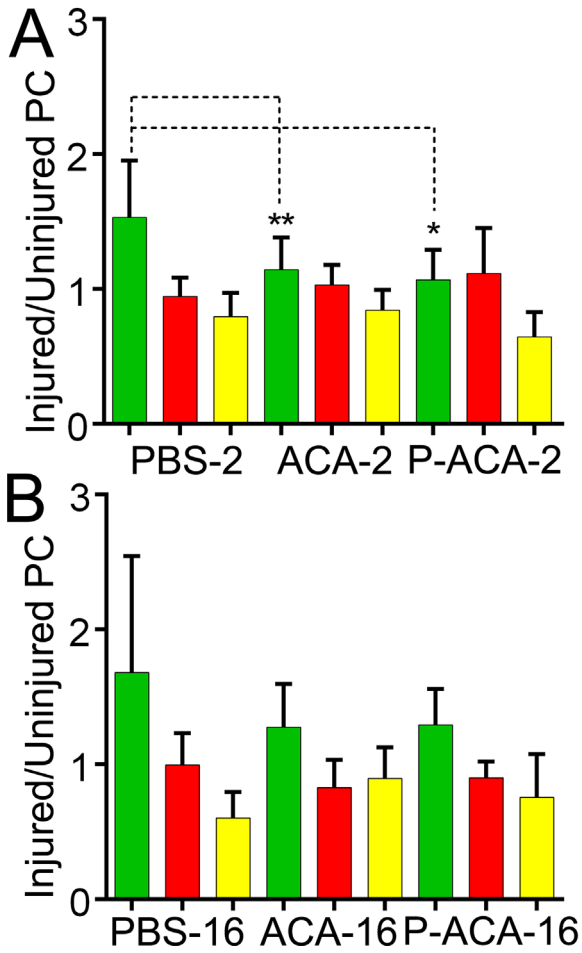

Figure 3. Graphic representations of results from quantitative microscopic assays of changes in subpopulations of collagen fibrils present in the PCs from injured versus uninjured knees: (A) 2-week recovery group; (B) 16-week recovery group. Bars represent the ratios of percent areas occupied by corresponding birefringence colors seen in injured and uninjured PCs. Asterisks indicate statistically significant differences between analyzed groups: $^{*}p < 0.05$, $^{**}p < 0.01$.

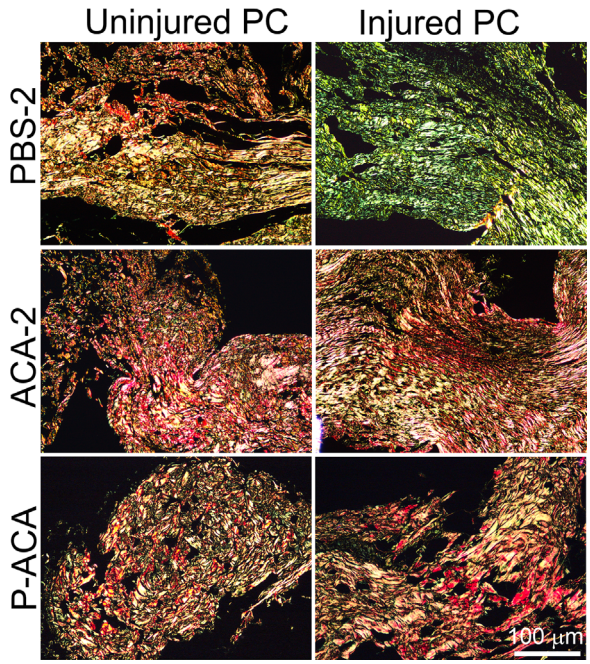

Figure 4. Representative images of collagen fibrils observed in the PCs with the use of a polarizing microscope.

Fibril Organization

While the anisotropy score was relatively high in the uninjured knees of all groups, thus indicating the preferred orientation of fibrils (Fig. 5), it was relatively low in the injured knees of the PBS-2 group. This low anisotropy score suggests non-preferential or more random organization of the fibrillar structures seen in the longitudinal sections of the injured PCs. Antibody-treated injured PCs in the 2-week groups showed similar anisotropy scores to those of their uninjured counterparts. As represented by the PBS-16 group, the anisotropy score values were comparable in the injured and uninjured PCs from the 16-week recovery groups (Fig. 5).

Relative Collagen Content

Assays indicated a relatively high content of collagenous proteins per unit of dry mass of the PCs isolated from the healthy and contracted knees (Fig. 6). When we compared the relative collagen content in uninjured PCs and injured PCs within the PBS-2, ACA-2, and P-ACA-2 groups, the findings indicated a statistically significant decrease of the collagen content in the injured PCs of the P-ACA-treated capsules ($p = 0.04$). There were no statistically significant differences between the collagen content in the PCs from the injured and uninjured knees from the 16-week recovery groups.

Collagen III Content

When we compared the relative contents of collagen III (Fig. 7A and B) in the uninjured and injured PCs, we found a 1.35-fold (±0.4 SD) increase in the PBS-2 group. The relative content of collagen III in injured

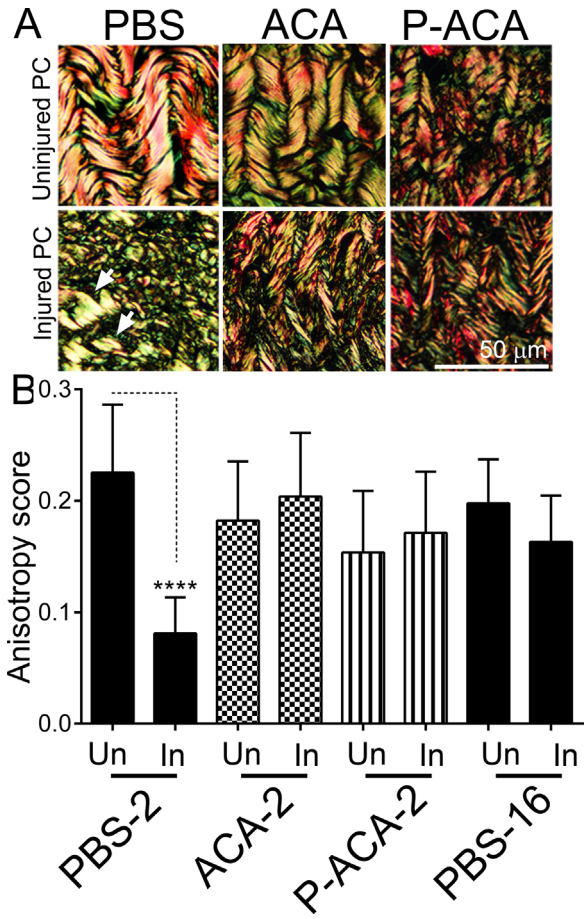

Figure 5. Analysis of the organization of collagen fibrils in the longitudinal sections of the uninjured (Un) and injured (In) PCs. (A) Representative images of collagen fibrils seen in selected PCs. Arrows indicates where the alignment of the fibrils in an injured PC resembles its uninjured counterpart. (B) A graphic representation of measurements of the anisotropy scores. Asterisks indicate statistically significant differences between analyzed groups: $^{****}p < 0.0001$.

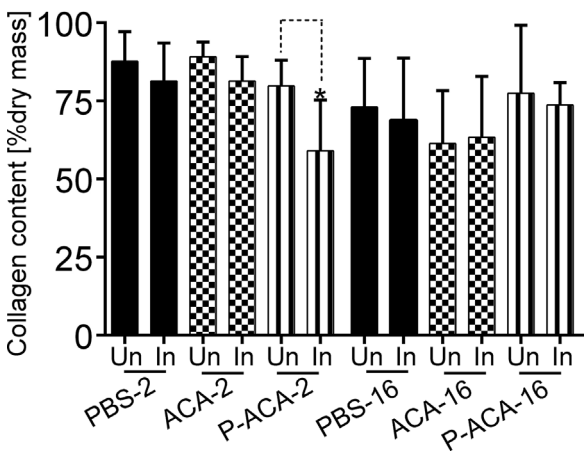

Figure 6. A graphic representation of the collagen content in the uninjured (Un) and injured (In) PCs. Asterisk indicates statistically significant differences between analyzed groups: $^{*}p < 0.05$.

PCs increased 1.06-fold (±0.2 SD) in the ACA-2 group and 1.10-fold (±0.3 SD) in the P-ACA-2 group. The relative content of collagen III in the uninjured and injured PCs indicated a 1.12-fold (±0.3 SD) increase in the PBS-16 group. The relative content of collagen III in injured PCs changed 0.91-fold (±0.2 SD) in the ACA-16 and 0.96-fold (±0.3 SD) in the P-ACA-16 groups (Fig. 7B).

Relative Amount of Cross-Linked Collagen Chains

When we analyzed the relative content of the cross-linked collagen chains in the uninjured PCs and in injured PCs, we discovered no changes across all analyzed groups (Fig. 7C).

DISCUSSION

Based on our earlier studies demonstrating that ACA inhibits collagen fibril formation in various experimental models of collagen fibril formation, we analyzed the feasibility of applying this antibody after knee injury to reduce post-traumatic joint contracture.[11–13] Mechanical tests carried out at the end of recovery periods demonstrated that the flexion contractures in the ACA-2 and P-ACA-2 groups were significantly reduced compared to the PBS-2 control. Although the flexion contractures decreased further in the ACA-16 and P-ACA-16 rabbits compared to the corresponding 2-week recovery groups, this decrease was not statistically significant. In contrast, the decrease in the flexion contracture in the PBS-16 group compared to the PBS-2 group was statistically significant. A spontaneous reduction of the flexion contracture during the 16-week recovery period in the non-treated PBS-16 group is consistent with results presented by Hildebrand et al.[24] The authors reported that, due to constant tissue remodeling and movement of joints, the flexion contracture of the rabbits' knees had decreased 10% at 8 weeks after K-wire removal. Then, 8 week later, that is, 16 weeks after removing K-wires, the flexion contracture had decreased to about 50% of value measured soon after K-wire removal. Measurements of the flexion contractures 16 weeks later, that is, 32 weeks after removing K-wires, did not show any additional reduction, thus indicating a plateau of spontaneous recovery.

Just 2 weeks following K-wire removal, we measured a 20% difference in the flexion contracture in the ACA-treated group and a 40% difference in the P-ACA-treated group compared to the control group. We propose that this reduction most likely resulted from limiting the development of full fibrotic change rather than accelerating spontaneous recovery.

Since earlier rabbit-based studies demonstrated that applying non-specific IgGs to the cavities of injured knees does not change the flexion contracture of the injured joints, we propose that the changes observed in the ACA-2 and P-ACA-2 groups are antibody-specific.[4] We analyzed the PCs to learn what caused the reduced flexion contracture in the

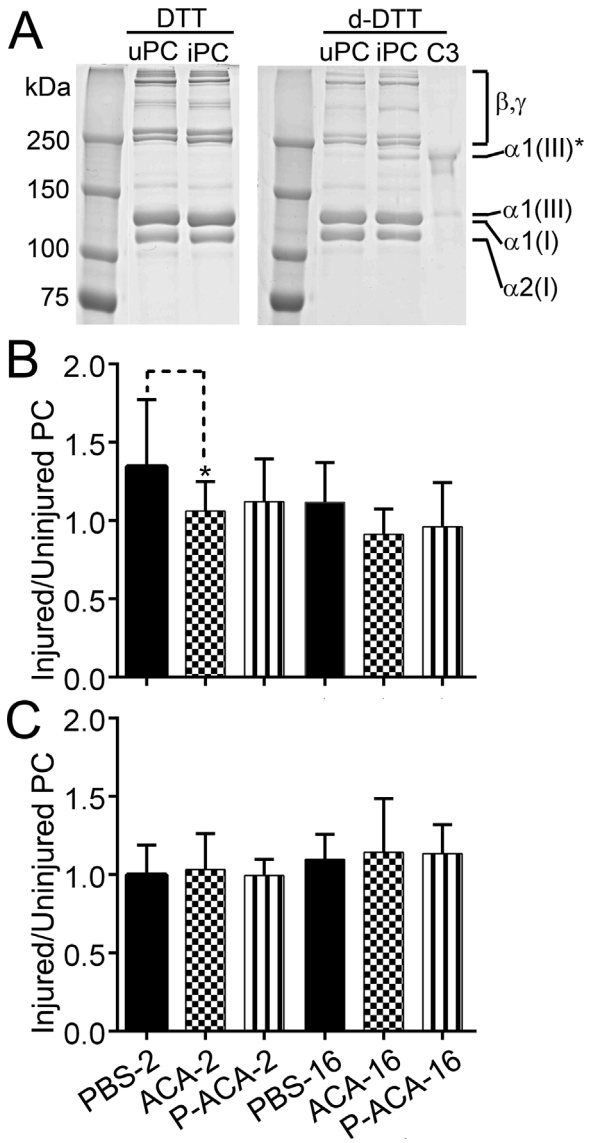

Figure 7. A graphic representation of the results from electrophoretic assays of collagens extracted from the PCs. (A) Patterns of migration of collagen I and collagen III chains separated in standard reducing conditions (DTT) and in delayed-reduction conditions (d-DTT) of electrophoresis. (B) A graphic representation of the proportions of the collagen III:collagen I ratios calculated for the injured and uninjured PCs ($^{*}p < 0.05$). (C) A graphic depiction of the results of measurements of the proportions of the βγ:α ratios; no statistically significant difference was observed. uPC, iPC—uninjured and injured PC; C3—collagen III marker; α1(I), α2(I), α1(III)—specific chains of collagen I and collagen III, respectively; α1(III)*—collagen III chains separated with the use of delayed-reduction conditions; β, γ—β and the γ oligomers consisting of cross-linked collagen α chains.

ACA-treated or P-ACA-treated groups. Since collagen fibril formation is the main target of the ACA and P-ACA, we first studied the fibrillar architecture of the treated PCs. In earlier studies, we had determined that an increase in green-birefringence thin collagen fibrils reflects an active process of collagen fibrillogenesis in injured PCs in response to injury.[2] Based on the reduced amount of green-birefringence fibrils in the injured PCs, we postulate that the ACA and P-ACA inhibited the collagen fibril formation process.

The anisotropy scores in the injured and uninjured PCs from the antibody-treated groups also indicate that the ACA and P-ACA may have reduced the formation of new fibrils. We propose that the relatively high anisotropy scores measured in the injured PCs reflect the organization of mature fibrils that existed before joint injury rather that improved organization of new fibrils formed de novo in response to joint trauma. In the injured PCs from the PBS-2 group, however, the general pattern of organization of the original fibrils is markedly disrupted by newly formed fibrillar assemblies.

To further elucidate the mechanisms that reduced flexion contracture in the ACA-treated and the P-ACA-treated groups, we analyzed the relative collagen content in the PCs. Although we were able to determine the relative content of collagen per unit of dry mass of the PCs, it is impossible to determine the total mass of a capsule or the total amount of collagen. Consequently, similar values for the relative collagen content per unit mass in the injured and uninjured PCs do not necessary indicate that the overall total masses of these capsules are identical. Thus, our collagen assays are not able to predict changes in the mass of fibrotic PCs as the function of the presence or the absence of the antibodies. These assays, however, provide useful information for antibody-dependent changes in collagen content relative to other macromolecules produced in the PCs.

We propose that the approximately 20% reduction of the collagen content in the injured PCs from the P-ACA-2 group resulted from substantially blocked incorporation of newly produced collagen molecules into fibrils and their degradation. Studies by Leikina et al. support this notion.[25] Specifically, the authors demonstrated that the triple-helical structure of individual collagen molecules, that is, not incorporated into a fibril, is quite unstable at body temperature, allowing their degradation by proteolytic enzymes. In contrast, the stability of collagen molecules increases upon their incorporation into fibrils, thereby rendering them more resistant to enzymatic degradation.

We have also analyzed potential effects of applied treatments on the collagen III:collagen I ratio in the injured PCs and on the relative amount of cross-links present in the pepsin-extracted fraction of collagenous proteins. Consistent with our earlier studies, the relative amount of collagen III increased in the injured PCs from the PBS-2 group[2]. Although the collagen III: collagen I ratio trended lower in the antibody-treated groups, a statistically significant difference was observed only between the PBS-2 and ACA-2 groups.

We determined that the relative amounts of the cross-linked fractions of pepsin-extracted collagen in the injured and healthy capsules were similar in all analyzed groups. Since we employed the pepsin-soluble fraction in which a portion of cross-linked chains is converted to monomers, we cannot exclude the possibility of changes in the quality and quantity of cross-links present in the initial collagen pool.

Considering the similar quantities of the cross-linked collagen chains present in the pepsin-extracted collagen pool, we postulate that the aberrant architectural arrangement of the collagen fibrils, formed de novo in response to injury, mostly likely contributed to joint stiffness. In a study of muscle fibrosis that supports this postulation, Chapman et al. suggested that a change in collagen fibril orientation, rather than increased cross-linking, was a decisive factor in developing muscle stiffness.[26] Similarly, studies have also indicated that aberrations in the microarchitecture of collagen fibrils contribute to the mechanism of joint contracture in a rabbit-based model.[27]

Our results showed that the P-ACA causes stronger effects compared to the ACA. We speculate that these stronger effects were likely due to greater stability of the PEG-modified antibody and perhaps to its prolonged residence in the injury sites. These qualities of PEG-modified molecules are well documented in studies where PEGylated peptides are utilized as therapeutic compounds.[28,29] Still, we cannot exclude the possibility that the stronger inhibitory effects were, in part, a consequence of a greater steric hindrance imposed by the bulkier architecture of the P-AFA which resulted from the presence of the PEG molecules on the surface of the AFA.

As rapid recovery of joint motion improves the quality of life of affected patients including reduction of chronic pain, early return to work, less need for physiotherapy, less need for surgery, we suggest that collagen fibril formation could be considered a valid target to reduce post-traumatic joint contracture. Unlike anti-fibrotic approaches that target broad intracellular processes associated with inflammation and cell proliferation, the anti-fibrotic approach tested here targets a well-defined extracellular process, thereby potentially reducing the chance for unwanted side effects. In this sense, our experimental approach is similar to targeting extracellular lysyl oxidase, or $\alpha v\beta 6$ integrin that activates pro-fibrotic TGF-$\beta 1$.[30,31]

Since we still observed flexion contracture despite the antibody treatment, our model approach was not able to fully prevent development of joint stiffness. We propose the following key reasons to explain this limitation: (i) antibodies do not reach fully effective concentration; (ii) antibodies are not able to access all sites of fibrosis; and (iii) antibodies do not target the formation of non-collagenous elements of fibrotic tissue.

In view of these limitations, we propose that future efforts to target extracellular processes of fibrotic tissue formation should include optimization of antibody concentration, employing more effective antibody delivery systems, and designing small molecular mass inhibitors able to penetrate fibrotic tissues more effectively than antibodies.

AUTHORS' CONTRIBUTIONS

A. Steplewski and J. Fertala: Substantial contribution to the acquisition, analysis, and interpretation of data. Contributed to drafting the manuscript. A. Fertala, P. Beredjiklian, and J. Abboud, J. Barlow: Substantial contribution to research design and execution of key experiments. M. Wang, S. Namdari, M. Rivlin, and W. Arnold: Substantial contribution to the acquisition and interpretation of data form an animal model. J. Kostas and C. Hou: Substantial contribution to the acquisition and interpretation of data. All authors have read and approved the final submitted manuscript.

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