The objective of the study was to determine whether strenuous running exercise produces a significant amount of fibrosis in the infrapatellar fat pad (IFP) of the knee and whether intraarticular injections of hyaluronan (HA) protect fibrosis in the IFP in a rat running model. Strenuous running exercise was performed on a rodent treadmill with 5% inclination using Wistar rats. After the exercise, hyaluronan was injected into the right knee joint once per week in the HA injection group. In the treatment control group of the left knee, no injection was done. Also, the running exercise group and the sedentary control group received no HA injection. The IFP was analyzed histologically and immunohistochemically after 1 week (5 km), 2 weeks (10 km), 3 weeks (15 km) and 6 weeks (30 km). The inflammatory reaction in the IFP was not prominent at each running stage. A slightly, but significantly increased amount of inflammatory cell infiltration appeared during strenuous running. The amount of collagen fibers increased significantly at 30 km stage. Histological scores showed less fibrosis of the IFP in the HA injection group than in the control group at 30 km run. Strenuous running exercise may cause fibrosis in the IFP of runners. Intraarticular HA injection will inhibit arthrofibrosis of painful runners.

Key words: infrapatellar fat pad, strenuous running, arthrofibrosis, hyaluronan injection

Introduction

Running is one of the most popular sporting activities. Overuse injuries are frequent in the knee joints of runners. Among the various types of disorders of the knee, anterior knee pain occurs most frequently, namely runner’s knee. Recently, magnetic resonance imaging has become a primarily and important instrument for the clinical diagnosis of runner’s knee based on the meticulous changes of soft tissue.

A relatively small amount of information is available regarding the long-term effects of running on the cartilage in animal models. Long-term running load has been reported to cause early and mild or moderate osteoarthritis in animal studies. However, to the best of our knowledge, the effect of long-term load running exercise focusing on the change of synovial tissue in an animal model has not been analyzed yet. Moreover, preventive treatment for runner’s knee has not been reported either. The analysis of the infrapatellar fat pad (IFP), a part of the synovial capsule of the knee, is thought to be important for producing effective treatment methods for runner’s knee with pain as its chief complaint.

Arthrofibrosis is thought to result from abnormal fibrous tissue hyperplasia and usually follows injury or...
surgery to the joint. Trauma initiates the clotting cascade and is followed by migration of inflammatory cells, then fibrosis occurs in the injured tissue. The ensuing surge in collagen synthesis is regulated by an incompletely understood interaction of growth factors, including platelet-derived growth factor, fibroblast growth factor, insulin-like growth factor-1, and transforming growth factor-beta, and so forth. An abnormal expression and concentration of these proteins may be dependent upon the degree of fibrous tissue formation following joint trauma or surgery.

The objective of this study has two purposes. One was to investigate the influence of extremely excessive running on the development of fibrous change of the IFP using a rat running model both histologically and immunohistochemically. The other was to investigate the effect of intraarticular hyaluronan (HA) injection on the change of the IFP due to strenuous running exercise.

Materials and methods

1. Animals and strenuous running exercise

All experiments were conducted in accordance with the institutional guidelines for the care and use of experimental animals of Tokyo Medical and Dental University. Male Wistar rats (16–18 weeks of age, weighing 350–400g) were used for the whole study. They were brought in from Sankyo Labo Service, Tokyo, Japan, and served as subjects in the study. Rats were housed in an environmentally controlled animal facility on a 12:12 light/dark cycle with food and water available ad libitum in the laboratory animal section of Orthopaedic Surgery, Tokyo Medical and Dental University.

Rats were divided into three groups as follows: running exercise group (5 km run, n = 3; 10 km run, n = 3; 15 km run, n = 3; 30 km run, n = 3), sedentary control group (0 km run, n = 5). In the HA injection group (n = 5), a rat received an intraarticular HA injection per week in the right knee joint after strenuous running exercise and no injection was done into the left knee as a control for HA treatment. The running distance was 30 km for six weeks in the HA injection group.

Running exercise was performed on a rodent treadmill (MK-680R5, ME Service., Tokyo, Japan) with 5% inclination of the running lane all the time following previously reported running protocol in rats. The MK-680R5 has been designed to compulsively make animals exercise by electrical shock delivered to the animals without failure by adoption of a shock generator scrambler. The rats were acclimated to the treadmill by gradually increasing running speed and time. Running distance was up to 30 km for six weeks (Fig. 1).

In the HA injection group, an intraarticular injection of 1 mg hyaluronan, total amount of 100 µl (MW = 8×10⁵ Daltons; Seikagaku Corp., Tokyo, Japan) was given initially at 5 days to the right knee, and followed every 7 days under anesthesia by an intra-peritoneal injection of 10 mg sodium pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan). The effect of HA injection was evaluated at 30 km with 5-time injection.

2. Histological examination

The rats were sacrificed with an overdose of sodium pentobarbital at each running stage (total: n = 17) and they were sacrificed at 30 km in the HA injection group (n = 5). The whole knee joint was fixed in 4% paraformaldehyde at pH 7.4 for 3 days, and decalcified in 20% EDTA solution for 21 days at 4°C, then embedded in paraffin wax. The whole knee joint was sectioned sagittaly with a section width of 5 µm and stained with Hematoxylin-eosin (H&E) and Masson trichrome.

3. Immunohistochemical analysis

Sections were deparaffinized, washed in 0.1 mol/l phosphate-buffered saline (PBS), and pretreated with 0.4 mg/ml proteinase (DAKO, Carpinteria, CA, USA) in Tris-HCl buffer for 15 minutes at room temperature for optimal antigen retrieval. Endogenous peroxidases were quenched using 0.3% hydrogen peroxide in methanol for 20 minutes at room temperature. The sections were rinsed once in PBS for 5 minutes and briefly blocked with 10% normal horse serum and 10% normal goat serum (Vector Laboratories, CA, USA) in Tris-HCl buffer for 30 minutes at room temperature. Sections were incubated with primary antibodies. After washing, the sections were incubated for 30 minutes at room temperature with a secondary antibody (DAKO) followed by chemiluminescence (ECL Western Blotting Detection Reagents; Amersham, Buckinghamshire, UK).

Fig. 1. The study design of rat strenuous running exercise with hyaluronan injection.

The rats were divided into three groups, the sedentary control group, the running exercise groups and the hyaluronan (HA) injection group. For the latter group, a HA injection of 100 µl was performed to the right knee joint once a week (every arrow). Running distance was up to 30 km during a six week period based on the running protocol. The effect of HA injection was evaluated only at 30 km run.
Burlingame, CA, USA) to avoid non-specific binding of the antibody. The sections were then incubated in mouse monoclonal anti-alpha-SMA antibody (1:200 dilution with PBS containing 1% BSA; Dako, Carpinteria, CA, USA) or ED1 mouse-anti-rat monoclonal antibody (1:100 dilution with PBS containing 1% BSA; Funakoshi Co., Ltd., Japan) or rabbit anti-calctonin gene related peptide (CGRP) polyclonal antibody (1:4000 dilution with PBS containing 1% BSA; Chemicon International a Serologicals Company, USA) at room temperature for 1 hour or 2 hours. After rinsing in PBS, the tissues were incubated with biotinylated horse anti-mouse IgG or goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature. The slides were again immersed in PBS and incubated for another 30 minutes with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, USA). Finally, the sections were shortly counterstained with methylene-green or hematoxylin, dehydrated and mounted in a xylol-soluble mount (Vitro-Clud, R. Langenbrinck, Emmendingen, Germany).

4. Semi-quantitative analysis of collagen fibers, ED1, α-SMA and CGRP

Histological sections were scanned into a computer using an Olympus IX71 microscope (Olympus, Tokyo, Japan) at a resolution of 10,000 (Fig. 2) or 20,000 (Fig. 3,4,5) pixels per millimeter. Each section was viewed using Adobe Photoshop 7.0 software in threshold configuration. The area of the whole IFP, the blue stained area of collagen fibers, ED1 for monocyte/macrophage, α-SMA positive cell number, CGRP positive nerve fiber number and α-SMA positive vessel number were measured and counted by Scion Image analysis. The average value of the percent area of collagen fibers stained with Masson trichrome to the whole IFP area was calculated. The number of ED1 positive cells, α-SMA positive cells, α-SMA positive vessels and CGRP positive nerve fibers was counted and expressed as a number per mm².

5. Statistical Analysis

The StatView 5.0 program (SAS Institute, Cary, NC) was employed for statistical analyses for the study. The Kruskal-Wallis test was used for the whole running groups to analyze each set of semiquantitative data evaluated by ED1, α-SMA and CGRP immunohistochemical staining. The Mann-Whitney U-test was used to analyze semiquantitative data of the IFP fibrous area of the control group between 0 km and 30 km evaluated by Masson trichrome stain, ED1, α-SMA and CGRP immunohistochemical staining. The Wilcoxon signed rank test was performed between both knees at 30 km for HA injection group. Statistical significance was considered when the p value was less than 0.05.

Results

1. Fibrosis of the infrapatellar fat-pad (IFP) after strenuous running

The severity of fibrous change of the IFP in the rats was clearly dependent upon the running distance (Fig. 2 A,B). In the IFP of the no running control, no fibrous change of the IFP was detected. After strenuous running exercise of 5 km, 10 km, 15 km and 30 km, Hematoxylin-eosin and Masson trichrome stains showed that the area of fibrous change of the IFP increased each time depending on increased running distance. The fibrous area at 30 km running stage without HA injection was statistically significantly greater than that of no running control (Fig. 2 B,C). The percentage of the fibrous area of the IFP averaged 8.6±0.6% (mean±standard deviation) in the 0 km control, and 68.7±4.7% in the 30 km group.

2. Evaluation of inflammatory reaction in the IFP

ED1 immunohistochemical staining showed that ED1 positive cells per mm² of the IFP at 0 km, 5 km, 10 km, 15 km and 30 km run was statistically significantly different (Fig. 3 A,C) (P < 0.05). The average ED1 positive cell number peaked at 10 km running stage.

3. Immunohistochemical analysis for angiogenesis, myofibroblast infiltration and CGRP positive nerve

Angiogenesis and number of myofibroblasts increased in the IFP which was indicated by α-smooth muscle actin (α-SMA) immunohistochemical staining (Fig. 4A). Statistical analysis revealed that the vessel number at 0 km, 5 km, 10 km, 15 km and 30 km run was statistically significantly different (Fig. 3 A,C) (P < 0.05). The average ED1 positive cell number peaked at 10 km running stage.

Also, cell number with positive α-SMA at 0 km, 5 km, 10 km, 15 km and 30 km run was statistically significantly different (Fig. 4C). The average α-SMA positive cell number was greatest at 30 km run. CGRP immunohistochemical staining revealed that the CGRP positive nerve fibers mostly surrounded blood vessels in the IFP (Fig. 5 A,B). Also, CGRP positive nerve fibers at 0 km, 5 km, 10 km, 15 km and 30 km (n
Fig. 2. Fibrosis of the infrapatellar fat pad (IFP) at each running distance and the effect of HA injection on the fibrosis of the IFP.
(A) Sagittal section of the anterior part of the knee joint. The whole knee joint sections were prepared at 5 μm with Hematoxylin-eosin staining. The change of the IFP can be seen at each running distance.
(B) Histological assessment of collagen fibers in the IFP after strenuous running of 30 km with or without HA injection was done using Masson trichrome staining.
(C) Scion image semiquantitative analysis of Masson trichrome stained area was expressed as a percentage of the fibrous area to the whole IFP area (0 km run control and right (injected) and left (no injection) joints of HA injection group). The fibrous area of the IFP at 30 km run was significantly larger than that at 0 km run control (n = 5). **: Mann-Whitney U-test showed a significant difference between the two groups (P < 0.01). The fibrous area of the IFP in the HA injection group was significantly smaller than that in the no injection control at 30 km run (n = 5). *: Wilcoxon signed rank test showed a significant difference between the two groups (P < 0.05). Scale bar = 300 μm.
Fig. 3. ED1 immunohistochemical staining

(A) The whole knee joint sections were prepared with ED1 immunohistochemical staining. Part of the IFP was shown at each running distance. Number of ED1 positive (+) cells was expressed in both synovial lining and sublining layers. ED1 (+) cells were indicated by arrows.

(B) Histological assessment of the ED1 (+) cells in the IFP after strenuous running of 30 km with or without HA injection was done using immunohistochemical staining. ED1 (+) cells were indicated by arrows.

(C) ED1 immunohistochemical staining showed that ED1 (+) cells per mm$^2$ of the IFP at 0 km, 5 km, 10 km, 15 km and 30 km run was statistically significantly different (n = 3 at each stage) (Fig. 3 A,C) (Kruskal-Wallis test; P < 0.05). The average ED1 (+) cell number peaked at 10 km running stage.

(D) Semiquantitative analysis of ED1 (+) cell number in the IFP at 30 km run was significantly larger than that at 0 km run (n = 5). (Mann-Whitney U-test; **: P < 0.01). The ED1 (+) cell number in the IFP of the HA injection group was significantly smaller than that in the no injection control at 30 km run (n = 5). (Wilcoxon signed rank test; *: P < 0.05). Scale bar = 50 μm.
Fig. 4. α-smooth muscle actin (α-SMA) immunohistochemical staining
(A) The whole knee joint sections were prepared with α-SMA immunohistochemical staining. Part of the IFP is shown at each running distance. Vessels were indicated by black arrows and α-SMA (+) cells with white arrows. Scale bar = 50 μm.
(B) Semiquantitative analysis expressed vessel number per mm² shown as α-SMA positive (+) lumen by Scion Image. The vessel number in the IFP at 0 km, 5 km, 10 km, 15 km and 30 km run was significantly different (n = 3). (Kruskal-Wallis test; P < 0.05). The vessel number was greatest at 30 km run.
(C) Also, semiquantitative analysis of α-SMA (+) cell number per mm² in the IFP at 0 km, 5 km, 10 km, 15 km and 30 km run was significantly different (n = 3). (Kruskal-Wallis test; P < 0.05). α-SMA (+) cells were indicated by white arrow heads. The α-SMA positive cell number was greatest at 30 km run.
(D) Histological assessment of the α-SMA (+) cells in the IFP after strenuous running of 30 km with or without HA injection was done using Immunohistochemical staining.
(E) The vessel number shown as α-SMA (+) lumen in the IFP at 30 km run was significantly larger than that at 0 km run control (n = 5). (Mann-Whitney U-test; **: P < 0.01). The vessel number shown as α-SMA (+) lumen in the IFP of the HA injection group was significantly smaller than that in the no injection control at 30 km run (n = 5). (Wilcoxon signed rank test; *: P < 0.05).
(F) The α-SMA (+) cell number in the IFP at 30 km run was significantly larger than that at 0 km run (n = 5). (Mann-Whitney U-test; **: P < 0.01). The α-SMA (+) cell number in the IFP of the HA injection group was significantly smaller than that in the no injection control at 30 km run (n = 5). (Wilcoxon signed rank test; *: P < 0.05).
HYALURONAN INHIBITS FIBROUS CHANGE OF IFP IN A RUNNER MODEL

Fig. 4.

Legend:
- D: Time points: 0 and 30 km.
- Hyaluronan: Control (-) and treated (+).
- Cell number: Alpha-SMA (+) cell number/mm².
- Vessel number: Alpha-SMA (+) vessel number/mm².

Significance:
- *: p < 0.05
- **: p < 0.01
Fig. 5. CGRP immunohistochemical staining
(A) The whole knee joint sections prepared with CGRP immunohistochemical staining were shown at each running stage. CGRP (+) nerve fibers were indicated by arrows.
(B) The part of the IFP was shown at 0 km control, 30 km run with or without HA injection.
(C) Semiquantitative analysis of CGRP (+) nerve fibers was expressed as number per mm\(^2\) by Scion Image. The number of CGRP (+) fibers in the IFP at 0 km, 5 km, 10 km, 15 km and 30 km run (n = 3) was significantly different (Kruskal-Wallis test; \(P < 0.01\)).
(D) The CGRP (+) nerve fibers number in the IFP at 30 km run was significantly larger than that at 0 km run (n = 5). (Mann-Whitney U-test; \(\ast\): \(P < 0.05\)). The CGRP (+) nerve fibers number in the IFP of the HA injection group was significantly smaller than that of no injection control at 30 km run (n = 5). (Wilcoxon signed rank test; \(\ast\): \(P < 0.05\)). Scale bar = 50 \(\mu\)m.
Effect of intra-articular hyaluronan (HA) injection on fibrosis of the IFP

To investigate the effect of intraarticular HA injection on fibrosis of the IFP, a histological examination was done using Masson trichrome stain. The injection effect was evaluated only at the 30 km running stage. The hyaluronan was injected into the right knee joint of rats. The blue stained fibrous area by Masson trichrome of the IFP was 68.7±4.7% (mean±standard deviation) of the whole IFP area of no injection control (left knee) and 33.7±3.6% of the whole IFP area in the HA injected joints (right knee) (Fig. 2 B,C). The difference of the fibrous area of the IFP between the control left knee and the HA injected right knee was statistically significant.

The number of ED1 positive cells at 30 km run without HA injection (10.6±4.9 per mm² of the IFP) increased statistically significantly to be compared with that at 0 km control (4.7±1.8 per mm² of the IFP) (Fig. 3 B,D). HA injection significantly decreased the number of ED1 positive cells at the 30 km run (5.5±2.6 per mm² of the IFP) than no HA injection control.

The α-SMA positive vessel number at 30 km run (102.7±15.1 per mm² of the IFP) with no HA injection was statistically significantly greater than that of no injection control at 0 km run (22.7±15.1 per mm² of the IFP) (Fig. 4 D,E). HA injection significantly decreased the number of α-SMA positive vessels (29.5±4.0 per mm² of the IFP) at the 30 km run to be compared with that of no HA injection control.

The α-SMA positive cell number (441.4±100.4 per mm² of the IFP) at 30 km in no injection control increased statistically significantly to be compared with that of 0 km run control (91.8±12.1 per mm² of the IFP) (Fig. 4 D,F). HA injection significantly decreased the number of α-SMA positive cells (145.3±29.4 per mm² of the IFP) to be compared with no HA injection control at the 30 km run.

The number of CGRP positive nerve fibers increased statistically significantly at 30 km run without HA injection (15.5±2.5 per mm² of the IFP) to be compared with that of 0 km run control (2.5±1.1 per mm² of the IFP) (Fig. 5 B,D). HA injection significantly decreased the number of CGRP positive nerve fibers at the 30 km run (7.1±1.5 per mm² of the IFP) to be compared with that of no HA injection control.

Discussion

In the present study, we used a rodent treadmill to motivate rats to freely do strenuous running exercise. The machine forces rats to run beyond their normal running activities by about 100 fold. This experimental condition with no surgery or medication is very similar to over strenuous running exercise or long-term running in humans. As shown, the running exercise model is meaningful in respect to stimulating rats to excessive running without artificial psychological or physiological distress on the animals. During running exercise, the body weight of the rats did not significantly change throughout the running stages. This running exercise protocol is thought to adapt to the natural running to the rat.

The first detailed study of strenuous running exercise in the rat knee was performed by Pap et al. They reported histological changes including fibrillations and clefts of joint cartilage. Another study also indicated osteoarthritic change of the knee. However, the studies of previous strenuous running exercise did not describe other changes of the joint structure including synovial membrane such as infrapatellar fat pad. The knee joint is clinically the most common site of overuse injury in runners. Runner’s knee includes various types of joint disorders. Anterior knee pain syndrome and Hoffa’s disease reportedly occur most frequently in runners. Therefore, the current study focused on pathological changes of the IFP and its preventative treatment using a rat running model. The pathological changes were investigated with regard to fibrosis of the synovial tissue with related inflammatory change, proliferation of small vessels and pain related nerves.

In this study, we described for the first time that fibrous change of the IFP became significantly changed. The average amount of collagen fiber increased along the running distance (Fig. 2A). The amount at 30 km run was significantly greater than that at 0 km run control. The fibrous change did not follow strong inflammatory reaction without a large amount of ED1 positive cell infiltration. But, the results of the peak number of ED1 (+) cell at 10 km run indicated that the changes of joint structure in the running model are less, but significantly, influenced by inflammatory reaction than usual joint arthritis model such as carrageenan induced arthritis and collagen induced arthritis.

The α-smooth muscle actin immunohistochemical staining showed that myofibroblast and blood vessels increased at 30 km of running. It is speculated that over...
strenuous running or long-term running habit has a risk of arthrofibrosis with little evidence of inflammation. Although tissue healing process includes new vessel proliferation phase which is thought to be necessary for the normal healing, the proliferation of small vessels accompanying free nerve endings caused tissue fibrosis with decreased threshold of pain sensation. CGRP positive nerve fibers were increased following strenuous running dependent upon the running distance. The findings of arthrofibrosis accompanying distance-dependent, increasing CGRP positive nerve fibers will partly explain the reasons for the complaints related to runner’s knee. Also, Fulkerson et al. indicated in clinical cases that the fibrous change of the patellar retinacular tissue with increased nerves and small vessels in the anterior pain with patellofemoral malalignment. For the complex problems of runner’s knee, the current study suggests that intraarticular hyaluronan injection is a potent candidate for treatment options. The study revealed that the number of myofibroblasts and blood vessels was decreased to be compared with that of the control in the IFP at 30 km of running after intraarticular HA injection (Fig. 4 B,C). The amount of collagen fibers was also decreased evaluated by Masson trichrome staining (Fig. 2 B,C). The number of CGRP positive nerve fiber also decreased to be compared with that of the 30 km run control without HA injection.

Hyaluronan is the major hydrodynamic non-protein component of joint synovial fluid (SF). Previous studies reported that hyaluronan restricts the entry of large plasma proteins and cells into SF but facilitates solute exchange between the synovial capillaries, cartilage and other joint tissues. In joint fluid, the high concentration of HA is responsible for the viscoelastic properties of SF. The viscoelasticity of HA is crucial for the maintenance of joint homeostasis. Since HA behaves as a viscous liquid at low shear rates and as an elastic solid at high shear rates, SF acts as a viscous lubricant during low impact movement of the joint and as an elastic shock absorber during high impact movement. The SF can help to lubricate and protect whole joint tissues, and to absorb joint load, which will prevent joints from repetitive minor injuries and degenerative changes. And intraarticular injected HA can decrease pain which is partly suggested by inhibited proliferation of CGRP positive nerve fibers. A previous study reported that intraarticular hyaluronan injection has some analgesic effects in vivo OA model. Hyaluronan has reportedly possessed some anti-inflammatory effects on joint inflammation in vitro and in vivo animal models.

In summary, the current study using a rat running model demonstrated that (1) strenuous running exercise can cause prominent fibrosis of the IFP without strong inflammatory reaction, and (2) intraarticular injection of hyaluronan can suppress progression of IFP fibrosis with an inhibition of CGRP positive nerve fibers. Intraarticular injection of hyaluronan may be a potent and promising option for treatment of runner’s knee.

Acknowledgments

This study is supported in part by grants from “the Japan Society for the Promotion of Science (19591752)” and from “the Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone in Tokyo Medical and Dental University” to TM, and from “the Japan Society for the Promotion of Science (18591657)” to IS.

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