Experimental osteoarthritis induced by surgical realignment of the patella in BALB/c mice

The patellofemoral joint is an important site of symptoms in osteoarthritis of the knee. We have used a newly designed surgical model of patellar strengthening to induce osteoarthritis in BALB/c mice and to establish markers by investigating the relationship between osteoarthritis and synovial levels of matrix metalloproteinases (MMPs).

Osteoarthritis was induced by using this microsurgical technique under direct vision without involving the cavity of the knee. Degeneration of cartilage was assessed by the Mankin score and synovial tissue was used to determine the mRNA expression levels of MMPs. Irrigation fluid from the knee was used to measure the concentrations of MMP-3 and MMP-9. Analysis of cartilage degeneration was correlated with the levels of expression of MMP.

After operation the patellofemoral joint showed evidence of mild osteoarthritis at eight weeks and further degenerative changes by 12 weeks. The level of synovial MMP-9 mRNA correlated with the Mankin score at eight weeks, but not at 12 weeks. The levels of MMP-2, MMP-3 and MMP-14 mRNA correlated with the Mankin score at 12 weeks. An increase in MMP-3 was observed from four weeks up to 16 weeks. MMP-9 was notably increased at eight weeks, but the concentration at 16 weeks had decreased to the level observed at four weeks.

Our observations suggest that MMP-2, MMP-3 and MMP-14 could be used as markers of the progression of osteoarthritic change.

Osteoarthritis (OA) is a slowly progressing condition resulting in fibrillation and loss of the articular cartilage. Up to 50% of the elderly population are affected of whom 25% are disabled because of joint symptoms. Risk factors for OA include age, a history of trauma, occupation and gender. Since these factors are closely related to mechanical loading of the joints, it has been assumed that OA is induced by accumulated mechanical stress. The patellofemoral joint is an important site of symptoms associated with OA of the knee. Pain in the knee is significantly associated with osteophytes in the patellofemoral joint, but not with those in the tibiofemoral joint.

In a community-based study of OA of the knee in patients with a mean age of 65.5 years, radiologically detected osteoarthritis in the patellofemoral joint in 65% of the knees and 53% in the tibiofemoral joint. In patients with pain in the knee, the compartmental distribution of radiologically visible OA was in the tibiofemoral and patellofemoral joints in 40%, followed by the latter only in 24% and of the former only in 4%. Within the patellofemoral joint, the lateral compartment was more frequently affected than the medial side.

Upregulation of all the matrix metalloproteinase (MMP) genes has been identified in the cartilage of STR/ort mice, serving as a murine model of spontaneous OA, but expressions of MMP-2, MMP-7, MMP-9 and MMP-13 were not detected in murine chondrocytes. We have previously shown the presence of interleukin (IL)-1β in the irrigation fluid and expression of mRNA in the synovium of the knee, and have demonstrated the role of MMP-9 mRNA as a therapeutic marker in the acute and chronic stages of arthritis induced by type-II collagen antibody.

In this study, we evaluated a newly designed model of patellar strengthening for inducing OA in BALB/c mice without invasion of the knee. We also attempted to establish markers for OA by investigating the relationship between MMPs in the synovium and degeneration of the cartilage.

Materials and Methods

Male BALB/c mice (aged six to eight weeks), were purchased from the National Laboratory...
Animal Centre, Taiwan. They were subsequently bred and maintained under pathogen-free conditions at the Laboratory Animal Centre of the National Defence Medical Centre, Taiwan. The experiments were approved by the local institutional review board and conducted in accordance with the National Institute of Health guidelines.

In order to test the new murine model, the mice were operated on by either surgical patellar strengthening or a sham procedure, divided into three batches and then killed at four (n = 6), eight (n = 6) and 12 weeks (n = 7 in the sham-operated group; n = 8 in the patellar strengthening group). The synovium and knees of all the mice were retrieved for examination and analysis.

**Induction of the surgical patellar strengthening model.** The mice were anaesthetised using pentobarbiturate (0.5 mg/10 g of body weight, intraperitoneally; Sigma, St. Louis, Missouri), and their hind limbs were shaved and prepared for aseptic surgery. The microsurgical techniques were carried out on the right knee. A 3 mm longitudinal incision was made from the proximal patella to the proximal tibia (Fig. 1a). The lateral muscle group of the femur was detached from the lateral aspect of the knee and the suprapatellar region. Using a microdissector the muscle flap was mobilised (Fig. 1b) and fixed with 7-0 vicryl sutures to the anterior aspect of the patella with the knee extended, in order to strengthen the patella (Fig. 1c). Sutures were placed at the medial side of the extensor muscle groups and along the medial pre-tibial region (Fig. 1d). The wound was flushed with sterile saline and the incision was closed in layers.

**Sham operation.** This involved elevation of the muscle flap and re-suturing it to its original insertion.

**Histological examination.** The knees were fixed in 10% formalin, decalcified, trimmed and embedded in paraffin. Sections were prepared from the tissue blocks and stained with haematoxylin and eosin for histopathological scoring. Sagittal sections were obtained from the middle, medial and lateral compartments. Each slide comprised four tissue sections in one embedded block, and we collected four every 80 μM. Each sample of 16 sections was examined according to a modification of the system of Mankin et al.\(^ {21} \) and included the following features: structure of the articular surface, cellular organisation, clone formation and the integrity of the tidemark (Table I).\(^ {22} \) Grading was performed on a scale of 0 to 9, in which normal healthy cartilage received a score of 0. Each slide was scored by two independent observers and the mean was used.

**RNA isolation and quantitative amplification of the polymerase chain reaction (PCR).** The total RNA was isolated using the Trizol method\(^ {23} \) with homogenisation of the synovium of the knee in Trizol lysis buffer followed by chloroform extraction (Life Technologies Corporation, Carlsbad,
The RNA was eluted with 20 μl of RNase-free water. All RNAs were quantified by spectrophotometry and the optical density 260:280 nm ratios were determined. For synthesis of complementary DNA, 5 μg of the total RNA were reverse-transcribed at 50°C for 60 minutes by using 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, California). The primer sequences for PCR amplification were as follows: MMP-2, 5'-ATGCCATCCCTGATAACCTG (forward) and 5'-CACATCCTTCACCTGGTG (reverse); MMP-3, 5'-CAGACCTGCCTTTCCCAT (forward) and 5'-GGAGAGATGGCCGAAARGA (reverse); MMP-9 5'-GGAACTCAGACGAATCTTTCCA-3' (forward) and 5'-GAAACTCACACGCGTATTTT-3' (reverse); MMP-13, 5'-CGAACTCACTCAGCATT (forward) and 5'-GCTGGTCTTCTCCATGTG (reverse); MMP-14, 5'-GCCCAAGGACACTTCAG (forward) and 5'-AGCGCTTCCTCCTCCAG (reverse). The SYBR Green master mix kit (BioRad, Hercules, California) was used for all real-time PCR reactions. The PCR was performed as follows: initiation at 94°C for two minutes, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 64°C for 30 seconds, extension at 72°C for 45 seconds and a final extension at 72°C for ten minutes. PCR amplifications for each sample were carried out in triplicate for all the products and the control. For each sample, the proportions of each product mRNA to GAPDH mRNA were calculated, and the data were expressed as a fold increase or decrease in the level of mRNA.

Cytokine assay. The knees were irrigated with 100 μl of phosphate-buffered saline and the fluid examined by an enzyme-linked immunosorbent assay using purified monoclonal antibody-coated plates to measure the concentration of MMP-3 and MMP-9. All the procedures followed the manufacturer’s protocol (Uscnlife, Nuhan, China). The cytokine concentration was measured using an MRX microplate reader (Dynex Technologies, Chantilly, Vermont) at 450 nm (reference 540 nm).

Statistical analysis. Statistical significance was determined using the Mann-Whitney non-parametric U test.
were expressed as the median with the 95% confidence interval (CI). A p-value $\leq 0.05$ was considered to be statistically significant. Correlations between the Mankin score and mRNA expression levels of the MMPs were assessed by using Spearman’s rank correlation test with SPSS version 14.0 software for Windows (SPSS Inc., Chicago, Illinois).

Results

Histopathological changes. After four weeks no significant degenerative changes were observed in either group. The median Mankin scores of the patellar strengthening group were 0.16 (95% CI not applicable (N/A)), 1.93 (95% CI 1.18 to 2.71), and 2.3 (95% CI 1.47 to 3.13) at four (n = 10), eight (n = 6) and 12 weeks (n = 8), respectively. Those of the sham group were 0.17 (95% CI N/A) at four weeks, 0.17 (95% CI N/A) at eight weeks and 0.15 (95% CI N/A) at 12 weeks. There were significant differences between the two groups at eight (p = 0.002) and 12 weeks (p = 0.0002) (Fig. 2). The median Mankin score at 12 weeks was greater than that at eight weeks, but without significant differences (p > 0.05) between the groups (Fig. 2d). There was no evidence of OA of the tibiofemoral joint or meniscal degeneration at 12 weeks in either group (Fig. 2c). The gross appearance of the synovial tissue was normal in both groups.

Levels of MMP expression. Synovium from the knee was harvested and prepared for determination of the relative levels of expression of mRNA in the MMPs (Fig. 3). There was no significant difference for MMP-2, MMP-3, MMP-9, MMP-13, and MMP-14 between the groups at four weeks. However, at eight weeks significant differences were seen in the levels of expression of MMP-2 (p = 0.037), MMP-3 (p = 0.0016), MMP-9 (p = 0.006) and MMP-14 (p = 0.019). At 12 weeks, there were also significant differences between the groups in the levels of expression of MMP-2 (p = 0.016), MMP-3 (p = 0.03) and MMP-14 (p = 0.016). After 16 weeks there were significant differences between the groups in the levels of expression of MMP-2 (p = 0.007), MMP-3 (p = 0.047), MMP-9 (p = 0.047) and MMP-14 (p = 0.007) but not for levels of
expression of MMP-13 at any time. The levels of expression of MMP-9 did not correlate with progression of degeneration of the joint. There was little expression of MMP-9 at 12 and 16 weeks in both groups (Table II).

**Correlations between levels of expression of MMP and histopathological changes in the surgical patellar strengthening group.** The cartilage and synovium were analysed. The correlations between the levels of expression of the MMPs and histopathological changes at eight and 12 weeks in the surgical patellar strengthening groups are shown in Figure 4. The histopathological changes in the surgical patellar strengthening group occurred in a time-dependent manner, and the levels of expression of MMP in the synovium were correlated with these (Table III).

**The concentration of MMP-3 and MMP-9 in the irrigation from the knee fluid in surgical patellar strengthening.** The concentration of MMP-3 and MMP-9 in the irrigation fluid from the knees in both groups at four, eight and 16 weeks is shown in Figure 5 and Table IV. After eight weeks there were significant differences between the groups in the concentrations of MMP-3 (p = 0.037) and MMP-9 (p = 0.019) in the irrigation fluid and also after 16 weeks (MMP-3 p = 0.005; MMP-9 p = 0.002). No significant differences were seen at four weeks (Table IV).

**Discussion**

We have designed a new surgical technique to modify the homeostasis of the patellofemoral joint without damaging the rest of the knee. The changes in the levels of expression of the MMPs showed some correlation with the time-dependent histopathological changes in the joint.

The patellar strengthening model focuses on the histopathological changes in the patellofemoral joint. The changes, in terms of the Mankin score, were seen in four mice after 12 weeks. Destruction of cartilage had occurred almost throughout the whole layer. Although not significant, the histopathological changes at 12 weeks were greater than those at eight weeks, possibly due to the short interval between the observations. This may also account for the normal histological appearance of the synovial tissue and the lack of formation of osteophytes in the patellofemoral joint.

In the levels of expression of mRNA of the synovial MMPs, MMP-9 showed a high level at eight weeks but this had disappeared after 12 weeks. We suggest that the expression of MMP-9 in the synovium is correlated with the arthritic process and may play a role in the initial phase of OA, MMP-9 may have disappeared at 12 weeks because of the absence of synovial inflammation. The levels of MMP-2, MMP-3 and MMP-14 were significantly elevated after both eight and 12 weeks. The levels of MMP-2 and MMP-14 were significantly higher at 12 weeks, unlike those of MMP-3. MMP-13 showed little expression at each time point. A previous study has shown a strong decrease in the levels of expression of MMP-3 and MMP-9 in the synovium, but not in the cartilage, of macrophage-depleted joints in collagenase-induced murine OA. The relative levels of expression of MMP-2, MMP-3 and MMP-9 were found to be higher on day seven than on day three, but no

### Table II. Levels of MMPs relative expression

<table>
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<tr>
<th>Weeks</th>
<th>Group</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>MMP-9</th>
<th>MMP-14</th>
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<td>4</td>
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<td>4.27</td>
<td>9.04</td>
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<tr>
<td></td>
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<tr>
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<tr>
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<tr>
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statistical evaluation was performed in that study. The observation period was shorter than that of our study, possibly accounting for the overexpression of synovial MMP-9 observed. In another study, MMP-2, MMP-3, MMP-13 and MMP-14 mRNAs were detected in the chondrocytes of tibial articular cartilage of STR/ort mice at 12, 18, 24, 30 and 35 weeks, but immunolocalisation was consistently detected only for MMP-3 and MMP-14 in the articular cartilage and MMP-13 in the calcified cartilage. These results are consistent with our findings that the mRNA levels in MMP-2, MMP-3 and MMP-14 were high in the synovium as OA progressed.

Significant correlations were observed between the relative levels of expression of mRNA and the histopathological changes for synovial MMP-9 after eight weeks and synovial MMP-2, MMP-3, and MMP-14 after 12 weeks. Inhibition of the production of MMP-14 has been found to significantly reduce the invasiveness of synovial fibroblasts in rheumatoid arthritis in a severe combined immunodeficiency mouse model. Sato et al. suggested that MMP-14 may play a role in the migratory and attachment activities of osteoclasts. It has been identified as an important activator of other MMPs, and is expressed in articular cartilage. Therefore, we postulate that MMP-14 plays a crucial role in the initiation of OA and the progression of changes in the synovium.

Our study has some limitations. We used a limited number of animals and did not undertake a power analysis, but the results showed significant differences. We did not assess loss of proteoglycan as part of the histopathological scoring. The tissue sections showed clear destruction of cartilage without obvious involvement of subchondral bone. We were unable to determine the relationship between the surgical patellar strengthening and the synovium. Since the
sham-group showed little expression of MMPs in the synovium, we conclude that there was no relationship between it and the surgical procedure.

This experimental model could examine different aspects of the development of OA. It is the first murine model induced by a surgical procedure of muscle transfer. Other murine models for OA have mimicked human traumatic OA and have been induced by division of the anterior cruciate ligament, surgical destruction of menisci or lesions of the collateral ligament.9-22 Our model may present a more realistic means of assessing patellofemoral OA in man. The surgical procedure is straightforward and quick. It does not destroy the synovium of the knee or any other structures within the joint and the pathological changes in the synovium and other structures within the joint can be followed during the disease process. The levels of expression of some MMPs in the synovium could be used as prognostic markers for OA.

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References